

Solid Phase Synthesis of Peptide C-Terminal Thioesters by Fmoc/*t*-Bu Chemistry

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Abstract: Peptide C-terminal thioesters are key intermediates in a variety of applications, most notably the recently developed native chemical ligation methods for the total chemical synthesis of proteins. So far they have been prepared only by the use of the least prevalent Boc/benzyl solid-phase method on ad hoc prepared resin supports. We describe here a novel method for the solid phase synthesis of thioesters by the most prevalent Fmoc/*t*-Bu method. The method is based on the use of a 3-carboxypropanesulfonamide safety-catch linker, which is fully stable to repetitive exposure to the basic conditions needed for Fmoc cleavage. Activation with diazomethane or iodoacetonitrile followed by displacement with a suitable thiol produces the thioester in good to excellent yields. The method is also compatible with Boc/benzyl chemistry. Moreover, all the necessary reagents are commercially available.

Introduction

Peptide C-terminal thioesters/thio acids have long since represented one of the most useful peptide reagents, whose applications range from fragment coupling¹ to enzyme-mediated condensation,² synthesis of proteins with backbone-engineered³ or non-native architecture,⁴ peptide dendrimers,⁵ cyclic peptides,⁶ and substrates for serine and metallopeptidases.⁷ Their usefulness has been greatly increased by the appearance in the past few years of native chemical ligation,^{8,9} which is establishing itself as a method for reproducible, practical total chemical synthesis of small-to-medium size proteins.¹⁰ According to this method, two unprotected peptides, the first one with a C-terminal thioester and the second one with an N-terminal cysteine, are joined together by a chemoselective reaction in aqueous solution at neutral pH. Successful applications include more than 200 proteins from numerous families.^{10,11} Template-assisted native

chemical ligation has also been used to implement self-replicative peptide systems addressing the origin-of-life problem.¹² The scope of the method is expanding, since the initial, absolute need for a cysteine residue N-terminal to one of the fragments has been recently relaxed, and Gly,¹³ His,¹⁴ and Met¹⁵ have been shown to be possible substitutes for Cys. When the reaction is conformationally assisted, many more N-terminal amino acids should be compatible.¹⁶ Finally, initial attempts have been described to perform the ligation directly on a solid support.¹⁷ The above-mentioned reasons have made more pressing the need for efficient synthetic procedures of the peptide intermediates. To this regard, the synthesis of the crucial thioester-containing fragments has been performed so far essentially by the least prevalent Boc methodology.^{5,15,18–21} This is because the thioester linkage attached to a resin support is

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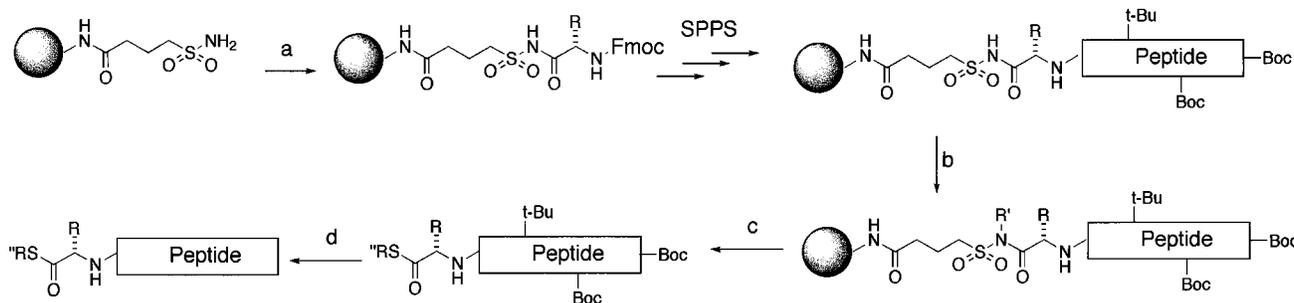
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Scheme 1^a

^a (a) Fmoc-aa, PyBOP, *i*-Pr₂EtN; (b) TMS-CHN₂ (R' = CH₃) or ICH₂CN, *i*-Pr₂EtN (R' = CH₂CN); (c) HSR'/NaSPh; (d) TFA, scavengers.

susceptible to strong nucleophiles such as the piperidine used for removal of Fmoc groups. The only reports of Fmoc-based thioester synthesis are from Futaki et al.,^{4b} where the thioester was formed in solution using a partially protected precursor, and by Li et al.,²² who used a thioester-compatible Fmoc-cleaving reagent to produce a 25-aa peptide in 24% yield. We describe here a novel method for the solid phase synthesis of thioesters by Fmoc/*t*-Bu chemistry. The method is based on the use of a safety-catch linker, which is fully stable to repetitive exposure to the basic conditions needed for Fmoc cleavage, yet can be activated for cleavage upon completion of the synthesis.

