Highly Efficient Synthetic Method on Pyroacm Resin Using the Boc SPPS Protocol for C-terminal Cysteine Peptide Synthesis

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A very effective process on Pyroacm resin was developed for solid-phase peptide synthesis (SPPS) of C-terminal cysteine and cysteine ester peptides. The process uses cysteine side chain anchoring to the Pyroacm resin and the Boc protocol for SPPS. The Pyroacm resin showed remarkable stability under standard trifluoromethanesulfonic acid (TFMSA) cleavage condition. TFMSA cleavage of protecting groups generates a peptide-linked resin, which can be subjected to peptide modification reactions. Finally, the peptide can be cleaved from the resin using methoxycarbonylsulfenyl chloride. The utility of this protocol was demonstrated by its applications to the synthesis of model peptides, key intermediates in the preparation of natural products riparin 1.2 and a-factor.

Keywords: Side chain anchoring, Solid-phase peptide synthesis, C-terminal cysteine, Thiol-sulfenyl protection, β-Elimination

Introduction

C-terminal cysteine and cysteine ester peptides hold a prominent position in peptide chemistry because of their wide occurrence in nature and applications as important bioactive agents.[1] Consequently, the development of methods to form solid-phase synthesis of these peptides is an important component of studies in this area. Boc-based solid-phase peptide synthesis (SPPS) found instant acceptance in the early years of peptide chemistry owing to its simplicity and adaptability to automation.[2,2a–c] However, the need for strong acids (*e.g.*, Hydrofluoric acid (HF)) for the final cleavage step requires the use of special expensive apparatus in order to account for safety issues.[2d,e] Use of HF in the final cleavage process also affects the ability to tag peptides with certain types of fluorophores or saccharides (oligo). As a result of these drawbacks, the Fmoc SPPS procedure, in which the Fmoc group is readily cleaved using mild base (piperidine) treatment followed by treatment with a Trifluoro acetic acid (TFA) cocktail,[3] has become prominent.

Nevertheless, the synthesis of peptides containing C-terminal cysteine and cysteine esters employing the Fmoc SPPS method remains problematic. For example, in most cases, these peptides undergo ready β-elimination and racemization (*ca.* 0.5%) during the piperidine treatment step.[4] In recent years, a few protocols to avoid the formation of C-terminal dehydroalanine β-elimination induced elimination have been developed including masking the C-terminal carboxylic group (Figure 1, **1**),[5] and anchoring the cysteine side chain through a trityl (**2a**),[6,6a,b] chlorotrityl (**2b**),[6b] Xanthenyl amide linker (XAL) (**2c**),[6c] or Pyroacm-based resins (Figure 1, **2d**).[6d]

Unfortunately, the masked carboxylic acid group approach is not compatible with cysteine ester peptide synthesis and the side chain anchoring method displays decreased yields owing to piperidine-promoted β-elimination and racemization. Finally, a technique, which involves initial preparation of the peptide sequence attached to the C-terminal cysteine followed by introduction of the cysteine using native chemical ligation methods, has been developed.[7] However, this protocol requires additional chemical steps.

In the previous efforts, we designed and synthesized an acetamidomethyl (Acm)-derived resin named Pyroacm resin (**2d**). In addition, we demonstrated that the new resin can be used for anchoring cysteine through its side chain thiol as part of an Fmoc protocol for synthesis of C-terminal cysteine peptides.[6d] Furthermore, we found that the inexpensive and mild reagent methoxycarbonylsulfenyl chloride (ScmCl) can be used for resin cleavage to produce the crude peptide with a high degree of purity.[8] However, prolong treatment with piperidine in the Fmoc removal step was found to result in decreased yields as a result of β-elimination. And also, during Fmoc deprotection of second residue leads to diketopiperazine (DKP) side reaction. To avoid the use of extended piperidine treatment required in the Fmoc protocol and to lower the atom economy of this approach, we continued in detail study of the Boc protocol in conjunction with the Pyroacm resin for the synthesis of peptides containing C-terminal cysteine and cysteine esters. In addition, to make the process have wide-ranging applications, we searched for an alternate to HF for promoting the Boc-based SPPS.[9] This effort led to the discovery that trifluoromethanesulfonic acid (TFMSA) is viable alternative to HF for this purpose.[10] As TFMSA does not require the use of a specialized apparatus, it compatible

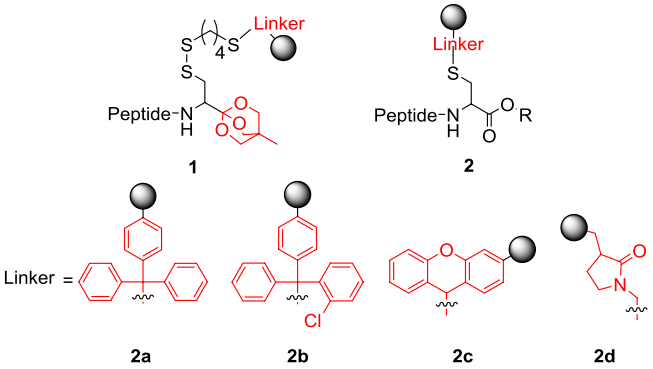


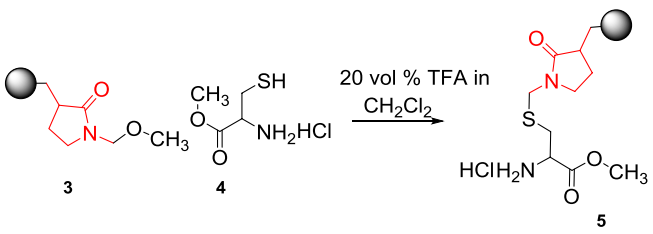
Figure 1. Strategies for C-terminal cysteine peptide synthesis.

with both small- and large-scale SPPS. Below, we describe the results of this investigation, which have led to the development of Boc and Pyroacm-based SPPS using both a one and two step cleavage protocol for the preparation of C-terminal cysteine peptides.

