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Studies on deprotection of cysteine and selenocysteine side-chain protecting groups

KATHARINE M. HARRIS, STEVENSON FLEMER Jr and ROBERT J. HONDAL*
Department of Biochemistry, 89 Beaumont Ave, Given Laboratory, Room B413, Burlington, VT 05405, USA

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Abstract: We present here a simple method for deprotecting *p*-methoxybenzyl groups and acetamidomethyl groups from the side-chains of cysteine and selenocysteine. This method uses the highly elecrophilic, aromatic disulfides 2,2′-dithiobis(5-nitropyridine) (DTNP) and 2,2′-dithiodipyridine (DTP) dissolved in TFA to effect removal of these heretofore difficult-to-remove protecting groups. The dissolution of these reagents in TFA, in fact, serves to 'activate' them for the deprotection reaction because protonation of the nitrogen atom of the pyridine ring makes the disulfide bond more electrophilic. Thus, these reagents can be added to any standard cleavage cocktail used in peptide synthesis.

The *p*-methoxybenzyl group of selenocysteine is easily removed by DTNP. Only sub-stoichiometric amounts of DTNP are required to cause full removal of the *p*-methoxybenzyl group, with as little as 0.2 equivalents necessary to effect 70% removal of the protecting group. In order to remove the *p*-methoxybenzyl group from cysteine, 2 equivalents of DTNP and the addition of thioanisole was required to effect removal. Thioanisole was absolutely required for the reaction in the case of the sulfur-containing amino acids, while it was not required for selenocysteine. The results were consistent with thioanisole acting as a catalyst. The acetamidomethyl group of cysteine could also be removed using DTNP, but required the addition of >15 equivalents to be effective. DTP was less robust as a deprotection reagent. We also demonstrate that this chemistry can be used in a simultaneous cyclization/deprotection reaction between selenocysteine and cysteine residues protected by *p*-methoxybenzyl groups to form a selenylsulfide bond, demonstrating future high utility of the deprotection method. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: selenocysteine; *p*-methoxybenzyl; acetamidomethyl; deprotection; electrophilic; aromatic; disulfide

INTRODUCTION

Our research efforts over the past several years have focused on synthesizing peptides containing selenocysteine (Sec, U) [1] (Abbreviations used in this paper follow the guidelines recommended by Jones JH) for use in peptide ligation reactions [2,3]. One problem with using Sec in solid-phase peptide synthesis (SPPS) is the requirement for using benzyl-type protecting groups for the selenol side-chain owing to the incompatibility of a trityl group (Trt) with this side-chain [4–7]. Benzyl (Bzl) and *p*-methoxybenzyl (Mob) protecting groups are difficult to remove and require harsh conditions, such as the use of hydrofluoric acid (HF), silyl-Lewis acids, or thiophilic heavy metals.

There are many problems associated with the use of these reagents. In the case of HF, a special apparatus must be used to deliver the HF gas to the peptide. This apparatus is expensive, not widely available, and the gas is highly toxic. Strong Lewis acids such as trimethylsilyl bromide (TMSBr) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) have also been used to remove Bzl and Mob groups from the side-chains of cysteine (Cys) and Sec [8]. These reagents are extremely harsh and can have side reactions with the peptide. TMSBr and TMSOTf have limited solubility in ether and they tend to precipitate with the target peptide, often causing the peptide to become oily and difficult to handle upon lyophilization. Heavy metals such as Hg^{2+}, Ag^{+}, and Tl^{3+} have been used to remove Mob and acetamidomethyl (Acm) groups from Cys and Sec side-chains [9–11]. These metals are toxic and difficult to handle, often precipitating in the solution conditions used to effect deprotection. Moreover, these metals effect removal of the protecting group by forming a very strong metal-sulfur or metal-selenium complex. The metal itself is then removed by the addition of a very large excess of thiol to the peptide in order to chelate the metal. The result is often poor yields because the strength of the metal-sulfur complex is such that incomplete removal of the metal is common.

Oxidative deprotection using I_2 and DMSO has also been used as a method to effect the removal of Bzl, Mob, and Acm groups [12–15]. In our experience, the use of I_2 is highly problematic as it is so reactive that reaction conditions must be optimized for each individual peptide. Formation of dehydroalanine and iodinated adducts are common side reactions. Moreover, the target peptide will be oxidized to a disulfide or diselenide with the use of this reagent [13]. The use of DMSO as an oxidative deprotection reagent for the removal of Cys(Mob) groups, however, has met with some success [16].

In this paper, we report a new, extremely mild method for removing Acm and Mob groups from cysteine and selenocysteine. The method presented here is related

*Correspondence to: R. J. Hondal, Department of Biochemistry, University of Vermont, College of Medicine. 89 Beaumont Ave, Given Laboratory, Room B413, Burlington, VT 05405, USA;
e-mail: Robert.Hondal@uvm.edu

to the work presented earlier by Galande *et al.* who reported on a new method for making disulfide bonds on-resin [17]. We have found that one reagent used by Galande *et al.*, 2,2′-dithiobis(5-nitropyridine) (DTNP), could be used directly to deprotect Sec(Mob) residues, and with the addition of thioanisole, Cys(Mob) and Cys(Acm) residues could also be deprotected. A related electrophilic, aromatic disulfide, 2,2′-dithiodipyridine (DTP), could also be used as a deprotection reagent. We believe that our results will find high utility for peptide chemists using cysteine. Our findings on the use of these new deprotection reagents and their applications are reported herein.

MATERIALS AND METHODS

Materials

Solvents for peptide synthesis were purchased from EMD Biosciences (San Diego, CA). Fmoc-amino acids were purchased from Synpep Corp (Dublin, CA). Resins for solid-phase synthesis were purchased from Applied Biosystems (Foster City, CA). All other chemicals were purchased from either Sigma-Aldrich (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA). The high pressure liquid chromatography (HPLC) system was from Shimadzu with a Symmetry® C_{18}-5 μm column from Waters (4.6 × 150 mm). A Voyager-DE™ PRO Workstation (Applied Biosystems) was used for mass spectral analysis of peptide samples.

Peptide Syntheses

Fmoc-selenocysteine(Mob)-OH was synthesized as had been reported previously by us [18,19]. All peptides were synthesized with a Burrell Model 75 wrist-action shaker (Pittsburgh, PA). For each peptide synthesis, the resin was first swelled in DCM for 30 min and washed six times with DMF. Deprotection of the N^{α}-Fmoc protecting group was carried out using one 15-min agitation with a solution of 20% piperidine/80% DMF. Success of all deblocking and amino acid couplings were monitored qualitatively using a ninhydrin test [20]. Elongation of the peptides used standard Fmoc SPPS chemistry with 4 equivalents (equivalents relative to peptide synthesis scale in mmoles) 2-(1H-benzotriazol-1-YL)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.05 M final concentration), 4 equivalents HOBt, 4 equivalents Fmoc-AA-OH, and 4 equivalents 2,4,6-trimethylpyridine (TMP) (collidine) in a 50% DCM/50% DMF solution with no preactivation time [21]. For the synthesis of peptides with a *C*-terminal carboxamide, Fmoc-PAL-Resin (0.35 mmol/g loading) was used, and each synthesis was done on a 0.15 mmole scale. For peptides with a *C*-terminal carboxylate, peptides were synthesized on a 0.2 mmole scale using Fmoc-Gly-PEG-PS-Resin (0.20 mmol/g loading). The composition of cleavage cocktails used to liberate the peptide from the solid support is listed in Tables 1 and 2. The cleavage cocktails were dripped into cold, anhydrous diethyl ether to precipitate the peptide. The peptide was collected by centrifugation at 3000 rpm for 10 min and then washed with diethyl ether twice. The peptide was dissolved in a minimal amount of a water/acetonitrile mixture and lyophilized to give the final product.

High Pressure Liquid Chromatography (HPLC)

All HPLC analysis was done on a Shimadzu analytical HPLC system with LC-10AD pumps, SPD-10A UV-Vis detector, and SCL-10A controller using a Symmetry® C_{18}-5 μm column from Waters (4.6 × 150 mm). Aqueous and organic phases were 0.1% TFA/H_2O and 0.1% TFA/acetonitrile, respectively. Gradient elution with the following profile was used to elute peptides from the column: 0% organic phase for 10 min, followed by a 1% increase in organic phase per min until the organic phase reached 60%. The organic phase was then ramped to 100% over 5 min and stayed at 100% for 10 min to complete the run. Peptides were detected at 214 nm and 254 nm.

