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The use of 2,2′-dithiobis(5-nitropyridine) (DTNP) for deprotection and diselenide formation in protected selenocysteine-containing peptides

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In contrast to the large number of sidechain protecting groups available for cysteine derivatives in solid phase peptide synthesis, there is a striking paucity of analogous selenocysteine *Se*-protecting groups in the literature. However, the growing interest in selenocysteine-containing peptides and proteins requires a corresponding increase in availability of synthetic routes into these target molecules. It therefore becomes important to design new sidechain protection strategies for selenocysteine as well as multiple and novel deprotection chemistry for their removal. In this paper, we outline the synthesis of two new Fmoc selenocysteine derivatives [Fmoc-Sec(Meb) and Fmoc-Sec(Bzl)] to accompany the commercially available Fmoc-Sec (Mob) derivative and incorporate them into two model peptides. Sec-deprotection assays were carried out on these peptides using 2,2′-dithiobis(5-nitropyridine) (DTNP) conditions previously described by our group. The deprotective methodology was further evaluated as to its suitability towards mediating concurrent diselenide formation in oxytocin-templated target peptides. Sec(Mob) and Sec(Meb) were found to be extremely labile to the DTNP conditions whether in the presence or absence of thioanisole, whereas Sec(Bzl) was robust to DTNP in the absence of thioanisole but quite labile in its presence. In multiple Sec-containing model peptides, it was shown that bis-Sec(Mob)-containing systems spontaneously cyclize to the diselenide using 1 eq DTNP, whereas bis-Sec(Meb) and Sec(Bzl) models required additional manipulation to induce cyclization.

Supporting information may be found in the online version of this article.

Keywords: selenocysteine; protecting group; deprotection; DTNP; thioanisole; selenol

Introduction

Selenocysteine (Sec, U) is the '21st' proteinogenic amino acid because it shares three key properties with the other 20 common amino acids: it has its own codon (UGA), it has its own unique tRNA, and it is co-translationally inserted at the ribosome [1,2]. Heterologous expression and production of selenoproteins is difficult because of the complexity of the UGA recoding machinery needed by the ribosome to translate UGA as a sense codon for Sec instead of as a stop codon [3,4]. A potential solution for overcoming the biological barriers for making Sec-containing proteins and peptides is by using solid phase peptide synthesis (SPPS). However, this route also presents its own set of problems including racemization, deselenization, a sparse choice of *Se*-protecting groups, and a lack of orthogonal methods for making selenosulfide and diselenide bonds [5–7]. Here, we report some advances in solving the latter two problems.

In contrast to the great number of sidechain *S*-protecting groups for cysteine in SPPS [8,9], there is a scarcity of sidechain *Se*-protecting groups for selenocysteine. With the exception of some novel sidechain protection strategy being advanced for Sec of late [10], the same general sets of benzyl-templated Sec sidechain protection functionality have persevered unadvanced for decades (Figure 1) [11–14]. In addition to the traditional benzyl functionality, electron-releasing *para* substituents give rise to the 4-methylbenzyl (Meb) group and the 4-methoxybenzyl (Mob) group. Moreover, although Mob *Se*-protection is utilized on Sec derivatives bearing both Boc and Fmoc $^{\alpha}N$ protection, the Bzl and Meb blocking motifs have so far only been utilized in Boc-protected Sec derivatives.

Traditionally, because of the robust nature of these benzyl-type protective moieties, conditions of deprotection have relied upon very harsh conditions such as the use of Hydroflouric Acid (HF) [15], molecular iodine [5], and metallic sodium in ammonia [11]. Recent research efforts in our laboratory have led to the discovery of a set of deprotection conditions for various Cys and Sec

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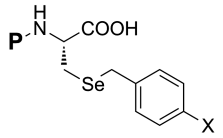

P	X	ref.
Boc	H	10
Boc	CH_3	11
Boc	OCH_3	12
Fmoc	OCH_3	13

Figure 1. Inventory of traditional benzyl-templated Sec-protected SPPS derivatives.

sidechain protecting groups that are much gentler than conventional approaches [16]. This methodology, which has been optimized for use with several Cys protecting groups [17], utilizes 2, 2′-dithiobis(5-nitropyridine) (DTNP) in a TFA/thioanisole cocktail to effect chalcogen deprotection under much less harsh conditions than traditionally possible (Figure 2). Although we previously reported some limited data on the enhanced effectiveness of these conditions towards Sec(Mob) deprotection [tenfold more effective than the corresponding Cys(Mob) deprotection] [16], we report here a comprehensive overview of the scope and of this deprotection methodology towards the three major benzyl-templated Sec sidechain blocking groups. Furthermore, because our laboratory is optimized towards Fmoc peptide synthesis rather than Boc synthesis, we needed to develop syntheses of the Fmoc Sec derivatives bearing Meb and Bzl protection at the selenol sidechain, compounds whose syntheses have not been published. The two peptides in which these Fmoc Sec derivatives were installed were carefully assayed as to their suitability to deprotection under DTNP-mediated conditions as well as to their abilities to cyclize into diselenide-containing targets.

Materials and Methods

Materials

N,N-dimethylformamide, HPLC-grade acetonitrile, and trifluoroacetic acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). NovaPEG® HMPB resin and 2-chlorotrityl resin were purchased from Novabiochem (San Diego, CA, USA). All standard Fmoc amino acids and *O*-benzotriazole-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) were purchased from RS Synthesis (Louisville, KY, USA). HATU was purchased from Oakwood Products (Jackson Hole, WY, USA). DTNP, thioanisole, and all other reagents were purchased from Sigma-Aldrich (Milwaukee, WI, USA).

Synthesis of Fmoc-Sec(X) Derivatives

Two procedures (1 and 2) were adapted with some modification for the synthesis of Fmoc-Sec derivatives (see Figure 3).

Procedure 1

This procedure is similar to that previously described [18] with some modification.