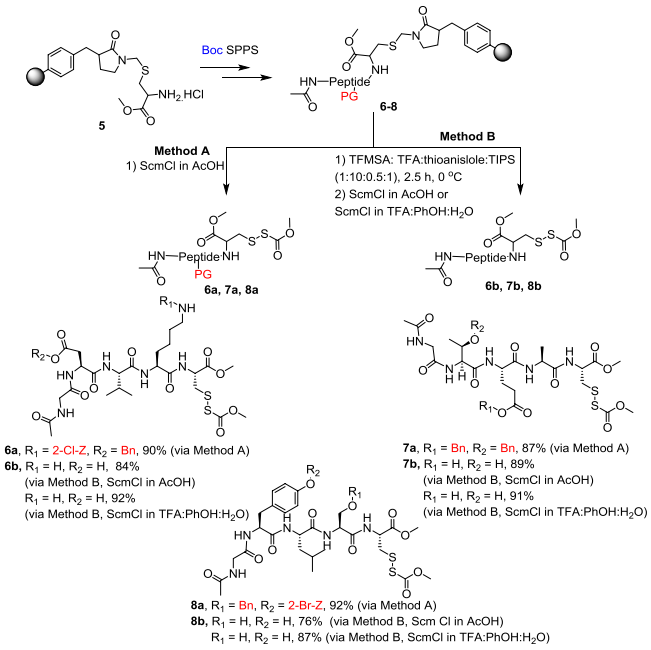
Results and Discussion

In order to evaluate the proposals presented above, we first immobilized HCl.H-Cys-OMe **4** (4 equiv) on Pyroacm resin **3** by using acidic conditions involving 20 vol % of TFA in CH_2Cl_2 for 2 h (Scheme 1). The extent of Cys-OMe loading in **5** was determined to be approximately 0.83 mmol/g using quantitative ninhydrin analysis. The ATR-FTIR spectrum of **5** contains a sharp peak at 1748 cm^{-1} (see Supporting Information) corresponding to the ester group in the amino acid linked to the resin.

To examine its uses, the Cys-OMe-linked resin **5** was employed to prepare resins containing the model pentapeptides **6**, **7**, and **8** utilizing the Boc SPPS protocol (Scheme 2). Method A, in which cleavage from the corresponding resins **6**, **7**, and **8** utilizing 2 equiv of ScmCl in AcOH gave the fully protected pentapeptides **6a**, **7a**, and **8a** in high yields and purities. In addition, Method B (in Scheme 2), we examined the procedure in which deprotection peptide side chain was carried out prior to cleavage from the resin. This process was performed on the Pyroacm-linked, protected pentapeptides **6**, **7**, and **8** using the standard TFMSA deprotection conditions with 1:10:1:0.5 TFMSA:TFA:thioanisole: Triisopropyl silane (TIPS) ratio for 2.5 h at 0°C. Under these conditions, the Pyroacm resin showed excellent stability. Finally, **6b**, **7b**, and **8b** (Scheme 2) are generated efficiently



Scheme 1. Anchoring cysteine onto the resin.

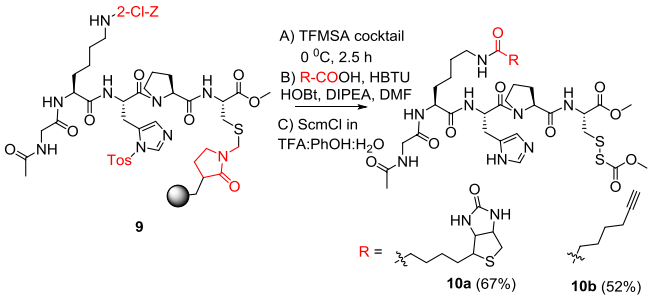


Scheme 2. Model peptides on resin using Pyroacm resin.

(76–84%) with high degrees of purity by cleavage from the resin using ScmCl in AcOH. When a 18:1:1 mixture of TFA: PhOH:H_2O containing 2 equiv of ScmCl was used for the cleavage process, the unprotected peptides were produced in yield in the 87–91% range.

Post-modification of peptides, such as the introduction of functional groups, taking place while they are attached to resins is typically efficient because it avoids difficulties associated with purification, characterization of intermediates, and extensive synthetic operations. In order to determine the effectiveness of this approach, model Pyroacm-linked peptide containing a Lys(2-Cl-Z) residue **9** (in Scheme 3) was prepared and subjected to the standard TFMSA deprotection conditions to obtain resin intermediate **10** (see Figure S1, Supporting Information). Introduction of a biotin or acetylene group was then carried out through use of lysine amide bond forming reactions. Finally, cleavage of both resins promoted using ScmCl in general TFA condition generated the respective biotin and acetylene modified peptides **10a** and **10b** in respective yields 67 and 52% after preparative High performance liquid chromatography (HPLC) purification.

In an earlier effort, we demonstrated that an Fmoc-based SPPS can be used to prepare riparin 1.2 in high purity but in low yield caused by competing β-elimination in the piperidine induced Fmoc removal step.[6d,11] In the current study, we probed the comparative efficiencies of Pyroacm resin approaches to intermediate of riparin 1.2 peptide **13** that utilize Boc and Fmoc-based SPPS methods. For this purpose, the Cys-allyl-linked resin **11** (Scheme 4) was prepared and subjected to the Boc and Fmoc protection and peptide assembly sequences. The allyl groups in both peptides prepared in this manner were cleaved using palladium to form intermediates **12a** and **12b**. Upon being subjected



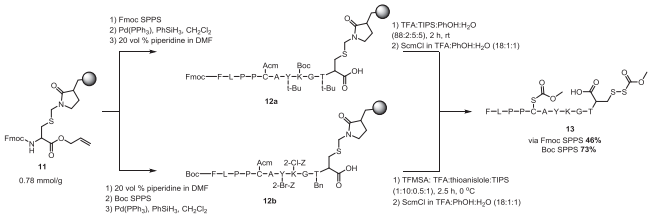
Scheme 3. Pre-cleavage modification on the resin.

to the two step protocol using first TFMSA and then ScmCl in TFA:PhOH:H_2O, the linked Boc-protected peptide **12b** produced polypeptide **13** in a 73% yield after preparative HPLC (Scheme 4). In contrast, the yield of **13** generated using the Fmoc protection and removal was 46%.