Mass Spectrometry

Mass spectrometry data was collected on a Voyager DE-Pro instrument in positive reflector mode. All samples were run using a matrix of 10 mg/ml 2,5-dihydroxybenzoic acid in 50% acetonitrile/50% water/0.05% TFA.

Formation of 5-Npys-Protected Cysteine Residues on-Resin and Conditions for on-Resin Cyclization

The use of a 5-nitropyridinsulfenyl (5-Npys) protected cysteine residue is incompatible for use in Fmoc SPPS [22]. In order to activate the sulfhydryl side-chain of Cys for cyclization, we used a procedure developed by Galande *et al.* for on-resin formation of 5-Npys-protected sulfhydryls [17]. After conversion of the Cys(StBu) derivative to a Cys(5-Npys) derivative, simultaneous cyclization/deprotection to form a selenylsulfide bond was then initiated by incubating the resin with 1% TFA in DCM, followed by the addition of neat TFA/scavenger cocktails as described in Table 1.

Use of DTNP and DTP as Deprotection Reagents in Solution

The Mob and Acm protecting groups were removed by treating lyophilized peptide with cocktails of TFA/thioanisole (97.5/2.5) plus electrophilic disulfide (DTNP or DTP). For selenocysteine-containing peptides, no thioanisole was required. The amount of DTNP or DTP added to the cocktails is described in 'Results'.

Peptide Yields with DTNP Cocktails

In order to determine the yields using DTNP in cleavage cocktails, peptides VTGGC(Acm)A-OH, VTGGC(Mob)A-OH, and VTGGU(Mob)A-OH were purified by preparative HPLC and then lyophilized. The peptides were then weighed and dissolved in cocktails containing DTNP. Peptide VTGGC(Acm)A-OH (21.0 mg) was treated with 15 equivalents of DTNP with 2% thioanisole in TFA for 2 h. Peptide VTGGC(Mob)A-OH (9.0 mg) was treated with 5 equivalents of DTNP with 2% thioanisole in TFA for 2 h. Peptide VTGGU(Mob)A-OH (9.0 mg) was treated with 5 equivalents of DTNP in TFA for 2 h. Following treatment with the cocktail, peptides were precipitated into ether, and allowed to dry. The peptides were then dissolved in water and purified by preparative HPLC. The product peak was collected and then lyophilized and weighed to determine the yield.

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Table 1 On-resin cyclization of vicinal Cys/Sec peptides to form a selenylsulfide bridge

Peptide[a]	Cleavage and cyclization conditions	Percent cyclized
C(Trt)U(Mob)G-PAL-Resin	95/2.5/2.5 TFA/EDT/H_2O (2 h)	5.5
C(StBu)U(Mob)G-PAL-Resin	95/5 TFA/H_2O (2 h)	38
C(StBu)U(Mob)G-PAL-Resin	90/7.5/2.5 TFA/thioanisole/phenol (20 h)	75
C(5-Npys)U(Mob)G-PAL-Resin	% TFA in DCM followed by washing with DCM and cleavage with 95/5 TFA/H_2O (2 h)	100
C(StBu)U(Mob)G-PAL-Resin	95/5 TFA/H_2O (20 h)	52.5
C(StBu)U(Mob)G-PAL-Resin	80/20 TFA/H_2O (2 h)	0
C(StBu)C(Mob)G-PAL-Resin	90/7.5/2.5 TFA/thioanisole/phenol (20 h)	0
C(StBu)AU(Mob)G-PAL-Resin	90/7.5/2.5 TFA/thioanisole/phenol (2 h)	20
C(StBu)AAU(Mob)G-PAL-Resin	90/7.5/2.5 TFA/thioanisole/phenol (2 h)	5

[a] U is the one letter abbreviation for selenocysteine.

Table 2 Deprotection of Sec(Mob) by DTNP/TFA cocktails[a]

Peptide	Cleavage conditions	Percent deprotected[b]
VTGGU(Mob)G-OH	90/7.5/2.5 TFA/phenol/thioanisole (2 h)	15
VTGGU(Mob)G-OH	TFA (2 h)	5–10
VTGGU(Mob)G-OH	TFA + 1.3 equivalents DTNP (2 h)	100
VTGGU(Mob)G-OH	90/7.5/2.5 TFA/phenol/thioanisole/1.3 equivalents DTNP (2 h)	100
VTGGU(Mob)G-OH	97.5/2.5 TFA/thioanisole + 1.3 equivalents DTNP (2 h)	100
VTGGU(Mob)G-OH	92.5/7.5 TFA/phenol + 1.3 equivalents DTNP (2 h)	100
VTGGU(Mob)G-OH	On-resin incubation with 10 equivalents DTNP in DCM[c]	5–10
VTGGU(Mob)G-OH	On-resin incubation with 10 equivalents DTNP in DCM[d]	5–10
VTGGU(Mob)G-NH_2	95/5 TFA/H_2O (2 h)	5–10
VTGGC(Mob)G-NH_2	90/7.5/2.5 TFA/phenol/thioanisole (2 h)	3

[a] The reactions footnoted in c and d serve as controls to ensure that the DTNP cannot react with Sec(Mob) in the absence of TFA and that we are able to wash all DTNP off the column respectively.
[b] The term 'deprotection' here refers to the amount of the Mob group removed from Sec. Depending on the stoichiometry of the reaction, the Sec residue will either exist as the selenol, or as a 5-Npys protected Sec residue.
[c] This step was followed by extensive washing with DMF and DCM and then cleaved with 95/5 – TFA/H_2O.
[d] This step was followed by extensive washing with DMF and DCM after which the resin was incubated with 1% TFA, washed again with DCM, and then cleavage with 95/5 – TFA/H_2O.

Model Cyclization Reaction Involving Sec(Mob) and Cys(Mob)

A peptide of the sequence AEAU(Mob)GPC(Mob)K-OH was synthesized and purified by preparative HPLC. Stock solutions of 3.5 mg (2.49 μmol) peptide in 700 μL TFA, and 11.7 mg (35.4 μmol) DTNP in 10.0 mL of TFA were prepared. In 15-mL conical tubes, three reactions were then carried out. To all three tubes were added 200 μL (0.71 μmol, 1 equivalent) of peptide solution, 200 μL (0.71 μmol, 1 equivalent) DTNP solution, and 1 mL of TFA. The reactions were then allowed to sit for 1 h. At the end of this time, 40 μL (340 μmol) thioanisole was added to one of the tubes, and 40 μL thioanisole (340 μmol) and an additional 200 μL DTNP (1 equivalent) solution was added to another. The third reaction tube was left alone. After an additional hour, the TFA supernatant in all the reaction vessels was evaporated by gently bubbling N_2 through the solution. To the residue in each was added 1 mL H_2O and 2 mL diethyl ether. The solutions were mixed by vortexing. After the aqueous and organic layers separated, the lower, aqueous layer was carefully pipetted out and deposited into 1 mL vials for HPLC analysis of the product mixture.

RESULTS AND DISCUSSION

Cyclization by Neighboring Group Participation

While searching for an efficient method for removing the Mob group of selenocysteine in our target peptide of sequence Cys-Sec-Gly-NH_2 (this peptide corresponds

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to the *C*-terminus of mammalian thioredoxin reductase) [2], we decided to attempt oxidative deprotection using I_2. Analytical HPLC analyses of these crude product mixtures resulted in extremely complex chromatograms. Some of the peaks were identified as iodinated adducts or as a dehydroalanine-containing peptide (data not shown), consistent with previous reports [15,23]. We then attempted to improve this procedure by protecting the neighboring group cysteine as a disulfide using a StBu group. It had been previously demonstrated by van der Donk and coworkers. that the oxidative deprotection of Mob groups with I_2 could be improved if the peptide also contained a disulfide bond [23]. We noticed that when performing a control reaction in which we cleaved the target Sec(Mob) containing peptide with a vicinal disulfide bond (Cys(StBu)-Sec(Mob)-Gly-PAL-Resin) using a cleavage cocktail containing TFA/water (95/5) that more peaks than expected were present in the analytical HPLC chromatogram, especially a large peak that eluted very early in the profile (Figure 1(A)). This early peak (labeled as peak 1 in Figure 1(A)) was identified by MALDI-TOF mass spectrometry as the fully oxidized and deprotected tripeptide with a m/z of 327 Da. Moreover, the peak area of the target product was 38%. We postulated that a neighboring group effect was driving deprotection of the Mob group of Sec because in previous attempts at synthesizing this tripeptide, we used a trityl (Trt) group to protect the neighboring Cys residue [24]. The synthesis of tripeptide Cys(Trt)-Sec(Mob)-Gly-PAL-Resin, followed by deprotection with a cocktail of TFA/water/ethanedithiol (95/2.5/2.5) yielded, according to MALDI-TOF-MS analysis, a mixture containing 95% Sec(Mob) containing peptide and 5% of the cyclized product (Figure 1(B), peaks 2 and 1, respectively). A postulated mechanism for this spontaneous cyclization and simultaneous deprotection reaction is shown in Figure 2. The cyclization reaction (with the neighboring Cys residue protected as StBu) could not be driven to completion by increasing the incubation time with cleavage cocktail or by adding scavengers such as phenol and thioanisole (HPLC trace not shown).