*Synthesis of H-Sec(X)-OH intermediates (**2a–c**)*

In a 250-ml round-bottom flask equipped with a stirring bar under inert N_2 atmosphere, selenocystine **1** (2.8 g, 8.3 mmol) was slurried in degassed 0.5 M aqueous NaOH solution (10 ml). Sodium borohydride (1.9 g, 49.7 mmol) dissolved in degassed water (20 ml) was added slowly using syringe to the selenocystine slurry. After stirring for 30 min at 0 °C, excess borohydride was quenched via dropwise addition of glacial acetic acid until all bubbling had ceased. Of the benzyl chloride, 16.6 mmol of the benzyl chloride was then added dropwise over 20 min, and the reaction was stirred under N_2 at 0 °C for 2 h. Concentrated HCl (4 ml) was then slowly added, and the reaction was maintained at 4 °C overnight. The crude Se-protected derivative was then isolated via suction filtration and used in the next step without further purification.

*Synthesis of Fmoc-Sec(X)-OH intermediates (**3a–c**)*

6.2 mmol of Sec(X)-OH [where X = Mob (**2a**), Meb (**2b**), and Bzl (**2c**)] was slurried in water (10 ml) in a 250-ml round-bottom flask with stirring. After the slurry was brought to 0 °C, triethylamine (1.70 ml, 12.4 mmol) was added in one portion followed by Fmoc-*O*-succinimide (2.3 g, 6.8 mmol) dissolved in acetonitrile (10 ml) with stirring. The reaction was then allowed to proceed for 2 h at room temperature, monitored using TLC. The reaction was quenched by the addition of 1 *N* HCl (50 ml), and the product was extracted with ethyl acetate (2 × 75 ml). The organic portions were combined and back-extracted with 1 *N* HCl (75 ml). The organic extract was dried over $MgSO_4$ and concentrated to a yellow oil under reduced pressure. The Fmoc-protected product was then purified over silica gel using 0.1 : 2.9 : 97 AcOH/MeOH/DCM as an eluent. The yields (over two steps) of protected Fmoc derivatives were as follows: Fmoc-Sec(Mob)-OH (**3a**, 10%); Fmoc-Sec (Meb)-OH (**3b**, 38%); and Fmoc-Sec(Bzl)-OH (**3c**, 80%).

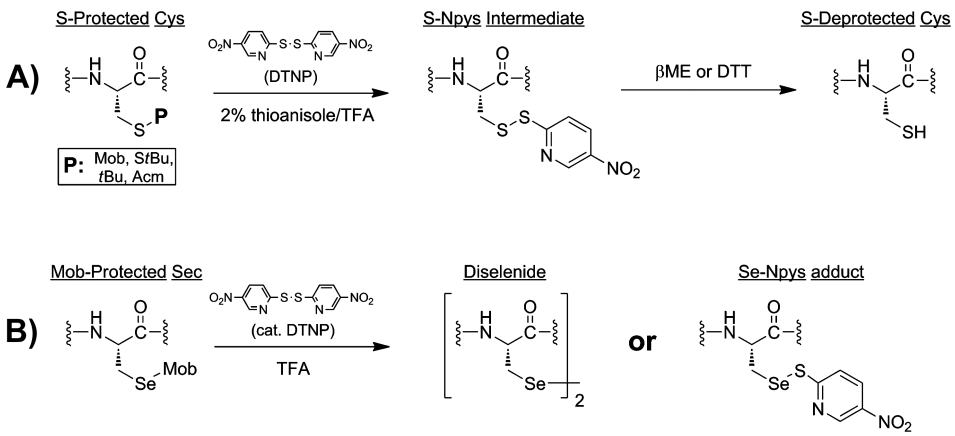

Figure 2. Prior results on protected Cys-containing and Sec-containing peptides [16,17]. (A) DTNP methodology allows facile deprotection of a number of common Cys S-protectants in the presence or absence of thioanisole. (B) Sec(Mob) deprotection required only catalytic amounts of DTNP in the absence of thioanisole.

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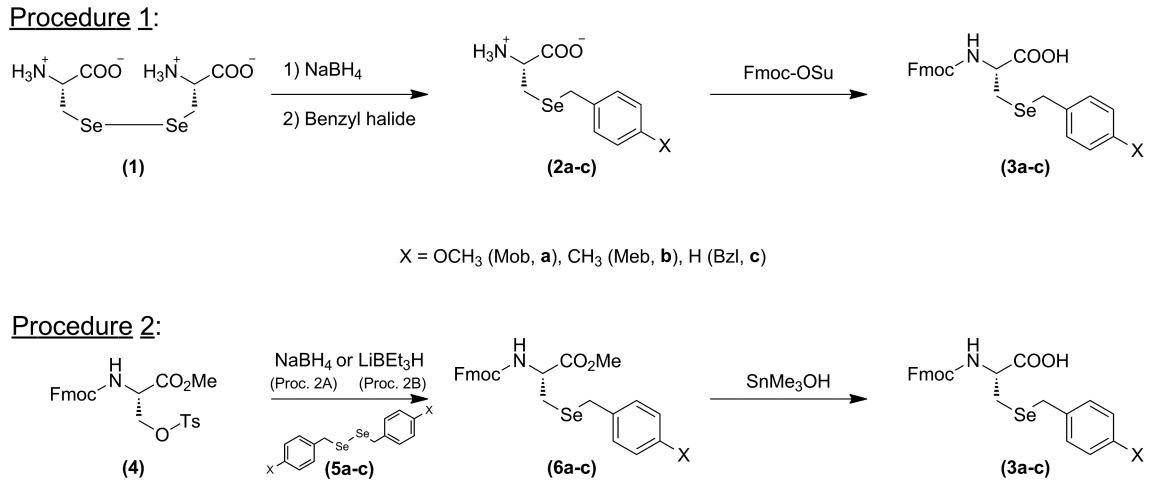

	Fmoc-Sec(Mob)-OH (**3a**) (% yield for 2 steps)	Fmoc-Sec(Meb)-OH (**3b**) (% yield for 2 steps)	Fmoc-Sec(Bzl)-OH (**3c**) (% yield for 2 steps)
Procedure 1	10	38	80
Procedure 2A ($NaBH_4$)	58	30	75
Procedure 2B ($LiBEt_3H$)	87	83	89

Figure 3. Synthetic routes to Sec derivatives **3a–c**.