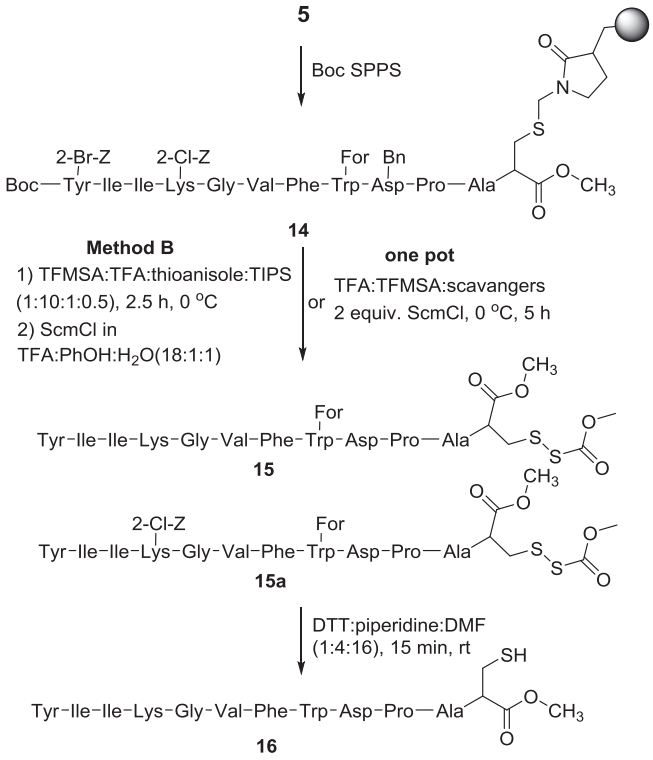
The two step Boc-based protocol described above was employed to synthesize the key intermediate **16** (Scheme 5) in the pathway employed for the preparation of the a-factor, which contains a tryptophan residue.[12] Typically, unprotected tryptophan residues in peptides undergo sulfenylation under ScmCl cleavage conditions.[13] However, we observed incorporation of a formyl protecting group on the tryptophan residue in the Pyroacm-linked peptide **14** prevents this process. Consequently, Method B in which treatment of **14** with first TFMSA and then ScmCl in TFA:PhOH:H_2O efficiently (83%) generated the highly pure *S*-sulfenyl formyl-protected peptide **15**. Peptide **16** then produced with high crude purity using a one-pot reaction involving treatment of **15** with 5 vol % DTT in piperidine:DMF (1:5) (Scheme 5). The crude purified via HPLC to obtain pure **16** (Figure 2).

The one-pot cleavage procedure can be employed to form the side chain deprotected target **15** directly from **14** (Scheme 5). However, we observed that treatment of **14** with a 9:1 mixture of TFA:TFMSA containing 2 equiv of ScmCl generated **15** in only a 9% crude purity along with lysin (2-Cl-Z) containing peptide **15a** (Scheme 5) in a 91% crude purity (Table 1, entry 1). Addition of 10 vol % of scavengers such as thioanisole or dimethylsulfide (DMS) resulted in deactivation of ScmCl (entries 2 and 3).

Although addition of 10 vol % anisole or 10 vol % *p*-cresol as scavengers did not result in deactivation of ScmCl, **15a** was still the major product (71 and 75%, respectively, entries 4 and 5) in crude. Likewise, the



Scheme 4. Comparison study for synthesis of riparin 1.2 intermediate on Pyroacm resin with Boc vs. Fmoc protocols.



Scheme 5. Synthesis of a key intermediate in the preparation of a-factor.

presence of 10 vol % TIPS does not lead to an improvement of the efficiency of the process (entry 6). Finally, we found that increasing the ratio of TFMSA in the cleavage cocktail led to generation of **15** in a highly pure state (Table 1, entries 7 and 8). The main drawback of one-pot cleavage process utilizes 70 vol % TFMSA (entry 8) due to Lys(2-Cl-Z) can be avoided, if orthogonal Lys(Z) or Lys(Alloc) side chain protection could be exploited instead of Lys(2-Cl-Z) in which Lysine side chain protective group can easily cleaved prior to one-pot cleavage process

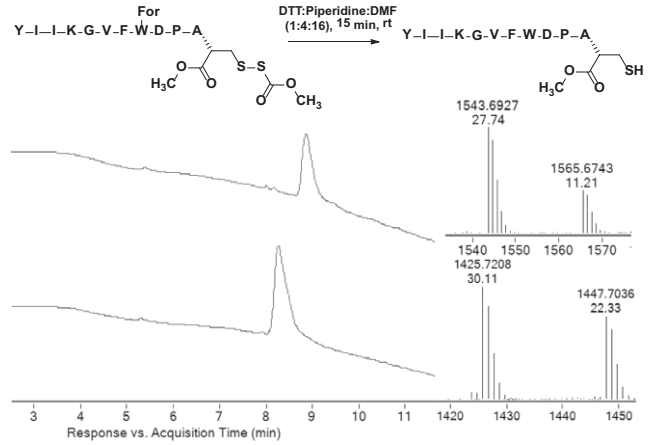


Figure 2. Liquid chromatography mass spectrometer (LCMS) chromatogram of purified **16** (Retention time (RT) = 8.4 min) after the addition of 5 vol % of DTT to **15** (RT = 8.9 min) in piperidine:DMF (1:4).