Inspired by a recent report by Galande *et al.* [17] who developed a new method for making disulfide bonds on-resin using 5-Npys-activated Cys residues, we then attempted to drive the cyclization reaction of the tripeptide to completion by using a 5-Npys-activated Cys residue in the adjacent position. We reasoned that the disulfide bond of a Cys(5-Npys) residue in the neighboring position to Sec(Mob) should be a much better electrophile and thus improve the cyclization. We used the procedure developed in [17] to convert a Cys(StBu) residue to a Cys(5-Npys) residue on-resin so that our peptide was of the sequence Cys(5-Npys)-Sec(Mob)-Gly-PAL-Resin.

In the original method described by Galande *et al.*, a Cys(StBu) residue was reduced to the free thiol,

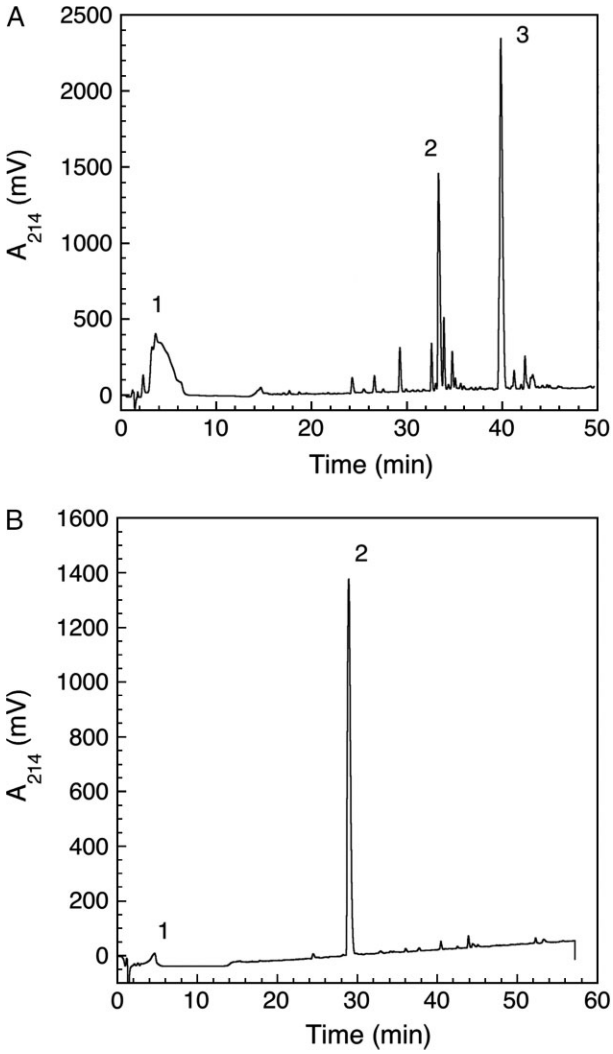

Figure 1 (A) Analytical HPLC chromatogram of peptide C(StBu)U(Mob)G-NH_2 that was treated with a cleavage cocktail containing TFA/H_2O (95/5). The peak labeled as 1 (37.9% of total peak area) is the fully deprotected and cyclic peptide. The material in peak 2 (17% of total peak area) was found to have a m/z of 831.7 and corresponds to a dimeric form of the target peptide in which the Mob groups were removed from each monomer. Peak 3 is the fully protected peptide with a m/z of 537.6 and is 37.8% of the total peak area. (B) Analytical HPLC chromatogram of peptide C(Trt)U(Mob)G-NH_2. that was treated with a cleavage cocktail containing TFA/EDT/H_2O (95/2.5/2.5). One major peak predominates (peak 2–94.5% of peak area) and MS analysis identified it as peptide CU(Mob)G-NH_2 (the trityl group is removed while the Mob group remains on Sec). Peak 1 was found to be the fully deprotected, cyclic peptide with a m/z of 327 and was 5.5% of the total peak area.

which was then converted to a Cys(5-Npys) derivative by reaction with the free thiol with excess DTNP. After derivatization, the resin was treated with a solution of 1%TFA/TIS so that a nearby Cys(Mmt) residue would be deprotected (the methoxytrityl group is removed by 1% TFA) and the newly liberated thiol would then attack

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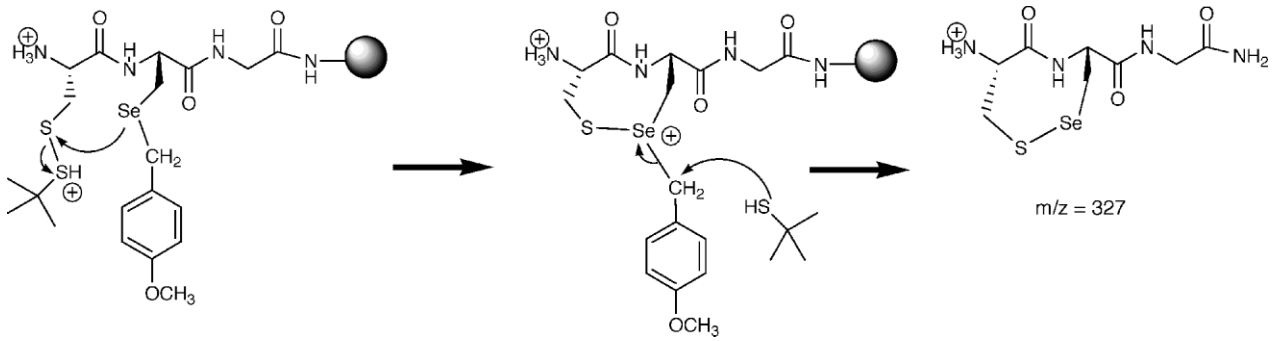

Figure 2 Our postulated mechanism for the spontaneous deprotection/cyclization reaction (on-resin) for peptide Cys(StBu)-Sec(Mob)-Gly-PAL-Resin. The reaction is dependent on high concentrations of acid, which would protonate the sulfur atom of the StBu group. Concomitant attack by the Se atom on the neighboring sulfur would result in a trivalent selenonium cation that would quickly break down in the presence of a scavenger. This scavenger could be the released StBu group or water present in the TFA. The shaded circle represents the resin bead.

the activated Cys(5-Npys) residue to form a disulfide bond on-resin.

In our case, we also converted the Cys(StBu) derivative to a Cys(5-Npys) derivative using the procedure developed by Galande after the completion of synthesis. Spontaneous cyclization/deprotection to form a selenylsulfide bond was then induced by incubating the resin with 1% TFA in DCM. After washing the solid support to remove the TFA solution, the peptide was treated with a cocktail of 95/5 TFA/H_2O for 2 h to liberate the peptide from the resin. However, here the purpose of the 1% TFA is not to remove a Mmt protecting group from Cys, but rather to activate the adjacent Cys(5-Npys) group by protonating the pyridine ring nitrogen. Here, the attacking nucleophile is the selenium atom from an adjacent Sec(Mob) residue instead of a naked sulfhydryl of Cys.