Procedure 2

This procedure is a methodology adapted and optimized from previous similar syntheses of Dawson [19] and van der Donk [20].

*Synthesis of Fmoc-Ser(Ts)-OMe (**4**)*

Fmoc-Ser-OH (25.0 g, 76.38 mmol) was dissolved in 300 ml DMF and DIEA (13.9 ml, 84.02 mmol, 1.1 eq) was added in one portion. The solution was cooled to 0 °C and MeI (5.23 ml, 84.02 mol, 1.1 eq) was added slowly over 20 min. The mixture was allowed to stir overnight at room temperature. The entire mixture was then poured into 500 ml of ice-cold 1% aq. HCl. To this slurry was added 400 ml EtOAc, and the new mixture was thoroughly shaken. The organic layer was separated, and the resulting aqueous layer was extracted once more with 200 ml EtOAc. The organic portions were combined and extracted with 2 × 500 ml 2% aq. $NaHCO_3$. The resulting organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to yield Fmoc-Ser-OMe as a dense amorphous colorless solid that was used without further purification directly in the next step.

Fmoc-Ser-OMe from the previous step (assumed 76.38 mmol) and TsCl (43.68 g, 0.229 mol, 3 eq) was brought to 0 °C and pyridine (135 ml) was added with stirring. This mixture was stirred at 0 °C for 7 h. At the end of this time, the mixture was poured into 600 ml Et_2O and extracted with water (2 × 300 ml) and 1% aq. HCl (2 × 300 ml). The organic portion was dried over $MgSO_4$ and concentrated *in vacuo* to afford crude Fmoc-Ser(Ts)-OMe, which was purified batchwise over silica gel (20–40% EtOAc/Hex) to yield the desired Fmoc-Ser(Ts)-OMe **4** as a colorless solid (24.6 g, 65% for two steps).

*Synthesis of benzyl-templated diselenides (**5a–c**)*

Mob-diselenide, Meb-diselenide, and Bzl-diselenide were synthesized through a previously established procedure [20].

*Synthesis of Fmoc-Sec(X)-OMe derivatives (**6a–c**)*

Benzyl-templated diselenide (16.35 mmol) was dissolved in THF (20 ml) in a 250-ml round-bottom flask, with a slow stream of nitrogen continually purging the reaction vessel. After cooling the reaction mixture to 0 °C, $NaBH_4$ (0.93 g, 24.53 mmol, 1.5 eq) dissolved in 7 ml water (Proc. A) or 19.62 ml (19.62 mmol, 1.2 eq) 1.0 M $LiBEt_3H$ in THF (Proc. B) was added dropwise over 20 min to the diselenide solution. Following the addition of borohydride solution, the mixture was allowed to stir at 0 °C for 15 min. At the end of this time, Fmoc-Ser(Ts)-OMe (**4**) (5.40 g, 10.90 mmol, 0.67 eq) dissolved in 20 ml THF was added dropwise to the reaction mixture. Following this addition, the reaction mixture was allowed to stir at 0 °C for 2 h. At the end of this time, AcOH was added dropwise to the reaction (at 0 °C) until no additional gas evolution was observed. The entire reaction contents were then poured into 150 ml water in a separatory funnel followed by 200 ml EtOAc. Following thorough mixing, the organic layer was then separated and washed further with 2 × 100 ml 0.5% HCl solution. The organic phase was separated, dried over $MgSO_4$, and concentrated *in vacuo* to afford the crude Se-protected methyl ester. The crude product was then purified over silica gel (20–40% EtOAC/Hex) to yield the desired methyl ester compounds **6a–c**.

Fmoc-Sec(Mob)-OMe (**6a**): (Proc. A: 3.32 g, 58%; Proc. B: 5.15 g, 90%) isolated as a colorless amorphous solid: melting point (mp) 76–77 °C; $[\alpha]_D^{28} = -27.54°$ (*c* 2.0, EtOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (dd, *J* = 2.8 Hz, *J* = 7.5 Hz, 2H), 7.60 (t, *J* = 7.0 Hz, 2H), 7.57–7.62

(m, 2H), 7.27–7.33 (m, 2H), 7.18 (d, $J = 8.3$ Hz, 2H), 6.80 (d, $J = 8.5$ Hz, 2H), 5.57 (d, $J = 7.8$ Hz, 1H), 4.66 (q, $J = 7.4$ Hz, 1H), 4.40 (d, $J = 7.0$ Hz, 2H), 4.23 (t, $J = 6.9$ Hz, 1H), 3.75 (s, 8H), 2.87–2.96 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) 171.3, 158.6, 155.6, 143.8, 143.7, 141.3, 130.4, 130.0, 127.7, 127.1, 125.1, 120.0, 114.0, 67.1, 55.2, 53.8, 52.6, 47.1, 27.5, 25.6 ppm; IR (film) 1682 cm^{-1}, 1510 cm^{-1}, 737 cm^{-1}; high resolution mass spectrometry (HRMS) calculated for $C_{27}H_{27}NO_5Se$: 525.1054. Found: 525.1056 (M + H).