Table 1. One-pot cleavage cocktail to obtain fully deprotected Scm-peptide.[a]

Entry	Cocktail composition	Ratio (%)[b] **15**:**15a**	Yield (%)[c]
1	TFA:TFMSA (9:1)	9:91	49
2	TFA:TFMSA:thioanisole (8:1:1)	—	Traces
3	TFA:TFMSA:DMS (8:1:1)	—	Traces
4	TFA:TFMSA:anisole (8:1:1)	29:71	57
5	TFA:TFMSA:*p*-cresol (8:1:1)	25:75	56
6	TFA:TFMSA:TIPS (8:1:1)	38:62	19
7	TFA:TFMSA:anisole (5:4:1)	89:11	52
8	TFA:TFMSA:anisole (2:7:1)	99.7:0.3	46

[a] Reactions carried out on 0.01 mmol scale.
[b] Percent product and byproducts in the crude mixtures (small peaks in HPLC have been ignored).
[c] Yields after preparative HPLC.

(Scheme 5). Resulting resin intermediate can be subjected to condition (Table 1, entry 4), which involves 10 vol % of TFMSA, to obtain target peptide with high crude purity.

Conclusion

In the study described above, we developed a mild protocol for SPPS of terminal cysteine and cysteine ester containing peptides in high purities. Moreover, we observed the Pyroacm resin showed excellent stability under standard TFMSA cleavage condition, which is useful for on resin peptide modification. The developed method eliminates unwanted β-elimination and racemization reactions that occur when using the Fmoc protocol. In addition, we observed that formyl groups on formyl-protected tryptophan residues in peptides are cleaved during the disulfide bond reduction process. Surprisingly, during the one-pot cleavage process, the stability of formyl group on tryptophan and very high cleavage potential of ScmCl observed even in the presence of the high concentrations of TFMSA. Finally, the one-pot cleavage process can be utilized in conjunction with Boc-based SPPS protocol employing the Acm protecting group, thus avoiding the need for tedious processes utilizing metals such as Hg^{2+}, Tl^{3+}, and Ag^{+}.[14]

Experimental

General Information. All reactions were conducted in flame- or oven-dried glassware under an atmosphere of argon. SPPS was carried out manually in a syringe containing a polyethylene frit. Solvents and soluble reagents were removed by using suction. Infrared (IR) spectra were recorded on a Smiths IdentityIR ATR-FTIR spectrometer. A Scinco S4100 PDA UV–vis spectrophotometer was used to record UV spectra. An Agilent 1260 infinity instrument, equipped with an Eclipse plus-C18 reversed-phase analytical column (4.6 mm × 250 mm, 5 μM), was used for HPLC analysis. HPLC separation used a flow rate of 1 mL/min and 40 min method, which involves an isocratic mixture of water: Acetonitrile (ACN): (95:5) containing 0.1% TFA for 5 min, gradient raised from 5% to 45% of ACN (0.1% TFA) from 5 to 30 min and from 30 to 40 min, and the gradient raised from 45% to 90% of ACN (0.1% TFA). An Agilent 6460 triple Quad LCMS coupled to an Agilent 1290 infinity LC instrument equipped with a Poroshell 120 EC-C18 analytical reversed-phase column (3.0 mm × 50 mm, 2.7 μm) was used for analysis. Linear gradients of ACN into water (5 mM NH_4HCO_3) were used at a flow rate of 0.3 mL/min in the ESI mode. An Agilent 6550 iFunnel Q-TOF coupled to an Agilent 1290 infinity II LC spectrometer, equipped with a Poroshell 120 EC-C18 analytical reversed-phase column (3.0 mm × 50 mm, 2.7 μm), was used for High resolution mass spectrometer (HRMS) measurements. Linear gradients of ACN into water (5 mM NH_4HCO_3) were used at a flow rate of 0.4 mL/min and a run time of 7 min in the ESI mode. All LC eluents were monitored at 220 nm. An Ilshin Biobase Co. Ltd, freeze dryer (Gyeonggi-do, Republic of Korea) was used for lyophilization. ^{1}H NMR spectra were recorded on a Bruker Ascend™500 instrument (Bruker, MA, USA) at 500 MHz. Residual chloroform (7.26 ppm) was used as an internal standard. ^{13}C NMR spectra were measured at 126 MHz with residual chloroform (77.16 ppm) as an internal standard. A Waters PrepHPLC containing Waters 2525 Binary Gradient System with a Waters 2487 dual wavelength UV–vis detector and reverse-phase column (19 mm × 250 mm, 5 μM) was used to purify peptides. Analytical thin layer chromatography (TLC) was performed using plates coated with a 0.25 mm thickness of silica gel, and compounds were visualized using UV light, iodine, or $KMnO_4$ staining. Normal phase flash chromatography was performed on an automated MPLC of Biotage IsoleraOne Instrument (Biotage, Uppsala, Sweden) (silica gel 230–400 mesh size).

SPPS Using the Boc Protocol. Resin was swollen in DMF for 10 min. Pre-activation of the amino acid involves HBTU/HOBt/DIPEA (4/4/8) treatment of 0.4 M Boc-protected amino acids (4.0 equiv) in DMF for 2 min. In each case, the mixture was taken into the resin and the syringe was tumbled on a shaker at 25°C and 160 rpm for 1 h. The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), and CH_2Cl_2 (5 × 1 min). A small portion of the resin was assayed using the Kaiser test. In the case of a positive test, the process was repeated. Removal of the Boc group was achieved by treatment of

the resin with 50 vol % TFA in CH_2Cl_2 containing 0.5% TIPS for (1 × 5 min) and (1 × 15 min). Washings between deprotection and coupling steps were performed with CH_2Cl_2 (5 × 1 min) and DMF (5 × 1 min). *In situ* neutralization Boc-chemistry for SPPS protocol was followed. After completion of the final coupling or deprotection step, the resin was washed with CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), and dried under high vacuum.

