Protocol

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On-resin conversion of Cys(Acm)-containing peptides to their corresponding Cys(Scm) congeners

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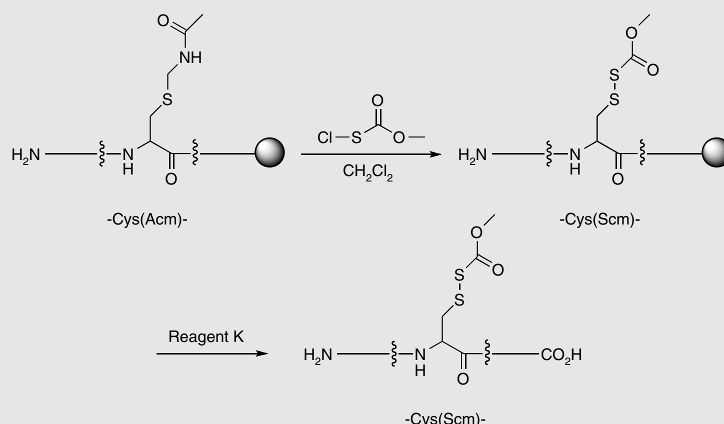
The Acm protecting group for the thiol functionality of cysteine is removed under conditions (Hg^{2+}) that are orthogonal to the acidic milieu used for global deprotection in Fmoc-based solid-phase peptide synthesis. This use of a toxic heavy metal for deprotection has limited the usefulness of Acm in peptide synthesis. The Acm group may be converted to the Scm derivative that can then be used as a reactive intermediate for unsymmetrical disulfide formation. It may also be removed by mild reductive conditions to generate unprotected cysteine. Conversion of Cys(Acm)-containing peptides to their corresponding Cys(Scm) derivatives in solution is often problematic because the sulfenyl chloride reagent used for this conversion may react with the sensitive amino acids tyrosine and tryptophan. In this protocol, we report a method for on-resin Acm to Scm conversion that allows the preparation of Cys(Scm)-containing peptides under conditions that do not modify other amino acids. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: acetamidomethyl; *S*-carbomethoxysulfenyl; Cys(Acm); Cys(Scm); on-resin conversion; methoxycarbonylsulfenyl chloride; SPPS

REACTION SCHEME



GENERAL OPTIMIZED PROCEDURE

Standard Fmoc SPPS is performed to yield fully protected, resin-bound peptide, and the *N*-terminal Fmoc protecting group is removed before Acm to Scm conversion since the Scm group is not stable to piperidine. Globally side chain protected peptide resin (0.025 mmol) is swollen in CH_2Cl_2 (2.5 mL), followed by the addition of methoxycarbonylsulfenyl chloride (0.030 mmol, 1.2 eq., 2.7 µL). The resin is allowed to tumble for 3 h, washed with CH_2Cl_2, and dried *in vacuo*. Freshly prepared Reagent K [TFA-phenol-thioanisole-water-ethanedithiol (82.5:5:5:5:2.5)] (2.0 mL) is added to the resin and the cleavage reaction is allowed to proceed for 1-2 h. The peptide is precipitated with Et_2O (40 mL) and isolated by centrifugation to form a pellet. The pellet is washed twice with Et_2O, dissolved, and purified by RP-HPLC.

Scope and Comments

The Acm protecting group for the side-chain thiol functionality of cysteine [1] is useful because it is stable to a variety of conditions used to cleave other protecting groups. The Acm group is stable to the acidic conditions (HF and TFA) used for global deprotection in Boc- and Fmoc-based chemistries. It is stable to the basic conditions used to remove the Fmoc group, to hydrazine, and

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Abbreviations used: *tBu, tert-butyl; Dc, decyl; DTT, dithiothreitol; HCTU, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate; Npys, 3-nitro-2-thiopyridine; Npys-Cl, 2-(2-nitropyridyl)sulfenyl chloride; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; Snip, S-(N-piperidylcarbamoyl)sulfenyl.*

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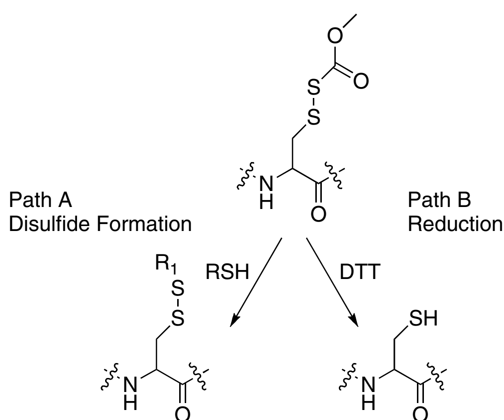
finally, to reductive (Zn in acetic acid) reaction conditions. The Acm group is typically removed by mercuric (Hg^{2+}) salts, conditions that are orthogonal to those necessary to remove Fmoc groups and *t*Bu-based protection. The necessity of using a toxic heavy metal and the difficulty in removing Hg^{2+} ions from product peptides are the main reasons that the Acm group is not more widely used in peptide chemistry.