Results and Discussion

General Scheme. Our method for C-terminal thioester synthesis is based on Kenner's acylsulfonamide safety-catch linker²³ as modified by Backes et al.^{24,25} The 3-carboxypropanesulfonamide linker^{24,25} in particular is completely stable to basic or strongly nucleophilic conditions and can be activated by treatment with diazomethane or iodoacetonitrile^{24,25} to provide the *N*-alkyl acylsulfonamide, which is then susceptible to nucleophilic attack. We reasoned that the *N*-alkyl-peptidyl-sulfonamide would yield a C-terminal peptide thioester upon cleavage with a suitable thiol and, since the peptide-resin linkage would be stable to repetitive basic treatment, the peptide thioester could be assembled by standard Fmoc chemistry.²⁶ The procedure for peptide thioester synthesis is illustrated in Scheme 1. Briefly, the first amino acid is attached to the handle-derivatized resin²⁷ by coupling with PyBOP/*i*-Pr₂EtN,²⁵ and then the peptide is assembled by standard Fmoc protocols.²⁸ At the end of the assembly, the resin is activated and then reacted with a thiol suitable for the subsequent ligation reaction. The cleaved peptide thioester is filtered, the solvent evaporated, and the cleavage cocktail for side-chain deprotection²⁹ is added directly

to the solid residue; the crude peptide is then recovered by standard workup procedures²⁸ and purified by preparative HPLC.

We envisaged and addressed two potential problems with this procedure: (a) efficiency of the thiol in the nucleophile-mediated cleavage step and (b) stability of the peptide during the acyl-sulfonamide activation step.

Activation of the Resin and Displacement by Thiols. To study the former issue, we activated the resin by either treatment with trimethylsilyldiazomethane (TMS-CHN₂) or ICH₂CN/*i*-Pr₂EtN. In our initial experiments only ICH₂CN/*i*-Pr₂EtN treatment (followed by thiol displacement) yielded the desired peptide thioester in reasonable (40–70%) yield. The displacement step was then modified by the addition of a catalytic amount of sodium thiophenolate.³⁰ We reasoned that the strongly nucleophilic NaSPh anion would initially cleave the peptide as α -phenyl-thioester, which would exchange in situ with the large excess of a suitable thiol (e.g. HS-(CH₂)₂-COOC₂H₅) to produce a more stable aliphatic α -thioester. The rationale for this procedure is analogous to the use of thiol additives to improve the kinetics of native chemical ligation.³¹ The modified displacement procedure led to a dramatic improvement (from near-zero to 75–85%) of the yields following diazomethane activation, while it was influential on the yields following ICH₂CN activation. This is in good agreement with previous observations showing that the diazomethane provides more efficient alkylation, while haloacetonitrile provides enhanced reactivity toward nucleophilic displacement.²⁴ In our case the use of thiophenolate circumvents the lower reactivity of methylated acylsulfonamides.

In Table 1 the yields are compared for the two activation protocols, followed by either amine or thiol nucleophilic displacement (compounds **1b–6b** and **1t–7t**, respectively). We found that while the two alkylation methods were equally effective for **1**, **2**, and **6**, diazomethane was more effective than iodoacetonitrile to alkylate the peptide sulfonamides **3–5**. Since the two treatments yield peptides of comparable quality (vide infra), we adopted TMS-CHN₂ followed by NaSPh (cat.)/HS-R³² as our standard treatment.

Whichever the preferred activation procedure, a side-by-side comparison of the cleavage of the same peptide with thiol and

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(26) The procedure described in this work is equally applicable to a peptide assembled by Boc chemistry; Fmoc chemistry is highlighted because, as discussed in the paper, a good method for thioester synthesis by Boc chemistry already exists.^{5,15,18–21}

(27) The resins used in our experiments were PEG-PS (Perkin-Elmer Perseptive), NovaSyn TG (NovaBiochem), and the already functionalized 3-carboxypropanesulfonamide-polystyrene (NovaBiochem).

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(29) Composition of the cleavage mixture and reaction time should be fine-tuned on the specific peptide sequence.²⁸ However, for all the peptides included in this study we used Reagent B (Sole, N. A.; Barany, G. *J. Org. Chem.* **1992**, *57*, 5399–5403) i.e., TFA-phenol-water-trisopropylsilane (88 5:5:2) and a cleavage time of 1.5 h.

(30) The rationale for this catalytic effect is that for both an aliphatic sulfonamide and an aliphatic mercaptan pK_a (DMSO) = 17, while for thiophenol pK_a = 10 (F. G. Bordwell *Acc. Chem. Res.* **1988**, *21*, 456–463). The thiophenol produced through the exchange is expected to immediately lose its proton, thus regenerating the thiophenolate anion.

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Table 1. Comparison of Trimethylsilyldiazomethane and Iodoacetone Activation Protocols for Peptides Released as C-Terminal Thioesters and C-Terminal Benzylamides

compd ^{a,b}	sequence	TMS-CH ₂ N ₂		ICH ₂ CN	
		t ^a	b ^b	t ^a	b ^b
1t-1b	MWAGADPA	85	80	82	78
2t-2b	AEGEFMRASRCNPN CPMWAGADPA	75	64	75	60
3t-3b	KKKSTWVLVGGVLA ALAAY	83	81	48	47
4t-4b	KMY	85	78	48	nd ^c
5t-5b	KML	75	77	51	nd
6t-6b	KMA	82	68	81	62
7t	KPA	80	nd	nd	nd

^a **t** = C-terminal thioester, -CO-S-(CH₂)₂-COOC₂H₅. ^b **b** = C-terminal benzylamide, -CO-NH-CH₂-C₆H₅. ^c Not done.

benzylamine (Table 1) shows that in all cases the thiol is equally or more effective in releasing the peptide from the resin as thioester than the primary amine as benzylamide (compare **t** peptides with **b** peptides in Table 1; the chromatograms of crude peptides **3t** and **3b** are shown in Figure 1).