Fmoc-Sec(Meb)-OMe (**6b**): (Proc. A: 1.83 g, 33%; Proc. B: 4.83 g, 87%) isolated as a colorless amorphous solid: mp 109–110 °C; $[\alpha]_D^{28} = -31.07°$ (*c* 2.0, EtOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.71 (d, $J = 7.5$ Hz, 2H), 7.58 (t, $J = 6.8$ Hz, 2H), 7.32–7.39 (m, 2H), 7.26 (t, $J = 7.4$ Hz, 2H), 7.12 (d, $J = 7.7$ Hz, 2H), 7.05 (d, $J = 7.8$ Hz, 2H), 5.64 (d, $J = 8.0$ Hz, 1H), 4.65 (q, $J = 5.1$ Hz, 1H), 4.38 (d, $J = 7.0$ Hz, 2H), 4.19 (t, $J = 6.9$ Hz, 1H), 3.71 (s, 2H), 3.69 (s, 3H), 2.82–2.87 (m, 2H), 2.26 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$) 171.2, 155.5, 143.7, 143.5, 141.1, 136.4, 135.5, 129.2, 128.6, 127.5, 126.9, 124.9, 119.8, 67.0, 53.7, 52.4, 46.9, 27.5, 25.5, 20.9 ppm; IR (film) 1717 cm^{-1}, 1497 cm^{-1}, 762 cm^{-1}; HRMS calculated for $C_{27}H_{27}NO_4Se$: 509.1105. Found: 509.1106 (M + H).

Fmoc-Sec(Bzl)-OMe (**6c**): (Proc. A: 4.48 g, 83%; Proc. B: 4.86 g, 90%) isolated as a light yellow amorphous solid: mp 70–72 °C; $[\alpha]_D^{28} = -33.04°$ (*c* 2.0, EtOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (dd, $J = 2.9$ Hz, $J = 7.5$, 2H), 7.60 (t, $J = 6.3$, 2H), 7.37–7.42 (m, 2H), 7.23–7.33 (m, 6H), 7.18–7.22 (m, 1H), 5.53 (d, $J = 7.3$ Hz, 1H), 4.66 (dd, $J = 5.0$ Hz, $J = 12.4$, 1H), 4.41 (d, $J = 7.0$ Hz, 2H), 4.23 (t, $J = 6.9$, 1H), 3.77 (s, 2H), 3.75 (s, 3H), 2.88–2.99 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) 171.2, 155.6, 143.7, 143.6, 141.2, 138.5, 128.8, 128.5, 127.6, 127.0, 126.9, 125.0, 119.9, 67.0, 53.7, 52.5, 47.0, 27.9, 25.6 ppm; IR (film) 1686 cm^{-1}, 1526 cm^{-1}, 1211 cm^{-1}; HRMS calculated for $C_{26}H_{25}NO_4Se$: 495.0949. Found: 495.0954 (M + H).

*General procedure for the synthesis of Fmoc-Sec(X)-OH **(3a-c)***

Method is adapted and optimized from the procedure of Nicolaou [21]. Fmoc-Sec(X)-OMe **6a–c** (6.48 mmol) and $SnMe_3OH$ (2.34 g, 12.96 mmol, 2 eq) was dissolved in a 250-ml round-bottom flask in 40-ml 1,2-dichloroethane. The reaction vessel was purged with N_2, fitted with a reflux condenser, and heated at reflux for 1 h. At the end of this time, TLC showed consumption of all starting material, and the reaction mixture was concentrated *in vacuo* and purified directly, without workup over silica gel (20–40% EtOAc/Hex) to yield the final carboxylate compounds **3a–c**.

Fmoc-Sec(Mob)-OH (**3a**): (3.21 g; 97%) isolated as a colorless amorphous solid [14].

Fmoc-Sec(Meb)-OH (**3b**): (3.05 g; 95%) isolated as a colorless amorphous solid: mp 143–145 °C; $[\alpha]_D^{28} = -21.89°$ (*c* 2.0, EtOH); ^{1}H NMR (500 MHz, d_6 acetone) δ 7.92 (d, $J = 7.5$ Hz, 2H), 7.81 (t, $J = 5.5$ Hz, 2H), 7.48 (t, $J = 7.3$ Hz, 2H), 7.30 (d, $J = 7.8$ Hz, 2H), 7.16 (d, $J = 7.8$ Hz, 2H), 6.92 (d, $J = 8.1$ Hz, 1H), 4.58–4.68 (m, 1H), 4.37–4.50 (m, 2H), 4.33 (t, $J = 7.0$ Hz, 1H), 3.95 (s, 2H), 3.06–3.15 (m, 1H), 2.95–3.03 (m, 1H), 2.34 (s, 3H); ^{13}C NMR (125 MHz, d_6 acetone) 172.2, 156.5, 144.7, 144.6, 141.7, 136.8, 136.6, 129.5, 129.4, 128.2, 127.6, 125.8, 120.4, 67.0, 54.8, 47.6, 30.3, 27.4, 25.4, 20.7 ppm; IR (film) 1684 cm^{-1}, 1533 cm^{-1}, 736 cm^{-1}; HRMS calculated for $C_{26}H_{25}NO_4Se$: 495.0951. Found: 495.0956 (M + H).

Fmoc-Sec(Bzl)-OH (**3c**): (3.09 g; 99%) isolated as a light yellow amorphous solid: mp 74–75 °C; $[\alpha]_D^{28} = -12.75°$ (*c* = 2.0, EtOH); ^{1}H NMR (500 MHz, $CDCl_3$) δ 11.48 (s, 1H), 7.69, (d, $J = 7.4$ Hz, 2H), 7.55 (t, $J = 7.6$ Hz, 2H), 7.33 (t, $J = 7.4$ Hz, 2H), 7.25 (t, $J = 7.4$ Hz, 2H), 7.18–7.23 (m, 3H), 7.12–7.17 (m, 2H), 5.62 (d, $J = 4.0$ Hz, 1H), 4.63–4.70 (m, 1H), 4.37 (d, $J = 4.2$ Hz, 2H), 4.17 (t, $J = 4.0$ Hz, 1H), 3.73 (s, 2H), 2.86–2.97 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) 175.2, 155.9, 143.6, 143.4, 141.2, 138.3, 128.8, 128.5, 127.6, 127.0, 126.9, 124.9, 124.6, 119.9, 67.2, 54.4, 53.6, 46.9, 28.0, 25.2, 24.7 ppm; IR (film) 1710 cm^{-1}, 1514 cm^{-1}, 737 cm^{-1}; HRMS calculated for $C_{25}H_{23}NO_4Se$: 481.0794. Found: 481.0811 (M + H).