The most common use of the Acm protecting group is for the direct oxidation of bis Cys(Acm)-containing peptides (or intermolecular variants) to the cyclic cystine derivatives by treatment with iodine [2–4]. This oxidative cyclization reaction has been shown to occur without scrambling other disulfides already present in the peptide [5]. A caveat of this reaction sequence is that the iodination of tyrosine and tryptophan residues may occur if long reaction times are necessary [3].

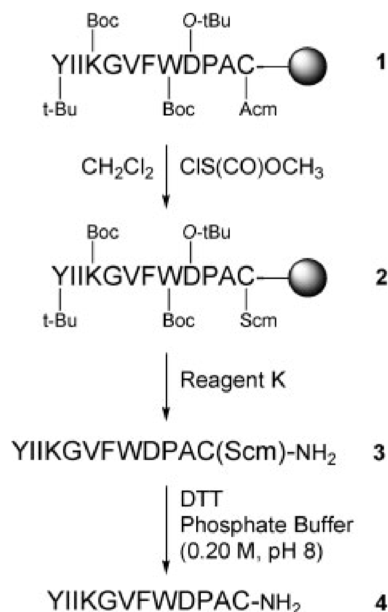
A second use of the Acm protecting group is the conversion to the *S*-carbomethoxysulfenyl (Scm) group with methoxycarbonylsulfenyl chloride. The Scm group has been used for directed unsymmetrical disulfide formation (Scheme 1) [2,6–9], and may be removed by treatment with DTT. This latter reaction sequence, conversion of Acm to Scm followed by treatment with DTT, is a milder, more environmentally benign method for removal of the Acm group. Finally, the conversion of Acm to Scm may be done without affecting other sensitive amino acid derivatives present in a peptide, such as prenylated cysteine [8].



Scheme 1. Conversion of Scm-containing peptides to unsymmetrical disulfides (Path A) or reduction to the free thiol with DTT (Path B).

The conversion of Acm to Scm in solution is often problematic. Acm-containing peptides often have very different solubilities than their Scm counterparts. We have found that methoxycarbonylsulfenyl chloride is not stable in methanol [8], a common solvent used for this conversion in the literature [6]. A large excess of sulfenyl chloride is usually used for the Acm to Scm conversion, yet sulfenyl halides are known to sulfenylate both tyrosine and tryptophan [3,10,11]. Finally, unless a peptide contains tryptophan (or other chromophore), the peptide concentration is often unknown, which makes controlling the exact stoichiometry of the reaction difficult.

A simple solution to these problems is to convert Acm to Scm while a peptide is still globally side chain-protected on the solid phase. On the solid support, peptide solubility is not an issue and stoichiometry is easy to control. Previous reports of on-resin Acm to Scm conversion in the literature have used large excesses of methoxycarbonylsulfenyl chloride [12]. We have found the conversion to proceed cleanly with only a slight excess of sulfenyl chloride reagent. These mild conditions for on-resin Acm to Scm conversion with methoxycarbonylsulfenyl chloride did not cause the modification of the Trp(Boc) and Tyr (*t*Bu) residues of peptide **1** (Scheme 2). Just as protected tyrosine, Z-Tyr(*t*Bu)-OMe, residues are not susceptible to modification by I_2 while Z-Tyr-OMe is rapidly iodinated [3], the bulky *t*Bu-based protecting groups protect tryptophan and tyrosine from sulfenylation.



Scheme 2. On-resin conversion of Cys(Acm) to Cys(Scm) and removal of the Scm group in solution by reduction with DTT.

A similar on-resin conversion of Cys(Acm) to Cys(Npys) with sulfenyl halide Npys-Cl has been reported [13]. Cys(Npys) derivatives have similar reactivity as compared to Cys(Scm). The advantage of Cys(Scm) over Cys(Npys) as an activated disulfide is that upon removal, Cys(Scm) produces COS and methanol as by-products, whereas Cys(Npys) releases 3-nitro-2-thiopyridine. The latter by-product can be difficult to separate from the product peptide by RP-HPLC. Methoxycarbonylsulfenyl chloride is also substantially cheaper than Npys-Cl. For these reasons, Cys(Scm) is a useful alternative over Cys(Npys).