It was hoped that cyclization would occur on-resin with the addition of 1% TFA/DCM (protonation of the pyridyl-nitrogen) and that the liberated Mob and thionitropyridine would be washed away. The solution was observed to turn yellow during this step, but we did not quantify the cyclization by UV-Vis. Upon liberation of the peptide from the solid support, the product was isolated by ether precipitation and then subject to analysis by LC-ESI-MS. The only mass detected in this experiment was the cyclized peptide with a m/z of 327 Da. We have postulated a similar mechanism for this spontaneous cyclization/deprotection as that given in Figure 2. However, the mixed disulfide that is formed in this case is much more reactive under acidic conditions because the nitrogen atom of the pyridine ring can be protonated, resulting in a very strong electron sink. Protonation of the pyridine nitrogen increases the electrophilicity of the mixed disulfide bond, and this increased elecrophilicity drives the reaction to completion (Figure 3). This neighboring group deprotection-cyclization reaction is driven by the presence of a nearby, highly elecrophilic disulfide bond and the high nucleophilicity of selenium. When the Sec(Mob) residue is replaced by a Cys(Mob) residue, there is no cyclization under the same deprotection conditions using a StBu group in the adjacent disulfide (Table 1). This effect is also dependent on the adjacency of a disulfide bond. When a single alanine residue is inserted inbetween the Cys(StBu) and the Sec(Mob)

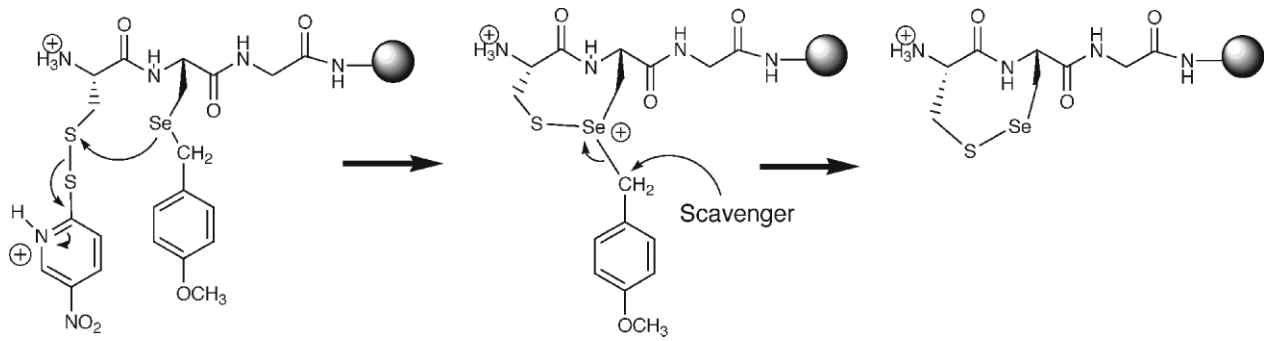

Figure 3 Our postulated mechanism for spontaneous deprotection/cyclization (on-resin) of a Sec(Mob) residue when a highly reactive Cys(5-Npys) residue is in the adjacent position. The propinquity of the highly electrophilic disulfide bond allows for easy attack by the Se atom onto the central S atom. This attack results in a trivalent selenonium cation that quickly breaks down to the cyclized product. This mechanism is nearly identical to the one in Figure 2, except that the peptidyl-disulfide bond is highly activated for nucleophilic attack by the presence of the 5-Npys group. The shaded circle represents the resin bead.

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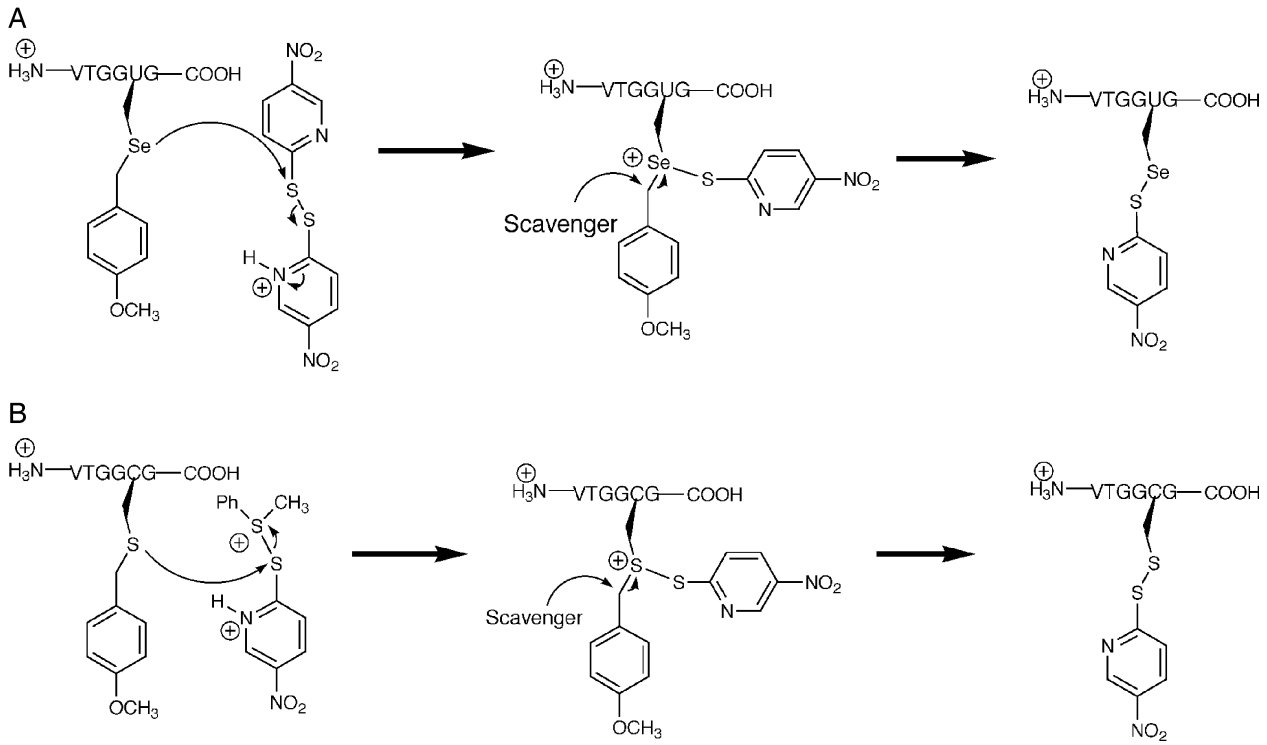

Figure 4 In (A), exogenous DTNP can be added to a peptide dissolved in TFA containing a Sec(Mob) group. The highly acidic solution activates DTNP by protonation of the nitrogen atom in the pyridine ring. This creates a very good electron sink and enhances the electrophilicity of the disulfide bond of DTNP. The highly nucleophilic selenium atom attacks this disulfide bond, resulting in a trivalent selenonium cation that breaks down with the scavenger to form a Sec(5-Npys) residue. As shown in (B), a Cys(Mob) residue can also be deprotected by DTNP, but it requires the addition of thioanisole. Thioanisole, most likely, forms a reactive, trivalent sulfonium cation when it attacks the disulfide bond of DTNP. This trivalent species is much more reactive than DTNP alone and we believe that it is this trivalent species that allows for the less nucleophilic sulfur atom (compared to selenium) to attack the resulting, reactive disulfide bond, causing deprotection and formation of a Cys(5-Npys) residue. This 5-Npys group can be removed by simple reduction with reagents such as dithiothreitol or β-mercaptoethanol.

residues, the cyclization efficiency drops to 20%, and when two alanine residues are inserted the efficiency is the same as that when the Cys residue is protected with a Trt group (5%). A summary of our findings of this neighboring group effect is presented in Table 1.