SPPS Using the Fmoc Protocol. Resin was swollen in DMF for 10 min. Amino acids were pre-activated for 2 min by HBTU/HOBt/DIPEA (4/4/8) treatment of 0.4 M Fmoc-protected amino acids (4.0 equiv) in DMF. In each case, the mixture was added to the resin and the syringe tumbled on a shaker for 1 h at 25°C and 160 rpm. The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), and DMF (5 × 1 min). A small portion of the resin was assayed using the Kaiser test. In the case of a positive test, the process was repeated. Removal of Fmoc groups was achieved by treatment of the resin with 20 vol % piperidine in DMF (2 × 10 min). Washings between deprotection and coupling steps were performed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), and DMF (5 × 1 min). Following the final coupling or deprotection step, the resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), and dried under high vacuum.

Removal of the Allyl Group. To the resin swollen in 50× dry CH_2Cl_2 for 15 min under argon atmosphere was slowly added *via* syringe $Pd(PPh_3)_4$ (0.75 equiv) and Ph_3SiH (30 equiv) in 20× CH_2Cl_2. The resin was tumbled in a shaker for 1.5 h at 25°C, 160 rpm and washed with CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), and dried under high vacuum. The process was repeated twice.

Two Step Cleavage Process

Standard TFMSA Deprotection Conditions. The peptide resin was placed in a glass vial with a stirring bar. Thioanisole/TIPS (2:1) was added (3 mL/g) and following placement of the glass vial in an ice bath, TFA (20 mL/g) was added. The mixture was stirred for 10 min and 2 mL of TFMSA (2 mL/g) was added very slowly. The mixture stirred for 2.5 h at 0°C and filtered to give the resin, which washed with TFA and CH_2Cl_2 (10 × 1 min) and dried under high vacuum.

General Cleavage Procedure Comprised of ScmCl in AcOH. To the resin (0.01 mmol) in 0.75 mL of AcOH (let resin swell for 2 min) in a syringe with a polyethylene frit was added 2 equiv of ScmCl (per Acm mimic) in 0.05 mL of AcOH. The syringe was tumbled at 25°C and 160 rpm on a shaker for 5 h. The resin was separated by using filtration and washed with AcOH (1 × 1 mL) and H_2O (1 × 1 mL). The filtrate was diluted with water (made 20% aqueous) and lyophilized to obtain the crude product, which was analyzed using HPLC and LCMS.

General Cleavage Procedure Using the General ScmCl in TFA Condition. To 0.01 mmol resin in a glass vial with a stirring bar was added a 1:1 mixture of water and phenol (3 mL/g) followed by TFA (30 mL/g). The mixture was stirred for 5 min and 2 equiv of ScmCl (per Acm mimic) in TFA was added very slowly. The mixture was stirred for 4 h at RT and filtered. The resin was washed with TFA, concentrated under reduced pressure, and precipitated using cold diethyl ether. The precipitate was centrifuged and lyophilized to obtain the crude peptide, which subjected to HPLC and LCMS analysis.

One-Pot Cleavage Protocol. To the peptide resin in a glass vial was added anisole (3 mL/g). The mixture was placed in an ice bath and TFA (30 mL/g) was added. The mixture was stirred for 10 min and TFMSA (3 mL/g) was added very slowly, followed by 2 equiv of ScmCl (per Acm mimic) in TFA. The mixture was stirred for 5 h at 0°C and filtered into cold diethyl ether. Centrifugation followed by lyophilization gave crude peptide, which subjected to HPLC and LCMS analysis prior to preparative HPLC purification.

Experimental Details

1-(Methoxymethyl)pyrrolidin-2-one (2e). To pyrrolidon-2-one (25 g, 0.294 mol) in 1 M KOH (0.01 equiv) at RT was slowly added 36% aqueous formaline (24.5 mL, 0.294 mol). The mixture was stirred at RT for 2 h, and concentrated under reduced pressure. The crude solid containing 1-(hydroxymethyl)pyrrolidin-2-one (11.5 g, 0.1 mol) was dissolved in THF (575 mL), and then NaH (4.4 g, 0.11 mol) was added in portions at 0°C. When H_2 evolution ceased, MeI (6.85 mL, 0.11 mol) was added dropwise. The mixture was stirred at 0°C overnight. After the consumption of starting material, MeOH (5 mL) was added dropwise to quench unreacted NaH. The mixture was concentrated under reduced pressure giving a residue that was mixed with CH_2Cl_2 (200 mL). The organic layer was filtered, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was subjected to flash silica chromatography eluting with a gradient of CH_2Cl_2:methanol (100:0 → 90:10) to yield the product **2e** as a pale yellow liquid (10.96 g, 84%). R_f = 0.25 (CH_2Cl_2:methanol, 9.5:0.5) ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.69 (s, 2H), 3.49 (t, J = 7.1 Hz, 2H), 3.29 (s, 2H), 2.45 (t, J = 8.1 Hz, 2H), 2.13–2.03 (m, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.3, 74.0, 56.1, 46.0, 31.2, 18.1.

Pyroacm Resin (3). To a solution of 1-(methoxymethyl) pyrrolidin-2-one (4.8 g, 37.07 mmol) in dry THF (90 mL) under an argon atmosphere was added 2 M LDA (18.5 mL, 37.07 mmol) at −78°C. The mixture was raised above the cooling bath for 10 min and then cooled to −78°C. After 10 min, 2.2 mmol/g of Merrifield resin (3.37 g, 7.41 mmol, 100–200 mesh size) was added in three portions with 5 min interval between additions. The mixture is stirred at −78°C and 200 rpm for 1 h and then the temperature was increased slowly to 25°C. The mixture was stirred for 4 h before the temperature was reduced to −20°C and 10% aqueous NH_4Cl was slowly added. The mixture was filtered and the resin was washed with THF (5 × 50 mL), DMF

(5 × 50 mL), and CH_2Cl_2 (5 × 50 mL) and then dried under high vacuum for overnight.