Peptide syntheses

All peptides were synthesized on a 60-μmole scale using 2-chlorotrityl chloride resin (1.51 mmol/g loading) as follows. A Symphony™ multiple peptide synthesizer (Protein Technologies Inc., Tucson, AZ, USA) was used for peptide syntheses via Fmoc protocol. Double coupling using 1 : 3 HATU/HBTU activation was employed for peptide elongation. A typical single coupling procedure is as follows: 20% piperidine/DMF (2 × 6 min); DMF washes (6 × 30 s); 5 eq each of standard Fmoc amino acid and coupling agent in 0.2 M NMM/DMF (2 × 40 min); DMF washes (3 × 30 s). Coupling of Fmoc-Sec derivatives was carried out using three times equivalents each of amino acid derivative, HOAt, and DIC with 5-min preincubation. Cleavage of peptides from their resins was accomplished through treatment of the resin with 96 : 2 : 2 TFA/Triisopropylsilane (TIPS)/H_2O for 1.5 h. Following filtration of the resin, the liquid cleavage mixture was evaporated to one tenth its original volume in a stream of nitrogen, followed by precipitation of the crude peptide into cold anhydrous diethyl ether.

High performance liquid chromatography

The HPLC analysis was carried out on a Shimadzu analytical HPLC system with LC-10 AD pumps, SPD-10A UV-VIS detector, and SCL-10A controller using a Symmetry™ C18 5-μm column from Waters (4.6 × 150 mm). Aqueous and organic phases were 0.1% TFA in water (Buffer A) and 0.1% TFA in HPLC-grade acetonitrile (Buffer B), respectively. Beginning with 100% Buffer A, a 1.4-ml/min gradient elution increase of 1% Buffer B/min for 55 min was used for all peptide chromatograms. Peptides were detected at both 214 and 254 nm. Preparative HPLC purification was carried out on a Shimadzu preparatory HPLC system utilizing LC-8A pumps, an SPD-10A UV-VIS detector, and an SCL-10A controller. A Waters SymmetryPrep™ C18 preparatory column (7-μm pore size, 1900 × 150 mm) was utilized in these separations. Beginning with 100% Buffer A, a 17 ml/min gradient elution increase of 1% Buffer B/min for 50 min was used for all preparative chromatograms.

Mass spectrometry

The MALDI-TOF mass spectrometry spectra were collected on a Voyager DE-Pro instrument under positive ionization and in reflectron mode. All samples were run using a matrix of 10 mg/ml 2,5-dihydroxybenzoic acid, vacuum-dried from a solution of 1 : 1 H_2O/ACN buffered to 0.1% TFA.

*DTNP deprotection assay conditions for VTGGU(X)A test peptides **7–9***

One-milligram (~1.7 μmol) aliquots of VTGGU(X)A test peptides were dissolved in 200 μl of either 100% TFA or 2% thioanisole/TFA to a final concentration of ~8.5 mM. Each of these solutions was incubated with different concentrations of DTNP with agitation at 25 °C for 1 h. At the end of this time, cold diethyl ether was added to each reaction and the crude precipitated product was isolated using centrifugation. Following drying of the pellets, the crude isolates were dissolved in 750 μl of 0.37 M aq. $NaBH_4$ for 20 min. At

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the end of this time, the mixture was brought to 0 °C and excess borohydride was destroyed with a few drops of AcOH. The solution was directly subjected to analytical HPLC analysis.

DTNP deprotection assay conditions for protected Sec-oxytocin test peptides ***10–12***

One-milligram (~0.8 μmol) aliquots of each protected Sec-oxytocin peptide were dissolved in 200 μl of either 100% TFA or 2% thioanisole/TFA to a final concentration of ~3.8 mM. Each of these solutions was incubated with different concentrations of DTNP with agitation with time and temperature parameters as reported in Table 1. At the end of the reaction time, cold diethyl ether was added to each reaction and the crude precipitated product was isolated using centrifugation. Following drying of the pellets, the crude isolates were dissolved in 0.75-ml 9 : 1 H_2O/ACN and the resulting solutions were evaluated using analytical HPLC.

DTNP-mediated diselenide cyclization conditions for Mob-protected Sec-oxytocin test peptide ***10***

5.0 mg (~4 μmol) of Mob-protected Sec-oxytocin peptide was dissolved in 1.0 ml of TFA. Two equivalents of DTNP dissolved in a minimal amount of TFA was then added with brief rapid stirring. The solution was incubated for 1 h, at the end of which cold diethyl ether was added to the reaction and the crude precipitated product was isolated using centrifugation. Following drying of the pellet, the crude isolate was dissolved in 0.75 ml 9 : 1 H_2O/ACN and evaluated using analytical HPLC [*m/z*: 1104.1 (M + H)].

DTNP/$NaBH_4$-mediated diselenide cyclization conditions for Meb-protected and Bzl-protected Sec-oxytocin test peptides ***11–12***

Of Meb-protected and Bzl-protected Sec-oxytocin peptides, 5.0 mg (~4 μmol) each were dissolved in 1.0 ml of 2% thioanisole/TFA. Each of these solutions was incubated with 20 eq DTNP with agitation for 1 h at 50 °C followed by 4 h at 25 °C. At the end of the reaction time, cold diethyl ether was added to the reaction and the crude precipitated product was isolated using centrifugation. Following drying of the pellets, the crude isolates were dissolved in 2-ml 0.37 M aq. $NaBH_4$ for 20 min. At the end of this time, the mixture was brought to 0 °C, and excess borohydride was destroyed with a few drops of AcOH. The solution was allowed to remain in contact with air for 30 min and then subjected to analytical HPLC analysis [*m/z*: 1104.1 (M + H)].