The Use of DTNP and DTP as Deprotection Reagents

Having developed a method for making this tripeptide that is crucial for our research, we wanted to develop a more general method for deprotecting Sec(Mob) residues. Our data in Table 1 demonstrates that the Mob group could be removed from Sec in the presence of a nearby, reactive disulfide bond. This neighboring group effect is obviously driven by the high effective concentration of the adjacent disulfide. Since a mixed disulfide bond between a Cys residue and thionitropyridine served as a good electrophile in this deprotection reaction, we wondered if we might use DTNP directly to effect the deprotection of Sec(Mob) residues under acidic conditions. To test this hypothesis, we synthesized a test peptide of sequence VTGGU(Mob)G-OH. We wanted to develop a cleavage cocktail that would be able to remove the

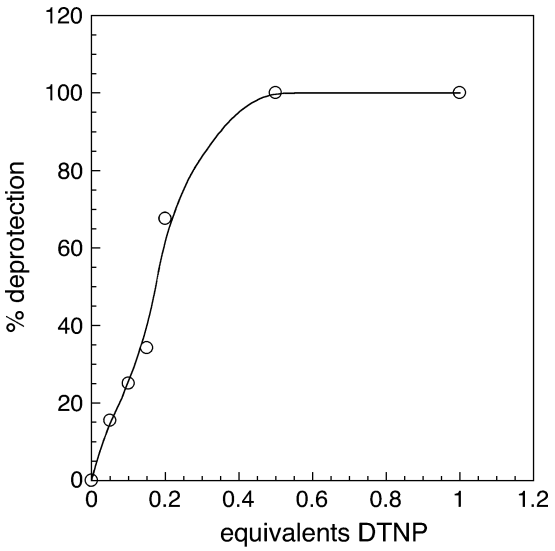

Figure 5 Plot of percent deprotection of a Sec(Mob) residue in target peptide VTGGU(Mob)G-OH *versus* equivalents of DTNP in the TFA cocktail. 100% deprotection can be achieved with less than stochiometric addition of DTNP (0.5 equivalents).

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peptide from the solid support while simultaneously removing the Mob group from Sec. We thus chose to add 1.3 equivalents of DTNP (based on the loading of the resin) to a cocktail of TFA/phenol/thioanisole (90/7.5/2.5) and incubated the resin with the cocktail for 2 h at RT. After isolating the peptide by ether precipitation, MALDI-TOF-MS analysis of the reaction product showed that the peptide was present as both the free selenol and the 5-Npys protected Sec derivative (data not shown). We then began to explore the mechanism of this deprotection reaction by varying the composition of our cleavage cocktail. The results of these experiments are summarized in Table 2. Control reactions were done without DTNP using cocktails of either TFA/water or TFA containing thioanisole and phenol. The Mob group was removed from the Sec residue in these control reactions at a level ranging from 5–15%. A last control was done on a peptide in which the Sec(Mob) residue was replaced with a Cys(Mob) residue. This control showed only 3% removal of the Mob group from Cys (Table 2).

These results demonstrate that all that is necessary to fully deprotect the Sec(Mob) residue is a cleavage cocktail consisting of 1.3 equivalents of DTNP dissolved in neat TFA. Thioanisole or other scavengers are not required for this reaction. The *p*-methoxybenzyl cation may be scavenged by the 2-thio-5-nitro-pyridine produced from the reaction with Sec(Mob) and DTNP, but is more likely scavenged by water that is absorbed by the TFA. Our proposed reaction mechanism based on *our initial data* for this deprotection reaction is shown in Figure 4(A). The deprotected peptide was found to exist in two forms, either as the free selenol, or as the Sec(5-Npys) protected derivative as determined by MALDI-TOF MS (data not shown). When 0.5 equivalents of DTNP are used in the reaction, the ratio of free selenol to 5-Npys protected Sec is 82/18. This ratio becomes 75/25 when 2 equivalents are added and is nearly equal (56/44) when 5 equivalents of DTNP are used.

Complete and full deprotection was achieved with 0.5 equivalents of DTNP in the cleavage cocktail and ~70% deprotection was achieved with as little as 0.2 equivalents of DTNP in the reaction (Figure 5). The results are consistent with DTNP acting in a catalytic fashion in which DTNP catalyzes the removal of the Mob group from the selenium atom, resulting in a Sec(5-Npys) derivative (Figure 6). The driving force for the catalytic behavior of DTNP acting in this fashion is the greater strength of the S–S bond of DTNP in relation

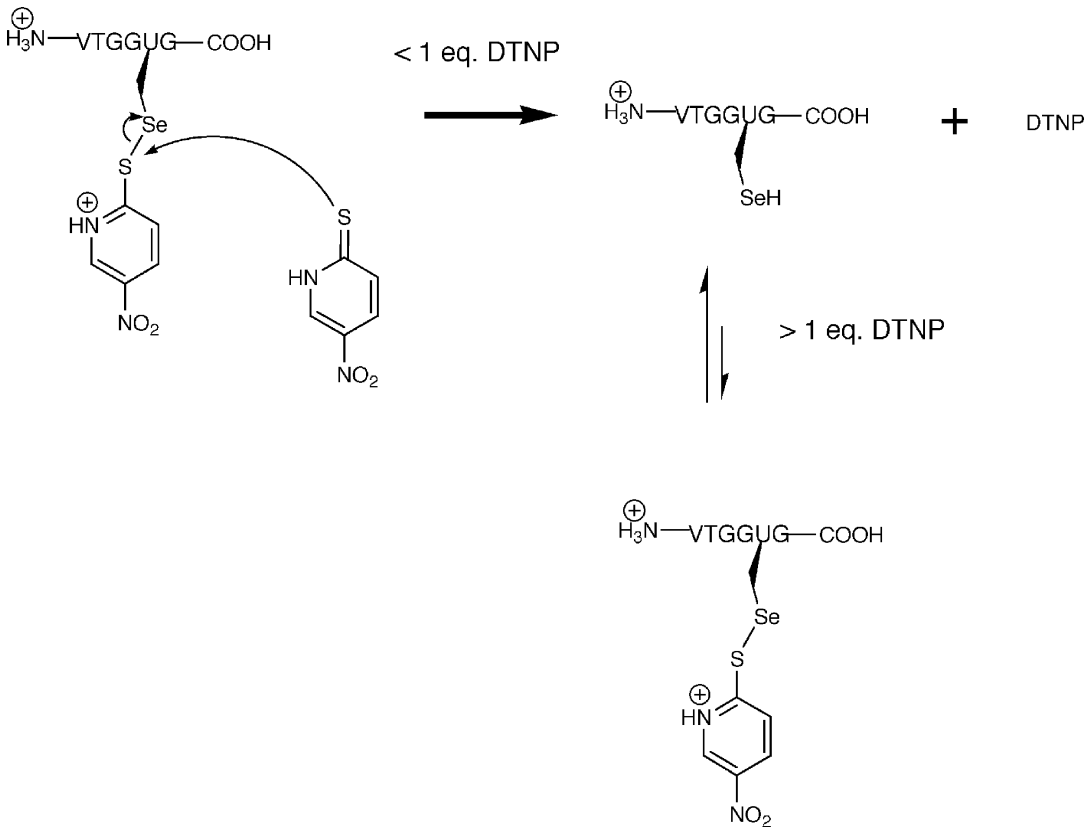

Figure 6 A catalytic role for DTNP is proposed here based on the results in Figure 5 that show that high levels of Mob removal can be achieved with less than 1 equivalent of DTNP. The free 2-thio-5-nitro-pyridine that is released in the deprotection reaction could cleave the Se–S bond of the Sec(5-Npys) derivative by attack on the sulfur atom resulting in a peptide with a free selenol and regenerating DTNP. Excess DTNP alters this equilibrium process resulting in the selenol being functionalized as the 5-Npys derivative. The thione form of 2-thio-5-nitro-pyridine most likely predominates in solution as is shown here [25].