MeO-Cys-NH₂·HCl (4). Acetyl chloride (16 mL, 220 mmol) was added to MeOH (100 mL) and the solution was cooled to 0°C. The solution stirred for 5 min. Cysteine (2.42 g, 20 mmol) was added to reaction mass and stirred at RT for 36 h under argon atmosphere. The reaction was evaporated under reduced pressure, added ether, and the solid thus obtained was filtered and washed with ether. Then dried under high vacuum to yield the product **4** as a pale green amorphous solid (3.28 g, 94%). ^{1}H NMR (500 MHz, MeOD) δ 4.34 (m, 1H), 3.83 (s, 3H), 3.09 (m, 2H). ^{13}C NMR (126 MHz, MeOD) δ 169.1, 55.7, 53.9, 25.1.

Anchoring Cysteine onto the Resin (5). To Pyroacm resin **3** (1 g, 1.82 mmol) under argon atmosphere was added **4** (1.25 g, 7.28 mmol) in CH_2Cl_2 (30 mL). After 15 min, 8 mL of TFA was slowly added. The mixture was shaken for 2 h before being filtered. The resin was washed with CH_2Cl_2 (5 × 20 mL), DMF (5 × 20 mL), and CH_2Cl_2 (5 × 20 mL). Loading of **5**, calculated using Ninhydrin-quantitative analysis, was found to be 0.83 mmol/g.

Ac-Gly-Asp(Bn)-Val-Lys(2-Cl-Z)-Cys(Scm)-OCH₃ (6a). Model pentapeptide **6a** synthesis started with **5**. Subsequent coupling with Boc-Lys(2-Cl-Z)-OH, Boc-Val-OH, Boc-Asp (Bn)-OH, and Ac-Gly-OH was carried out *via* the standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. *Cleavage via ScmCl in AcOH*: Resin **6** (0.011 mmol) was subjected to the general cleavage conditions using ScmCl in AcOH to obtain **6a**. The crude product was analyzed using HPLC and LCMS, and purified by using prepHPLC. This process yielded **6a** as a white solid (9.2 mg, 90%). ESI-MS: for $C_{40}H_{53}ClN_6O_{13}S_2$ $[M + H^+]^+$; cald 925.2874, found 925.2893 $[M + Na^+]^+$; cald 947.2693, found 947.2684.

Ac-Gly-Asp-Val-Lys-Cys(Scm)-OCH₃ (6b). Model pentapeptide **6b** synthesis started with **5**. Subsequent coupling with Boc-Lys(2-Cl-Z)-OH, Boc-Val-OH, Boc-Asp(Bn)-OH, and Ac-Gly-OH was carried out *via* the standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. 0.025 mmol resin **6** subjected to the standard TFMSA deprotection condition to remove side chain protective groups. *Cleavage via ScmCl in AcOH*: Resin (0.0114 mmol) was subjected to the general cleavage conditions using ScmCl in AcOH to obtain crude **6b**. The crude product was analyzed using HPLC, LCMS and purified by using prepHPLC. This process yielded **6b** as a white solid (6.4 mg, 84%). ESI-MS: for $C_{25}H_{42}N_6O_{11}S_2$ $[M + H^+]^+$; cald 667.2426, found 667.2453 $[M + Na^+]^+$; cald 689.2245, found 689.2235 *Cleavage via standard ScmCl in TFA*: The resin (0.01 mmol) was subjected to the general cleavage conditions using ScmCl in TFA to obtain crude **6b**. The crude product was analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **6b** as a white solid (5.9 mg, 88%). ESI-MS: for $C_{25}H_{42}N_6O_{11}S_2$ $[M + H^+]^+$; cald 667.2426, found 667.3 $[M + Na^+]^+$; cald 689.2245, found 689.3.

Ac-Gly-Thr(Bn)-Glu(Bn)-Ala-Cys(Scm)-OCH₃ (7a). Model pentapeptide **7a** synthesis started with **5**. Subsequent coupling with Boc-Ala-OH, Boc-Glu(Bn)-OH, Boc-Thr (Bn)-OH, and Ac-Gly-OH *via* standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. *Cleavage via ScmCl in AcOH*: Resin **7** (0.012 mmol) was subjected to the general cleavage condition using ScmCl in AcOH to obtain crude **7a**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **7a** as a white solid (8.9 mg, 93%). ESI-MS: for $C_{36}H_{47}N_5O_{12}S_2$ $[M + H^+]^+$; cald 806.2736, found 806.2736 $[M + Na^+]^+$; cald 828.2555, found 828.2547.

Ac-Gly-Thr-Glu-Ala-Cys(Scm)-OCH₃ (7b). Model pentapeptide **7b** synthesis started with **5**. Subsequent coupling with Boc-Ala-OH, Boc-Glu(Bn)-OH, Boc-Thr(Bn)-OH, and Ac-Gly-OH was carried out *via* standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. 0.025 mmol resin **7** subjected to standard TFMSA deprotection condition to remove side chain protective groups. *Cleavage via ScmCl in AcOH*: The resin (0.01 mmol) was subjected to the general cleavage conditions using ScmCl in AcOH to obtain crude **7b**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **7b** as a white solid (5.3, 84%). ESI-MS: for $C_{22}H_{35}N_5O_{12}S_2$ $[M + H^+]^+$; cald 626.1797, found 626.1790 $[M + Na^+]^+$; cald 648.1616, found 648.1604. *Cleavage via standard ScmCl in TFA*: The resin (0.0135 mmol) was subjected to the general cleavage conditions using ScmCl in TFA to obtain crude **7b**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **7b** as a white solid (8 mg, 91%). ESI-MS: for $C_{22}H_{35}N_5O_{12}S_2$ $[M + H^+]^+$; cald 626.1797, found 626.2 $[M + Na^+]^+$; cald 648.1616, found 648.2.