Results and Discussion

This research endeavor was composed of two distinct parts: (1) a synthetic component involving the construction of the Sec(Meb) and Sec(Bzl) Fmoc derivatives required to evaluate the effectiveness of this deprotection methodology and (2) an evaluation component in which peptide models bearing these protected Sec derivatives were assayed as to the effectiveness of their deprotection using DTNP. Because the achievement of the latter depended upon the successful chemical syntheses of the former, our efforts were first focused on the synthesis of the protected Fmoc derivatives.

Synthesis of Se-Protected Fmoc-Sec Derivatives

Our decision to carry out the synthesis of the model peptides for this study exclusively using Fmoc methodology necessitated the construction of the Fmoc-Sec(Meb) and Fmoc-Sec(Bzl) derivatives. We ultimately settled for two distinct synthetic schemes (Figure 3). The first synthesis (Procedure 1) was based upon an optimization of the procedure presented by Raines [18], in which selenocystine **1** was reduced with $NaBH_4$ and subsequently *Se*-alkylated with the corresponding benzyl chloride to afford intermediates **2a–c**. Subsequent Fmoc-protection yielded the completed Fmoc-Sec derivatives **3a–c**. Yields of **3a–c** varied reproducibly using this method, with the benzyl-protected **3c** typically affording the best yields, followed by more modest yields for the Meb-protected variant **3b** and low yields for the Mob-protected derivative **3a**.

Table 1. DTNP deprotection parameters and results on model Sec-containing peptide

Protecting group P	Peptide synthetic yield	Mass (*m/z*) M + H	Deprotection conditions		
			Equivalent (mM) DTNP/TFA	Reaction time (h)/temperature (°C)	% deprotection/cyclization (measured using HPLC)
		H_3N^+–Val-Thr-Gly-Gly–N(H)–CH(CH$_2$–Se–P)–CO–Ala–COOH			
Mob (**7**)	46	675.9	0.38 (3.5)	1/25	100
Meb (**8**)	72	659.8	3.3 (30)	1/25	93
Bzl (**9**)	71	646.0	11 (100) [a]	1/25	90
		H_3N^+–CH(CH$_2$–Se–P)–CO–Tyr-Ile-Gln-Asn–N(H)–CH(CH$_2$–Se–P)–CO–Pro-Leu-Gly–$CONH_2$			
Mob (**10**)	43	1346.0	2 (18)	1/25	98[b]
Meb (**11**)	17	1314.3	20 (183)[a]	1/50	95[c]
Bzl (**12**)	16	1286.0	20 (183)[a]	1/50	95[c]

[a]2% Thioanisole added.
[b]Yield of spontaneously cyclized peptide.
[c]Yield of two-step induced cyclization from bis-Npys intermediate.

Although the Fmoc-Sec(Mob) derivative **3a** is commercially available, its cost was a deterring factor for us, so we decided to seek another synthetic approach that would provide us the considerable amounts of the Mob-protected derivative that we would need at a much lower overall cost. We ultimately settled for an optimized combination of two synthetic approaches based upon the syntheses presented by Dawson [19] and van der Donk [20]. In this synthetic design, Fmoc-protected tosylate **4** became an advanced intermediate onto which selenium delivery was achieved via OT displacement of an *in situ*-generated benzyl-templated selenol functionality. Diselenides **5a–c** were synthesized according to the literature procedure [20] and used to deliver the protected selenium to intermediate **4** upon reduction with borohydride. Because of the lability of the Fmoc group towards basic conditions, we were concerned that the use of borohydride would lead to decomposition of our starting material **4**. However, diselenide reduction using two equivalents of borohydride while maintaining the reaction at 0 °C allowed clean conversion to the desired benzyl-templated Sec methyl esters **6a–c** without observed Fmoc degradation. Although we were again concerned about the integrity of the Fmoc group in the hydrolysis of methyl esters **6a–c** to their corresponding carboxylates **3a–c**, we found that treatment of these intermediates with unusual and effective reagent $SnMe_3OH$ in refluxing 1,2-dichloroethane [21] allowed for extremely facile transformation to carboxylates **3a–c**.

There was a marked difference in yields of intermediates **6a–c** dependent upon the medium of delivered borohydride. We noticed that the use of $NaBH_4$ in a partially aqueous medium, although giving reasonable yield for Sec(Bzl) intermediate **6c**, afforded moderate to poor yields of Sec(Mob) and Sec(Meb) intermediates **6a** and **6b**. The use of Super Hydride™ in THF gave much more satisfactory yields of intermediates **6a–c**.

Synthesis of Sec-Containing Model Peptides

With the protected Sec derivatives in hand, we then proceeded to use them in the syntheses of two model peptide systems. Each of these Sec-containing peptides was constructed to illustrate a different facet of the scope of this deprotection technique. We wished to utilize a peptide model that would compare the robustness of the three different protecting groups towards deprotection under the DTNP conditions. It was also of interest to utilize an additional peptide model that would demonstrate a merging of this basic deprotective process with concomitant diselenide formation.

As shown in Table 1, two model peptides were employed to illustrate the effectiveness of this deprotection methodology and its amenability towards further synthetic manipulation. Sec-containing hexamer test peptides **7–9** were chosen because of their use as successful models in the analogous prior publication by us [17] and were found to illustrate nicely the *Se*-deblocking proficiency of the DTNP conditions against the three protecting groups under study. Oxytocin analogs **10–12** were selected as models for concomitant diselenide formation subsequent to DTNP deprotection to further illustrate the practical use for the process. Syntheses of hexamer test peptides **7–9** proceeded cleanly and without difficulty. However, some difficulty was encountered in the syntheses of oxytocin analogs **10–12**. Although construction of the Sec(Mob) oxytocin **10** proceeded smoothly, Sec(Meb)- and Sec(Bzl) analogs **11** and **12** encountered problems with only partial attachment of one of the Sec residues as shown using crude HPLC of the product peptide (data not shown). Preparative HPLC allowed for clean excision of the desired peptide from the product mixture, albeit in diminished yield. Nevertheless, the recovered amounts of Sec(Meb)-oxytocin and Sec(Bzl)-oxytocin were sufficient for the DTNP deprotection assays to follow.