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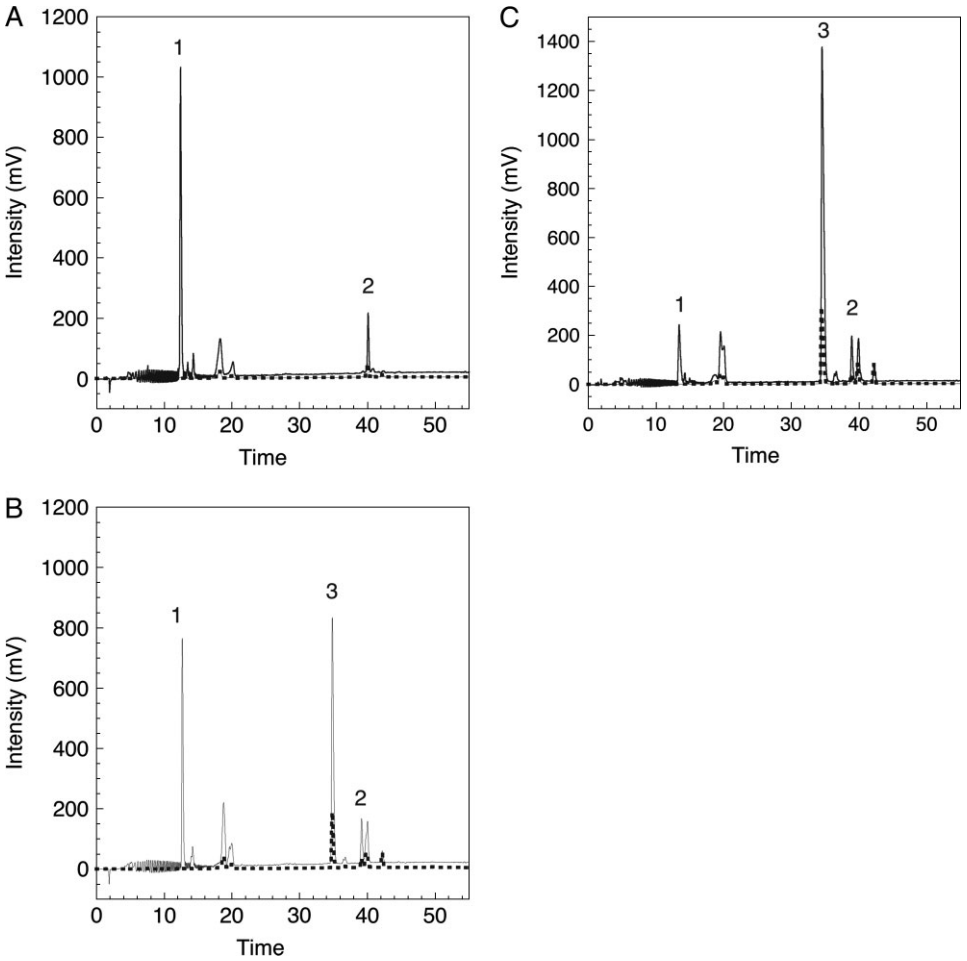

Figure 7 Progress of removal of the Acm group of the cysteine residue in test peptide VTGGC(Acm)A followed by analytical HPLC. In (A), the peptide has been treated with 2% thioanisole and TFA only as a control. Peak 1 is the Acm-protected peptide, and peak 2 is thioanisole. In (B), the peptide has been treated with 3 equivalents of DTNP in TFA with 2% thioanisole in the cocktail. A new peak is clearly visible in the trace at 35 min (labeled as peak 3). This peak was identified by MALDI-TOF MS as the 5-Npys protected peptide (VTGGC(5-Npys)A with $m/z = 658$). In (C), the peptide was treated with 15 equivalents of DTNP in TFA with 2% thioanisole in the cocktail. Near complete deprotection is observed, but there is still some Acm-protected peptide present. In all traces, detection at 214 nm is represented by the solid line and detection at 254 is represented by the dashed line.

to the comparative weakness of the Se–S bond of the Sec(5-Npys) derivative.

We next turned our attention to attempting the deprotection of Cys(Mob) and Cys(Acm) using DTNP as a deprotectant. These derivatives are much less nucleophilic than a Sec(Mob) residue, thus we hypothesized that Cys(Mob) might be able to be deprotected with longer reaction times, but Cys(Acm) would be unaffected. For these reactions, we synthesized test peptides of sequence VTGGC(Mob)A-OH and VTGGC(Acm)A-OH. We decided that we would include thioanisole in the cleavage cocktails as it might enhance deprotection as a sulfonium cation transfer catalyst [26,27]. The peptide derivatives were analyzed by HPLC and products were identified by MALDI-TOF MS. To our great surprise, the Acm group of cysteine could be removed by DTNP. The reaction of the Cys(Acm) derivative with DTNP is shown in Figure 7(A)–(C). The Mob group of cysteine is similarly removed with DTNP, but only 2 equivalents are required for full removal. The reaction of DTNP with Cys(Mob), unlike the reaction of DTNP with Sec(Mob), always resulted in a Cys(5-Npys) derivative. A plot of equivalents of DTNP *versus* percent deprotection is shown in Figure 8.

The final yields of target peptides VTGGC(Acm)A-OH, VTGGC(Mob)A-OH, and VTGGU(Mob)A-OH treated with DTNP-containing cocktails are reported in Table 3 and yields are in the range of 62–78%. Here, the yields are reported starting from material purified from preparative HPLC, treated with DTNP cocktails, precipitation into ether, and then subjected to preparative HPLC and lyophilization. Thus, the yields seem reasonable after undergoing several steps. The yield will vary depending on the choice of peptide; one factor in yield here is in recovery, as our test peptides were rather short and hydrophobic. While quantitative deprotection is observed for Sec(Mob) and Cys(Mob) peptides, we

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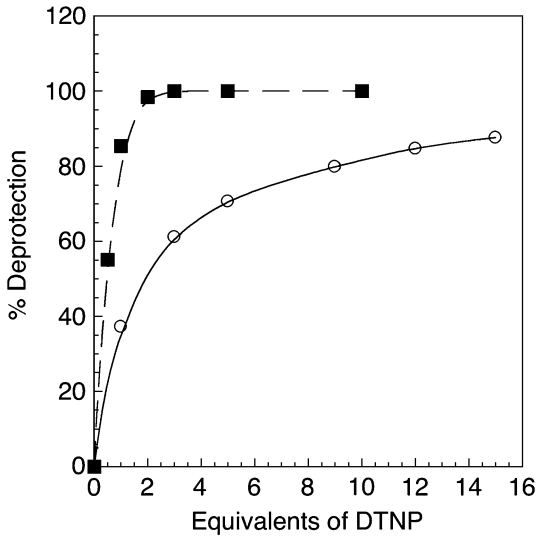

Figure 8 A plot of percent deprotection of the Mob group from Cys (closed squares) or the Acm group of Cys (open circles) *versus* equivalents of DTNP added to the peptide dissolved in TFA with 2% thioanisole. The test peptides here were either VTGGC(Acm)G-OH or VTGGC(Mob)G-OH. The Cys(Mob)-containing peptide can be fully deprotected with 2 equivalents of DTNP, while ~90% deprotection is achieved for the Cys(Acm)-containing peptide with 15 equivalents.

achieved only ~90% deprotection for Cys(Acm) peptides (based on HPLC analysis).

Our original hope was that there would be a large reactivity difference between Cys(Mob) and Cys(Acm) derivatives with DTNP. Although the data in Figure 8 seems to suggest a modest reactivity gap, we could not selectively remove a Mob group from cysteine in the presence of a Cys(Acm) derivative using DTNP (data not shown). Hoping to increase the reactivity gap between the two derivatives, we tested the ability of DTP as a deprotection reagent to further exploit differences in reactivity between Cys(Mob) and Cys(Acm) residues. DTP should be much less reactive as it lacks a nitro group in the *para* position and hence the disulfide bond of DTP should be much less electrophilic. The results show that DTP also causes deprotection of Cys(Mob) and Cys(Acm) derivatives, but its reaction with Cys(Acm) is much more sluggish than that of a Cys(Mob) derivative as shown in Figure 9. Using DTP as the deprotectant, the Mob group could be removed using >6 equivalents of DTP, while only ~40% deprotection was achieved with 15 equivalents of DTP with the Cys(Acm) derivative. The reaction of DTP with either Cys(Mob) or Cys(Acm) results in the formation of a mixed disulfide bond between the thiopyridyl group and the sulfur of cysteine, similar to that of the 5-Npys derivative that is formed when using DTNP. The data in Figure 9 shows that there is a much larger reactivity gap between the two derivatives when using DTP. However, we were not able to achieve orthogonal deprotection of Cys(Mob) and Cys(Acm) residues using DTP in the case of at least one test peptide [AC(Acm)GTTGC(Mob)A].

In the hope of developing orthogonal conditions for deprotecting Cys(Mob) in the presence of a Cys(Acm) derivatives, we explored the option of omitting thioanisole from the cleavage cocktail containing DTNP and TFA. The result was that no deprotection occurred using DTNP or DTP with either cysteine derivative when thioanisole was not present in the cocktail. Thus in the case of cysteine derivatives, thioanisole is crucial in catalyzing the deprotection reaction, most likely through a sulfonium cationic intermediate as shown by our postulated mechanism in Figure 4(B). Thus, clear reactivity differences can be seen between Sec(Mob) and Cys(Mob) when using DTNP as a deprotectant, and there are further differences in the reactivities of

Table 3 Deprotection conditions for Sec and Cys amino acid derivatives using DTNP

Amino acid derivative	Thioanisole required for deprotection	Equivalents of DTNP needed for effective deprotection	Percent yield of Deprotected target peptide[a]
Sec(Mob)	No	0.5–1.0	78
Cys(Mob)	Yes	2	70
Cys(Acm)	Yes	>15	62

[a] Target peptides were VTGGC(Acm)A-OH, VTGGC(Mob)A-OH, and VTGGU(Mob)A-OH. The procedure for deprotection and yield determination are described in 'Methods'.