Ac-Gly-Tyr(2-Br-Z)-Leu-Ser(Bn)-Cys(Scm)-OCH₃ (8a). Model pentapeptide **8a** synthesis started with **5**. Subsequent coupling with Boc-Ser(Bn)-OH, Boc-Leu-OH, Boc-Tyr(2-Br-Z)-OH, and Ac-Gly-OH was carried out *via* the standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. *Cleavage via ScmCl in AcOH*: Resin **8** (0.0098 mmol) was subjected to the general cleavage conditions using ScmCl in AcOH to obtain crude **8a**. The crude product was analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **8a** as a white solid (9 mg, 92%). ESI-MS: for $C_{43}H_{52}BrN_5O_{13}S_2$ $[M + H^+]^+$; cald 990.2259, found 990.2252 $[M + Na^+]^+$; cald 1012.2078, found 1012.2076.

Ac-Gly-Tyr-Leu-Ser-Cys(Scm)-OCH₃ (8b). Model pentapeptide **8b** synthesis started with **5**. Subsequent coupling

with Boc-Ser(Bn)-OH, Boc-Leu-OH, Boc-Tyr(2-Br-Z)-OH, and Ac-Gly-OH was carried out *via* the standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. 0.025 mmol resin **8** subjected to standard TFMSA deprotection condition to remove side chain protective groups. *Cleavage via ScmCl in AcOH*: Resin (0.0095 mmol) was subjected to the general cleavage conditions using ScmCl in AcOH to obtain crude **8b**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **8b** as a sticky solid (5 mg, 76%). ESI-MS: for $C_{28}H_{41}N_5O_{11}S_2$ $[M + H^+]^+$; cald 688.2317, found 688.2307 $[M + Na^+]^+$; cald 710.2136, found 710.2126. *Cleavage via standard ScmCl in TFA*: The resin (0.0089 mmol) was subjected to the general cleavage conditions using ScmCl in TFA to obtain crude **8b**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **8b** as a sticky solid (5.3 mg, 87%). ESI-MS: for $C_{28}H_{41}N_5O_{11}S_2$ $[M + H^+]^+$; cald 688.2317, found 688.3 $[M + Na^+]^+$; cald 710.2136, found 710.3.

Ac-Gly-Lys(2-Cl-Z)-His(Tos)-Pro-Cys-OMe (9). Model pentapeptide **9** synthesis started with **5**. Subsequent coupling with Boc-Pro-OH, Boc-His(Tos)-OH, Boc-Lys(2-Cl-Z)-OH, and Ac-Gly-OH was carried out *via* the standard Boc protocol. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group.

Ac-Gly-Lys(Biotin)-His-Pro-Cys-OMe (10a). The resin (0.02 mmol) was subjected to the standard TFMSA deprotection conditions to remove the side chain protective groups. To the dried resin in HBTU/HOBt/DIPEA (4/4/8) was added 0.4 M Biotin (4.0 equiv) in DMF. The mixture was tumbled on a shaker at 25°C and 160 rpm for 1 h. The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), and CH_2Cl_2 (5 × 1 min). A small portion of the resin was assayed using the Kaiser test and if the test was positive, the process was repeated. Finally, the resin (0.011 mmol) was subjected to the general cleavage conditions using ScmCl in TFA to obtain crude **10a**. The crude product was analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **10a** as a white solid (6.6 mg, 67%). ESI-MS: for $C_{37}H_{56}N_{10}O_{11}S_3$ $[M + H^+]^+$; cald 913.3365, found 913.3353 $[M + Na^+]^+$; cald 935.3184, found 935.3170.

Ac-Gly-Lys(Hexyn)-His-Pro-Cys-OMe (10b). The resin (0.02 mmol) was subjected to the standard TFMSA deprotection condition to remove side chain protective groups. The dried resin in HBTU/HOBt/DIPEA (4/4/8) was treated with 0.4 M hexynoic acid (4.0 equiv) in DMF for 2 min and tumbled on a shaker at 25°C and 160 rpm for 1 h. The resin was washed with DMF (5 × 1 min), CH_2Cl_2 (5 × 1 min), DMF (5 × 1 min), and CH_2Cl_2 (5 × 1 min). A small portion of the resin was assayed using the Kaiser test and if the test was positive, the process was repeated. Finally, the resin (0.0123 mmol) was subjected to the general cleavage conditions using ScmCl in TFA to obtain crude **10b**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **10b** as a sticky solid (5.1 mg, 52%). ESI-MS: for $C_{34}H_{50}N_8O_{10}S_2$ $[M + H^+]^+$; cald 795.3164, found 795.3152 $[M + Na^+]^+$; cald 817.2983, found 817.2963.

Ac-Gly-Lys-His-Pro-Cys-OMe (10c). The resin (0.02 mmol) was subjected to the standard TFMSA deprotection condition to remove side chain protective groups. Finally, the resin (0.0114 mmol) was subjected to the general cleavage condition using ScmCl in TFA to obtain crude **10c**. The crude product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **10c** as a sticky solid (6.5, 84%). ESI-MS: for $C_{27}H_{42}N_8O_9S_2$ $[M + H^+]^+$; cald 687.2589, found 687.2574 $[M + Na^+]^+$; cald 709.2408, found 709.2389.