Deprotection/Cyclization of Sec-Containing Model Peptides

Probing the general deprotective abilities of this methodology made use of hexamer peptide systems **7–9**. The general protocol of the deprotection assay involved incubation of the model peptide with varying concentrations of DTNP in TFA either in the

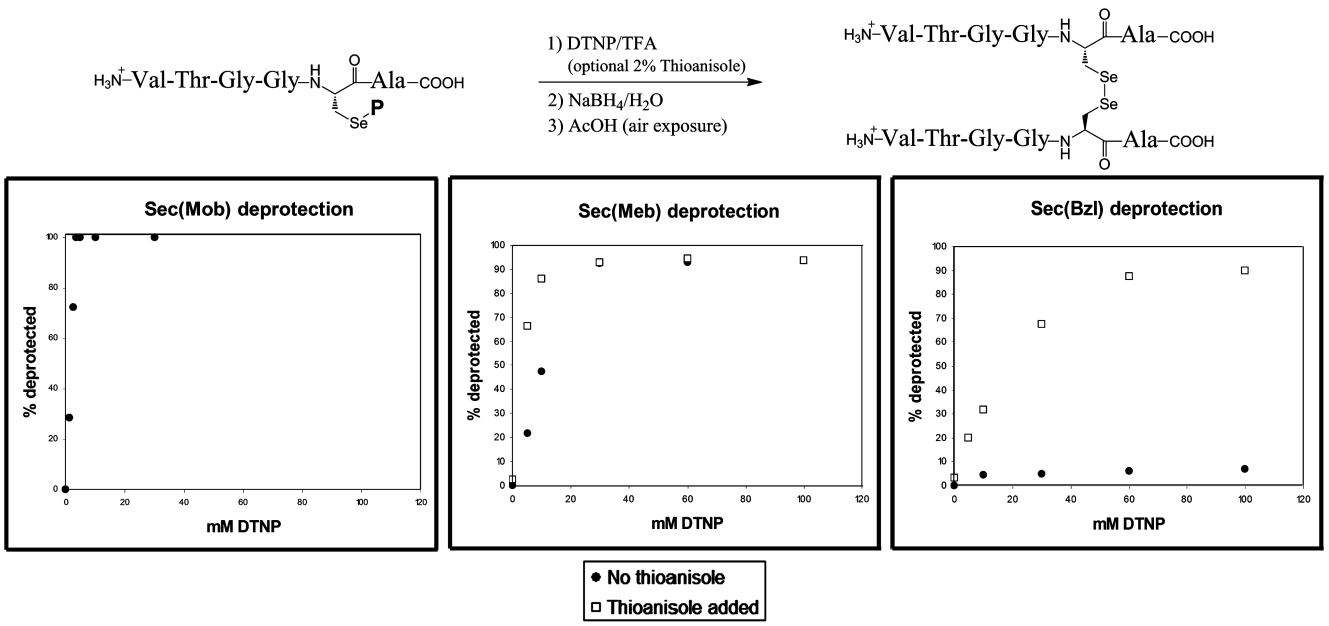

Figure 4. Results of DTNP assay on protected Sec-containing peptides **7–9**.

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presence or absence of thioanisole. Control reactions were carried out via incubation of the test peptides in 2% thioanisole/TFA without added DTNP. Following isolation of the crude peptide pellet via Et_2O trituration and centrifugation, treatment of the crude isolate with excess $NaBH_4$ in water was carried out to homogenize the complicated deprotection mixture. Following quenching of the $NaBH_4$, the free selenols quickly dimerized and were quantified as their corresponding diselenides via HPLC assay. As shown in Figure 4, the results of these general assays illustrate some interesting differences and parallels when compared with their corresponding Cys-protected analogs [17].

As reported elsewhere [16], the Sec(Mob) deprotection was effected using only stoichiometric amounts of DTNP in the absence of thioanisole, illustrating the effectiveness of this methodology towards what has traditionally been a very robust Sec protecting group. Sec(Meb) deprotection, although admittedly requiring somewhat higher DTNP concentration, was brought about in a comparatively facile fashion. It was found that the addition of thioanisole to the reaction mixture effected a somewhat superior deprotection, but similar to *Se*-Mob deprotection, ultimately full *Se*-Meb deprotection was achievable at low DTNP concentrations whether thioanisole was present or not. The Sec(Bzl) deprotection offered the most promising avenue of orthogonality to the process. It was found that *Se*-Bzl was quite robust to the deprotection conditions, even at very high concentrations of DTNP (<10% Bzl deprotection) in the absence of thioanisole, whereas it proved quite labile if 2% thioanisole was added to the reaction milieu (>90% deprotection). This 'orthogonality hole' in response to the different reaction conditions has potential to provide a very practical tool for peptide syntheses requiring post-synthetic stepwise preparation of multiple diselenide-containing peptides.

Oxytocin-based peptides **10–12** were employed to illustrate any propensity of this deprotective process to mediate concomitant diselenide formation following deprotection as part of a desired 'one-pot' procedure. Following some optimization, we were delighted to find that Mob-protected oxytocin system **10** underwent deprotection and spontaneous cyclization to the diselenide using 2 eq DTNP in the absence of thioanisole (Figure 5). This deprotection/cyclization sequence afforded a very pure product as shown in the HPLC trace in Figure 5. This result further marked the first time concomitant cyclization accompanied a deprotection using these conditions and stands in stark contrast to the behavior of the analogous Cys(Mob)-containing oxytocin analogs investigated previously [17], in which a (bis)-Npys intermediate instead formed and required further manipulation to induce cyclization to its corresponding disulfide.