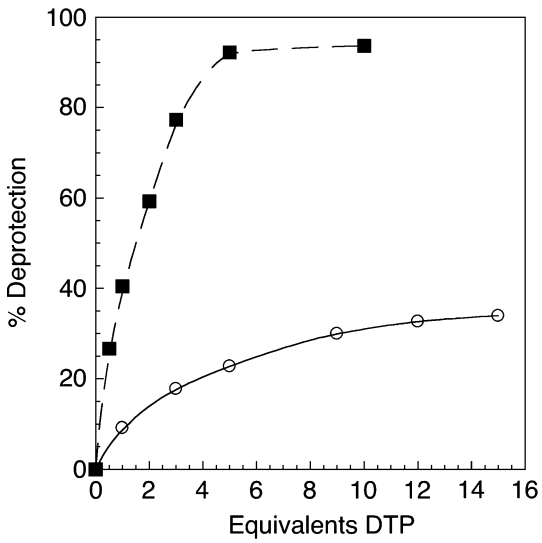

Figure 9 A plot of percent deprotection of the Mob group from Cys (closed squares) or the Acm group of Cys (open circles) *versus* equivalents of DTP added to the peptide dissolved in TFA with 2% thioanisole. The test peptides here were either VTGGC(Acm)G-OH or VTGGC(Mob)G-OH. As can be seen in the plot, DTP is a less effective deprotection reagent than DTNP under the same conditions (compare the plot in Figure 9 with the plot in Figure 8).

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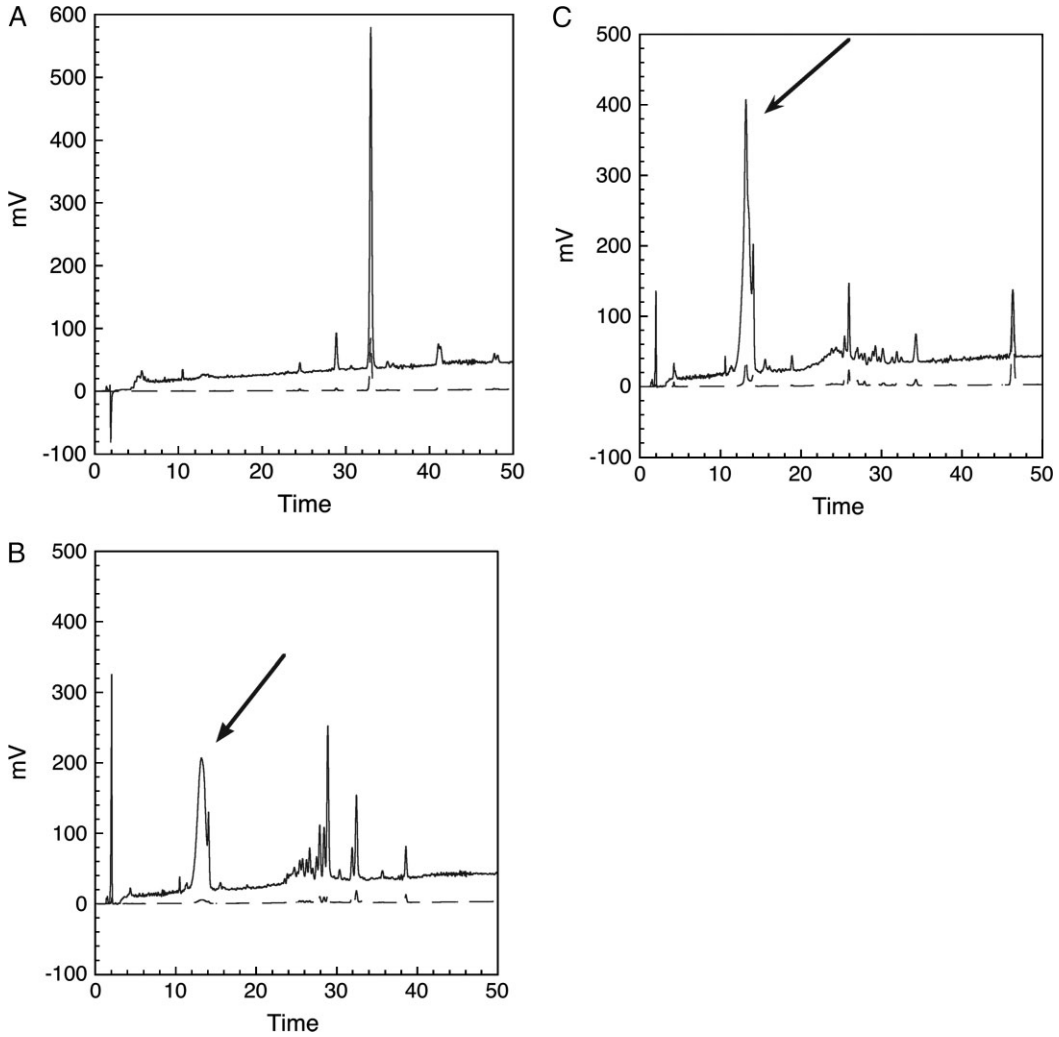

Figure 10 Selective deprotection of a Sec(Mob) residue in the presence of a Cys(Mob) residue, followed by the formation of a selenylsulfide bond. In (A), the purified peptide AEAU(Mob)GPC(Mob)K-OH is treated with TFA and incubated for 1 h at RT and then injected onto the HPLC column. A large peak at 33 min is present corresponding to the fully protected peptide [Sec(Mob) and Cys(Mob)]. In (B), the peptide has been treated with 1 equivalent of DTNP in TFA for 1 h at RT, followed by injection onto the HPLC column. A new peak centered at 13 min is present after treatment with DTNP. In (C), an aliquot of the peptide sample in B was taken after 1 h of reaction and an additional equivalent of DTNP plus 2% thioanisole was added to the aliquot. This aliquot was allowed to react for an additional hour and then injected onto the HPLC column. The peak at 13 min has grown larger while the peak at 33 min has nearly disappeared. MALDI-TOF MS analysis identified the peak at 13 min as the fully deprotected and cyclized peptide ($m/z = 824.4$) as shown in Figure 11. The solid line is the absorbance at 214 nm and the dashed line is the absorbance at 254 nm.

Cys(Mob) and Cys(Acm) derivatives that also could be exploited in future orthogonal protection/deprotection schemes. A summary of the conditions needed to effect deprotection for each derivative using DTNP is listed in Table 3.

Cyclization Reactions Involving Sec(Mob) and Cys(Mob)

Since we have shown that Cys(Mob) and Cys(Acm) residues require thioanisole for deprotection in the presence of DTNP, it should be practical to remove a Mob group from Sec in the presence of either Cys derivative, especially Cys(Acm). In the first part of this paper, we reported on a specific on-resin, cyclization reaction involving adjacent Cys(5-Npys) and Sec(Mob) residues. It is desirable to have cyclization conditions not requiring on-resin activation of Cys residues via a two-step process involving conversion of the Cys(StBu) derivative to a Cys(5-Npys) derivative by reduction using βME followed by addition of excess DTNP to the solid support. In this section, we report on conditions for direct cyclization between Sec(Mob) and Cys(Mob) without the need for an intermediary Cys derivative (conversion of Cys(StBu) to Cys(SH)). We reasoned that we should be able to form a selenylsulfide bond between Sec and Cys residue by