Synthesis of Intermediate Riparin 1.2 Resin (12a) via Fmoc SPPS. For synthesis of intermediate **12a**, the synthesis started with Fmoc cleavage of **11** using 20 vol % of piperidine in DMF wherein Fmoc SPPS protocol used to couple Fmoc-Thr(*t*-Bu)-OH, Fmoc-Gly-OH, Fmoc-Lys (Boc)-OH, Fmoc-Tyr(*t*-Bu)-OH, Fmoc-Ala-OH, Fmoc-Cys (Acm)-OH, twice Fmoc-Pro-OH, Fmoc-Leu-OH, and Fmoc-Phe-OH, respectively. 20% piperidine in DMF (2 × 10 min) treated with resin to remove Fmoc group. Allyl group is removed by given method followed by Fmoc removal with standard protocol.

Synthesis of Side Chain Deprotected Di-Sulfenyl Riparin 1.2 (13) via Two Step Cleavage Protocol. Resin **12a** (0.015 mmol) was swollen in 3 mL of TFA:TIPS:PhOH:H_2O of ratio (88:2:5:5) and stirred for 2.5 h at RT, filtered and washed with TFA (1 × 1 min) and CH_2Cl_2 (10 × 2 min). The resin (0.0065 mmol) was subjected to the general cleavage condition of ScmCl in TFA to obtain crude **13** as a sticky solid. The product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **13** as a white solid (4.1 mg, 46%). ESI-MS/MS: for $C_{59}H_{86}N_{12}O_{18}S_4$ $[M + H^+]^+$; cald 1379.5139, found 1379.5 $[M + Na^+]^+$; cald 1401.4958, found 1401.5.

Synthesis of Intermediate Riparin 1.2 Resin (12b) via Boc SPPS. The synthesis of resin **12b** started with Fmoc cleavage of **11** using 20 vol % of piperidine in DMF. The Boc SPPS protocol was used to couple Boc-Thr(Bn)-OH, Boc-Gly-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Tyr(2-Br-Z)-OH, Boc-Ala-OH, Boc-Cys(Acm)-OH, twice Boc-Pro-OH, Boc-Leu-OH, and Boc-Phe-OH, respectively. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group. The allyl group was removed by utilizing the previously reported method.

Synthesis of Side Chain Deprotected Di-Sulfenyl Riparin 1.2 (13) via Two Step Cleavage Protocol. Resin **12b** (0.015 mmol) was swollen in 3 mL of TFA:TFMSA:thioanisole:TIPS of ratio (9:1:1:0.5) and stirred for 2.5 h at 0°C, filtered and washed with TFA (1 × 1 min) and CH_2Cl_2 (10 × 2 min). The resin (0.0074 mmol) was subjected to

the general cleavage condition of ScmCl in TFA to obtain crude **13** as a sticky solid. The product analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **13** as a white solid (7.4 mg, 73%). ESI-MS/MS: for $C_{59}H_{86}N_{12}O_{18}S_4$ $[M + H^+]^+$; cald 1379.5139, found 1379.5117 $[M + Na^+]^+$; cald 1401.4958, found 1401.4944.

Intermediate a-Factor Resin (14). The synthesis of **14** started with **5**. The Boc SPPS protocol was used to couple Boc-Ala-OH, Boc-Asp(Bn)-OH, Boc-Trp(For)-OH, Boc-Phe-OH, Fmoc-Val-OH, Boc-Gly-OH, Boc-Lys(2-Cl-Z)-OH, twice Boc-Ile-OH, and Boc-Tyr(2-Cl-Z)-OH respectively. 50 vol % TFA in CH_2Cl_2 containing 0.5 vol % TIPS (1 × 5 min) and (1 × 15 min) treated with the resin to remove Boc group.

Synthesis of Side Chain Deprotected Trp(CHO) Sulfenyl a-Factor (15) via Two Step Cleavage Process. Resin **14** (0.015 mmol) was subjected to the standard TFMSA deprotection condition to deprotect side chain protective groups. The dried resin (0.007 mmol) was subjected to the general cleavage condition of ScmCl in TFA to obtain crude **15** as a white solid. The crude product was analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **15** as a white solid (9.1 mg, 83%). ESI-MS/MS: for $C_{73}H_{102}N_{14}O_{19}S_2$ $[M + H^+]^+$; cald 1543.6960, found 1543.6927 $[M + Na^+]^+$; cald 1565.6779, found 1565.6743.

Synthesis of Side Chain Deprotected Trp(CHO) Sulfenyl a-Factor (15) via One-Pot Cleavage Process. Resin **14** (0.01 mmol) was subjected to the general one-pot cleavage condition (*i.e.*, 2 equiv of ScmCl in TFMSA:TFA:anisole of ratio 7:2:1) to obtain crude **15** as a white solid. The crude product was analyzed using HPLC and LCMS, and purified by using prepHPLC. The process yielded **15** as a white solid (6.7 mg, 46%). ESI-MS/MS: for $C_{73}H_{102}N_{14}O_{19}S_2$ $[M + H^+]^+$; cald 1543.6960, found 1543.7 $[M + Na^+]^+$; cald 1565.6779, found 1565.7.

Synthesis of Trp, S-Scm Reduced a-Factor (16). The crude resin **15**, generated from 0.0015 mmol of **14** *via* two step cleavage process, was placed in 0.5 mL of argon purged 5 vol % DTT (*ca.*, 100 equiv) in piperidine:DMF of ratio (1:4). After 15 min, it was diluted with ACN and analyzed with HPLC before purification to obtain **16** of 95% purity. ESI-MS/MS: for $C_{70}H_{100}N_{14}O_{16}S$ $[M + H^+]^+$; cald 1425.7235, found 1425.7208 $[M + Na^+]^+$; cald 1447.7054, found 1447.7036.

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Supporting Information. The Supporting Information of characterized data of all reactions via NMR, IR, HPLC, LC/MS, HRMS data is available free of charge through Internet at http://onlinelibrary.wiley.com/journal.

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