In contrast to the ease with which Se-protection was removed in peptides **7–9**, (Meb)-protected and (Bzl)-protected oxytocins **11** and **12** required more forcing conditions to effect complete deprotection of both Sec residues. Indeed, 20 eq DTNP in 2% thioanisole/TFA at a temperature of 50 °C was required to fully deprotect both of these model systems. Moreover, the consistent isolated reaction product was not the cyclized product derived from **10** but rather the (bis)-Npys intermediate as shown in Figure 5. This intermediate, however, was converted to the desired cyclized seleno-oxytocin albeit in a decidedly less elegant fashion using aqueous $NaBH_4$ reduction, and following quenching of the borohydride environment, spontaneous cyclization afforded the desired diselenide product in a remarkably singular fashion.

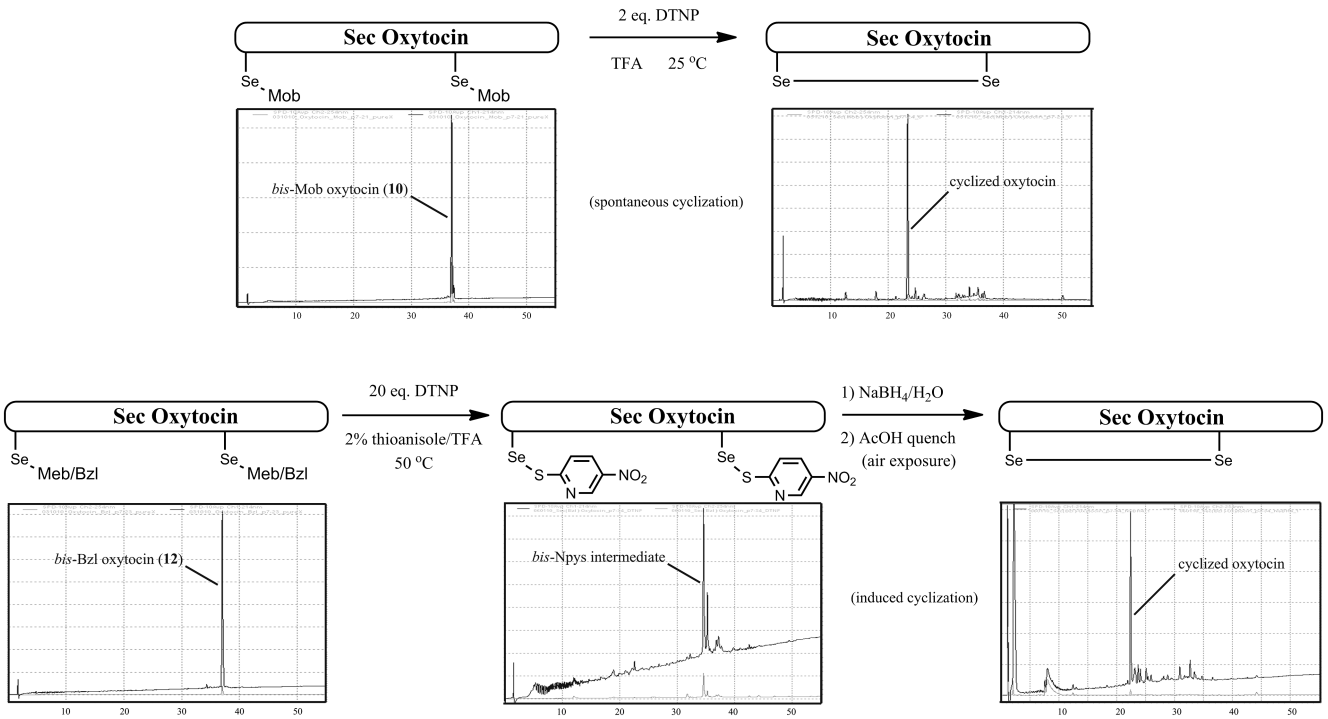

Figure 5. Deprotection/cyclization sequence for oxytocin peptides **10–12**, graphically illustrating the different requirements between the Sec(Mob)-bearing peptides and the Sec(Meb) or Sec(Bzl)-bearing peptides.

Conclusions

We have presented herein a gentle DTNP deprotection methodology applied to a standard selenocysteine *Se*-protecting group (Mob) that has traditionally required relatively harsh conditions for its removal. Two new Sec derivatives, Fmoc-Sec(Meb) and Fmoc-Sec(Bzl), were constructed via an optimized synthetic protocol to possess the building blocks necessary to synthesize via Fmoc protocol two model peptides upon which to assay the deprotective potential of this approach. These three new Fmoc-Sec derivatives were assayed as to their sidechain protecting groups' lability towards the deprotection conditions.

The DTNP deprotection assays were carried out at specific concentrations in TFA in either the presence or absence of thioanisole. On hexamer test peptides **7–9**, it was found that both Mob and Meb protecting groups were very labile to the DTNP conditions in the absence of thioanisole, with Mob requiring sub-stoichiometric amounts of DTNP and Meb requiring somewhat higher amounts to effect complete deprotection. The Bzl protecting group was remarkably robust when treated with high concentrations of DTNP in the absence of thioanisole, whereas in the presence of thioanisole, it was readily removable at higher DTNP concentrations. This orthogonal thioanisole dependency on deprotective effectiveness has potential for application towards iterative diselenide closure in chemical syntheses of multiple diselenide-containing peptides.

Oxytocin models **10–12** were used in testing the versatility of this deprotective methodology in tandem with cyclization of newly deprotected selenols to their corresponding diselenide. Sec(Mob) oxytocin **10**, while requiring minimal DTNP concentration to bring about bis-deprotection, cleanly cyclized into the corresponding diselenide spontaneously. Sec(Meb) and Sec(Bzl) analogs, by contrast, required more forcing conditions for their respective deprotection and required additional chemical manipulation to bring about their diselenide formation.

The HPLC chromatograms and MALDI mass spectra of all peptides are provided in the Supplemental Information section associated with this article.

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