Scm-containing peptides are easily obtained after standard acidic cleavage conditions with Reagent K [14], followed by RP-HPLC purification. The lyophilized powders are stable solids that need no special handling other than standard desiccated storage in a freezer. The mild and convenient procedure described here should simplify and potentially increase the utility of the Acm protecting group as an orthogonal alternative in both Fmoc and Boc SPPS.

Experimental Procedures

Materials and methods

Methoxycarbonylsulfenyl chloride was purchased from Aldrich. All solvents were of HPLC grade. DIEA and TFA were of Sequalog/peptide synthesis grade from Fisher. CLEAR® resins were purchased form Peptides International. Standard Fmoc/HCTU

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chemistry was used for SPPS of the peptides. Vydac 218TP54 and 218TP1010 columns were used for analytical and preparative RP-HPLC, respectively. All analytical and preparative RP-HPLC solvents, water and CH_3CN, contained 0.10% TFA. Retention times (T_r) are based on 1%/min linear gradients starting at 0% B (CH_3CN). The term 'overall yield' includes chain assembly, further on-resin or in solution conversions, Reagent K cleavage, and RP-HPLC purification steps.

Synthesis of YIIKGVFWDPAC(Scm)-NH_2 (3)

Y(*t*Bu)IIK(Boc)GVFW(Boc)D(*Ot*Bu)PAC(Acm)-CLEAR® amide resin (**1**) (0.025 mmol) was swollen in CH_2Cl_2 (2.5 mL). Then, methoxycarbonylsulfenyl chloride (0.030 mmol, 1.2 eq., 2.7 µL) was added. The resin was allowed to tumble for 3 h, washed with CH_2Cl_2, and dried *in vacuo*. Next, the peptide resin (**2**) was treated with freshly prepared Reagent K (2.0 mL) for 1 h. The peptide was precipitated from the cleavage solution by the addition of Et_2O (50 mL). The precipitate was centrifuged to form a pellet, which was washed twice with diethyl ether (Et_2o). The peptide pellet was dissolved in CH_3OH (5.0 mL), further diluted by the addition of 0.1% aqueous TFA (25 mL), filtered, and purified by preparative HPLC on the C18 column. Overall yield: 26%, 10.4 mg, $T_r = 37.4$ min. Purity by HPLC: 90%. ESI-MS: calculated 1499.7, found 1499.6.

Removal of Scm in solution to produce YIIKGVFWDPAC-NH_2 (4)

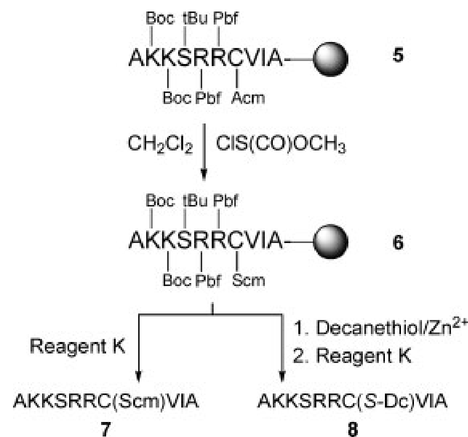
To the crude peptide (**3**, obtained from 0.025 mmol peptide resin) dissolved in CH_3OH (5.0 mL) was added 2.0 mL of 0.20 M phosphate buffer, pH 8.0. Sufficient H_2O was added to dissolve any precipitated phosphate. DTT (0.25 mmol, 10 eq., 38 mg) was added and the solution stirred for 10 min. The pH was adjusted to about 3 by the addition of neat TFA. The peptide solution was filtered and purified by HPLC on the C18 column. Overall yield: 18%, 6.2 mg, $T_r = 35.0$ min. Purity by HPLC: 94%. ESI-MS: calculated 1409.7, found 1409.6.

Synthesis of AKKSRRC(Scm)VIA (7)

AK(Boc)K(Boc)S(*t*Bu)R(Pbf)R(Pbf)C(Acm)VIA-Wang resin (**5**) (0.025 mmol) was swollen in CH_2Cl_2 (2.5 mL). Then, methoxycarbonylsulfenyl chloride (0.030 mmol, 1.2 eq., 2.7 µL) was added. The resin was allowed to tumble for 3 h, washed with CH_2Cl_2, and dried *in vacuo* to yield compound **6**. Next, the peptide resin was treated with freshly prepared Reagent K (2.0 mL) for 2 h. The peptide was precipitated from the cleavage solution by the addition of Et_2O (50 mL). The precipitate was centrifuged to form a pellet, which was washed twice with Et_2O. The peptide pellet was dissolved in 0.1% aqueous TFA (25 mL), filtered, and purified by preparative HPLC on the C18 column. Overall yield: 50%, 30.5 mg, $T_r = 25.4$ min. Purity by HPLC: 98%. ESI-MS: calculated 1220.6, found 1220.6.