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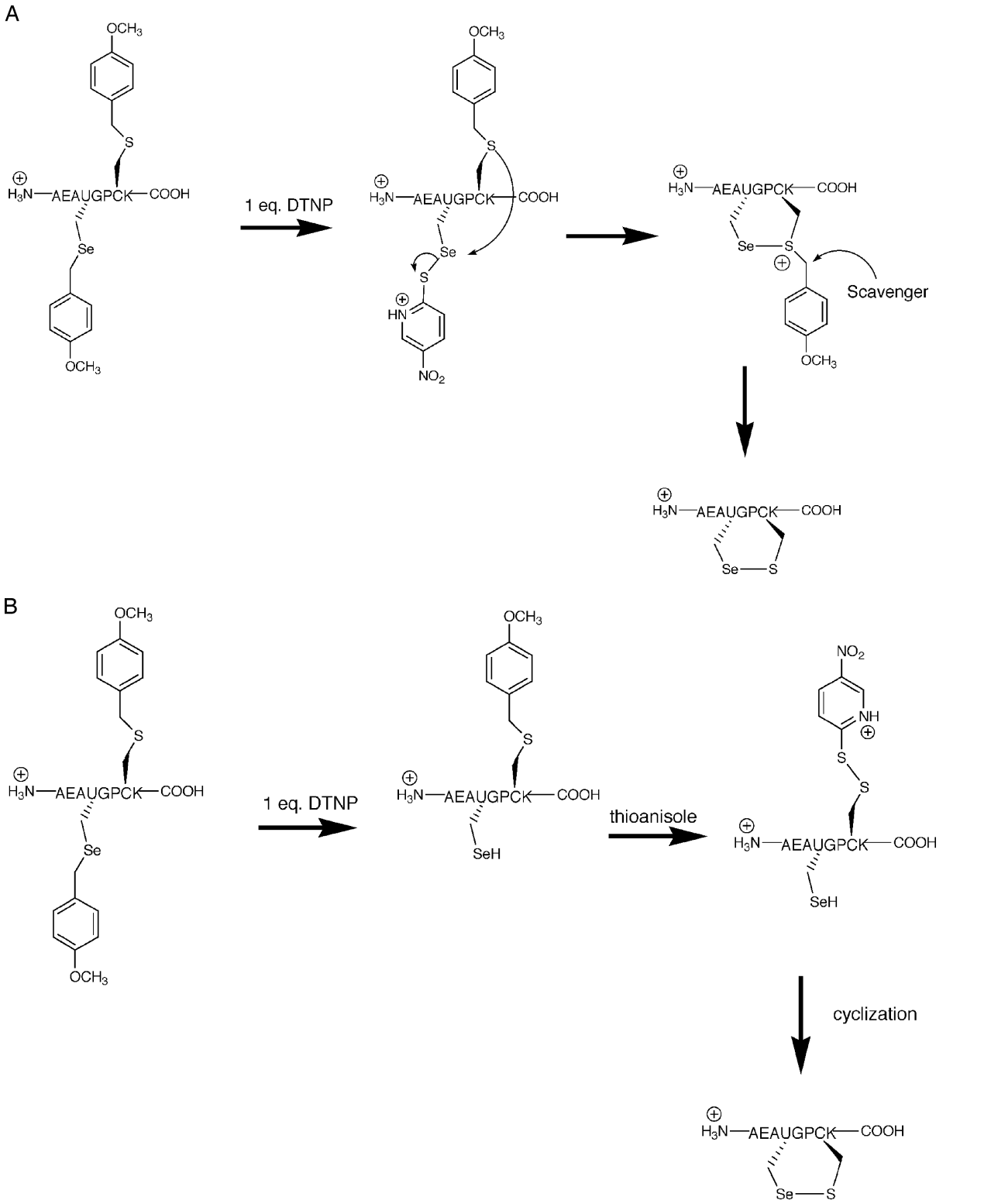

Figure 11 A postulated mechanism for selective deprotection of a Sec(Mob) residue in the presence of a Cys(Mob) residue followed by cyclization to form a selenylsulfide bond. There are two likely mechanisms for the cyclization reaction to occur. In (A), the Sec residue is deprotected with DTNP to form a Sec(5-Npys) residue. Since there is a nearby Cys(Mob) residue, the sulfur atom of Cys(Mob) can attack the highly reactive, neighboring selenylsulfide bond, with concomitant loss of the Mob group. In (B), some of the Sec residues will exist as the free selenol. The selenol would then be able to attack the disulfide of the neighboring Cys(5-Npys) residue that forms upon addition of a second equivalent of DTNP and thioanisole. In either case, the result is fully cyclic, deprotected peptide.

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first treating the peptide with a solution of TFA/DTNP. In theory, this reaction should selectively remove the Mob group from Sec, while keeping the Cys(Mob) derivative intact. Furthermore, we hypothesized that we should be able to drive cyclization with concomitant removal of the Mob group from Cys by the addition of catalytic thioanisole to the reaction containing TFA/DTNP. To test this hypothesis, we synthesized peptide AEAU(Mob)GPC(Mob)K and dissolved it in TFA with 1 equivalent of DTNP. In the example we have chosen, the Sec and Cys residue are separated by two amino acids so that we could show that there is not a requirement for adjacency. Figure 10(A)–(C) shows the course of the cyclization reaction as followed by HPLC. Analysis of the product shows that the peptide had been fully deprotected (removal of both Mob groups) and that the mass corresponds to the oxidized, cyclic form of the peptide containing a selenylsulfide bond ($m/z = 824.4$, data not shown). The near complete conversion to the cyclic product is clearly enhanced by the addition of thioanisole, which is shown by the collapse of the peak at 33 min and the growth of the product peak (comparing Figure 10(B) to 10(C)). The above example demonstrates the facile replacement of a Sec residue for a Cys residue in peptides and proteins and concomitant selenylsulfide bond formation. There are several possible pathways that could lead to the observed product using this new deprotection chemistry and these are illustrated in Figure 11.

The DTNP/TFA/thioanisole cleavage cocktail should find great use in situations where Acm is used to protect Cys because of its high stability to a wide range of conditions. For example, in the case of a peptide ligation reaction where three or more segments are to be joined together, at least one segment must be protected at the *N*-terminus with a Cys(Acm) group so that the *C*-terminal thioester can be ligated to another peptide with a free thiol. After ligation has taken place, the Cys(Acm) group must be deprotected, often with great difficulty [28]. The chemistry presented here should make the removal of the Acm group much easier.

It should be noted that van der Donk and coworkers have reported the use of 2-nitrobenzensulfenyl chloride (Nps-Cl) in 9 : 1 acetic acid/1 M HCl for the removal of the Mob group from Sec [29], which is a harsher condition than we report here. Nps-Cl and 2-pyridinesulfenyl chloride (Pys-Cl) have been used to remove Trt, Dpm, Acm, Bu^t, and StBu groups from cysteine [30–32]. However, Nps-Cl and Pys-Cl are extremely reactive, decompose in the presence of moisture, and tryptophan undergoes side reactions with these reagents [33]. Thus the use of these reagents is problematic [33]. They are not widely in use and are not among the list of reagents and methods that are recommended by Novabiochem (Novabiochem has an extensive reference section in their catalog that is widely referred to by peptide chemists. Please see pages 4.3 and 4.4 of their 2006–07 catalog for a discussion of deprotection reagents for Cys derivatives.). A solution of TFA/DTNP/thioanisole is extremely mild for side-chain deprotection with DTNP being very easy to handle and is also much less sensitive to moisture than Nps-Cl.

CONCLUSIONS

We have presented in this paper an extremely facile, and gentle method to remove Mob and Acm protecting groups from cysteine and selenocysteine after completion of SPPS. DTNP and DTP are solids and have good solubility in TFA and can thus be added to any existing cleavage cocktail. This method is simple and lacks the complications of other currently available methods for removing Mob and Acm groups, such as the use of heavy metals or the use of I_2.

This method has high utility. We have shown that we can form a selenylsulfide bond in a peptide with Sec(Mob) and Cys(Mob) residues without the need for going through an intermediary step. The method presented here has great potential for use in higher order orthogonal protection/deprotection schemes to allow the synthesis of complex peptides with many disulfide bonds [16]. We hope to further exploit the reactivity gap between Cys(Mob) and Cys(Acm) residues using DTP as a deprotection reagent, as DTP showed the largest difference in completeness of deprotection between the two derivatives.

The key property of DTNP is that it is a much milder electrophile than, for example, the iodonium cation. In fact, it has just enough reactivity to remove the Mob and Acm groups from Cys, without being reactive toward other peptidyl-nucleophiles. This milder reactivity is the basis for the success of DTNP as a deprotection reagent. This method of deprotection should find use where Sec is used as a replacement for Cys in peptides and proteins, which has become an increasingly popular choice where chemists wish to take advantage of the unique chemical properties of Sec [34,35].

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