On-resin directed disulfide formation, AKKSRRC(S-Dc)VIA (8)

AK(Boc)K(Boc)S(*t*Bu)R(Pbf)R(Pbf)C(Scm)VIA-Wang resin (**6**) (0.025 mmol), was swollen in DMF (1.6 mL). In a test tube was dissolved $Zn(OAc)_2 \cdot 2H_2O$ (0.125 mmol, 5 eq., 27 mg) in 0.1 M acetate buffer, pH 5.0 (0.40 mL). This solution was added to the peptide resin. Then, decanethiol (0.025 mmol, 1.0 eq., 5.1 µL) was added, and the solution allowed to tumble for 48 h. The peptide was cleaved from the resin with freshly prepared Reagent K for 2.5 h. After precipitation with Et_2O and washing the pellet as described earlier, the pellet was dissolved in about 5 mL of DMF, filtered, and purified on the C18 column. Overall yield: 9%, 3.0 mg, $T_r = 43.7$ min. Purity by HPLC: 95%. ESI-MS: calculated 1302.8, found 1302.8 (Scheme 3).



Scheme 3. On-resin unsymmetrical disulfide formation after Cys(Acm) to Cys(Scm) conversion.

Limitations

The data presented here, together with previously reported examples [8], suggest that this is a robust and versatile procedure. However, there are two issues which require discussion.

A reviewer of this work has pointed out that sulfenyl chlorides are known to react with free amino groups to form sulfenamide derivatives [4]. Any such resulting sulfenamides would be cleaved by Reagent K, and not be found in the product. This side reaction would consume the methoxycarbonylsulfenyl chloride reagent, which our procedure uses only in a small excess. As a result, one might expect a significant amount of unreacted Cys(Acm)-containing peptide in the crude product isolated after Reagent K cleavage. For the two examples of on-resin Cys(Acm) to Cys(Scm) conversion discussed here, analytical HPLC analysis of crude YIIKGVFWDPAC(Scm)-NH_2 (**3**) revealed none (<1%) of the corresponding YIIKGVFWDPAC(Acm)-NH_2 peptide, whereas crude AKKSRRC(Scm)VIA (**7**) did contain untransformed AKKSRRC(Acm)VIA (about 25%). From these data, we conclude that the sulfenyl chloride reagent, which is a soft electrophile, is kinetically much more reactive toward the corresponding soft sulfur atom of the Cys(Acm), as compared to the hard nucleophilic *N*-terminal amino group. Previous examples of this conversion on amino acids and peptides with unprotected amines have always started with trifluoroacetate or hydrochloride salts [8,15]. For a case such as peptide **7** where incomplete conversion of Cys(Acm) to Cys(Scm) is observed, it may be useful to wash the peptide resin with a dilute solution of acetic acid prior to treatment with methoxycarbonylsulfenyl chloride, to ensure that the amine is further deactivated by protonation.

The outcomes of on-resin reactions of the Scm group, either reduction with DTT or disulfide exchange with alkanethiols, are variable. An attempt to reduce Y(*t*Bu)IIK(Boc)GVFW(Boc) D(*Ot*Bu)PAC(Scm)-CLEAR® amide resin (**2**) with ten equivalents

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of DTT in DMF followed by Reagent K cleavage gave the Scm-containing peptide (**3**) as the main product. Initially, this result was surprising as the related Snip protected cysteine has been successfully reduced with DTT while still on resin [16]. However, in that case the Cys(Snip) residue was located at the N-terminus of the sequence, rather than at the C-terminus as reported here. Attempts at directed on-resin formation of unsymmetrical disulfides have also given variable results. When peptide AK(Boc)K(Boc)S(*t*Bu)R(Pbf)R(Pbf)C(Scm)VIA-Wang resin (**6**) was reacted with decanethiol in the presence of Zn^{2+} to promote disulfide exchange [8,17], the desired AKKSRRC(*S*-Dc)VIA peptide (**8**) was the main product found after Reagent K cleavage. But when the Cys(Scm)-containing CLEAR® amide resin (**2**) was treated with decanethiol under the same reaction conditions, YIIKGVFWDPAC-NH_2 (**4**) was the main product found after cleavage. The location of the Cys(Scm) residue in the sequence, and possibly the flanking residues, has a strong influence on the outcome of on-resin reactions of the Scm group. For these reasons, we prefer to first cleave peptides from the resin, and then react the Scm group in solution. As previously reported for Cys(Snip) [16], the activated disulfides of Scm-protected peptides are stable to Reagent K.

Acknowledgement

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