Stability of the Resin-Bound Peptide to Activation Treatments. The problem of the stability of peptide side-chain functionalities to the conditions used during activation had already been addressed for ICH₂CN treatment using dipeptides.²⁵ Although this study did not highlight major problems, a few residues (Cys, Asn, Met) gave lower yields. The peptides in Table 1 range from 3 to 24 amino acids in length and include combinations of all the potentially troublesome residues. We compared the purity and yields of a given peptide as both C-terminal thioester and C-terminal benzylamide (prepared by the new procedure) with the corresponding C-terminal carboxylate (prepared by standard methods); the peptides being compared were assembled with identical protocols starting with the first amino acid attached either to 3-carboxypropanesulfonamide resin or to a standard ALH resin. The syntheses were carried out on the resin supports more commonly used in peptide synthesis, i.e., poly(ethylene glycol)-polystyrene copolymers (brand names PEG-PS or Tentagel) or aminomethylated polystyrene (PS). A comparison of the chromatograms of crude peptide thioester **3t**, the benzylamide peptide **3b** (Table 1), and their C-terminal -COOH homologue is given in Figure 1; their yields were 61%, 49%, and 60%, respectively. Figure 1 also shows the analytical HPLC of the crude peptide thioester **2t** (Table 1). This was a particularly challenging case, since it included many of the most problematic and potentially labile amino acids residues, i.e., 2 methionines, 2 cysteines, and 1 tryptophan. As shown in Figure 1, the peak corresponding to the target compound represents 59% of the total peptide material, the same purity as the -COOH analogue (not shown).

Native Chemical Ligation. As a final confirmation of the suitability of the thioesters generated by the new method for native chemical ligation we reacted the thioesters **1t**, **2t**, and **3t** with the cysteine peptides **1c** (CRAIVGFR-NH₂), **2c** (CRAIVGFRVQWLRRYFVNGSR-NH₂), and **3c** (CLTTGSVVIVGRILSGRPAIVPDRELLYQEFDEMEECASHLPYKKK-NH₂), respectively, to produce products ranging from 17 to 71 amino acids. Ligation reactions between **1t-1c** and **2t-2c** are shown in Figures 2 and 3, respectively and all details are given in the Experimental Section. Peptide **3t** had been previously produced by Boc chemistry and used in the synthesis of the NS4A protein from human hepatitis C virus by native chemical ligation with peptide **3c**.³³ The peptide thioester produced by the new protocol (Table 1 and Figure 1) was similarly used in native chemical

ligation with **3c** and produced NS4A in the same yield as previously reported.³³

Conclusions. In conclusion, the method described in the present work allows for facile preparation of peptide thioesters without limitations of size or amino acid composition. The method is compatible with both Fmoc/*t*-Bu and Boc/benzyl SPPS and allows for a combination of the two (for example to prepare cyclic or branched peptide thioesters). It can be performed on the most commonly used SPPS resin supports and requires only low-cost, commercially available reagents, like the most recently developed Boc/benzyl procedure.²¹

Experimental Section

General Methods. Unless otherwise noted, all the materials were obtained from commercial suppliers and used without further purification. *i*-Pr₂EtN was distilled under N₂. All solvents were commercially available peptide synthesis grade. Protected amino acids were purchased from Novabiochem (Läufelfingen), Bachem (Bubendorf), and Neosystem (Strasbourg). Peptide synthesis was performed on a Pioneer Synthesizer (Perkin-Elmer-PerSeptive). Analytical HPLC was performed on a Beckman System Gold chromatograph equipped with a diode-array detector, operating flow rate 1 mL min⁻¹. Preparative HPLC was performed on a Waters 600E chromatograph equipped with a Jasco UV-975 detector (monitoring wavelength, 214 nm) and either a Phenomenex (Jupiter) C-4 column (21.2 × 250 mm, 10 μm), a Waters Delta-Pak C-18 column (100 × 250 mm, 15 μm), or a Waters Delta-Pak C-4 column (100 × 250 mm, 15 μm); the operating flow rate was 30 mL min⁻¹. For semipreparative work we used a Phenomenex (Jupiter) C-4 column (10 × 250 mm, 10 μm), operating flow rate 5 mL min⁻¹. The solvent system was always eluent A, water (0.1% TFA); eluent B, MeCN (0.095% TFA).

Preparation of the 3-Carboxypropanesulfonamide Resin. PEG-PS HCl resin (1 g, 0.19 mmol/g, PE PerSeptive) or Tentagel (NovaSyn TG, NovaBiochem, 0.8 g, 0.24 mmol/g) was weighed into a 35 mL polypropylene syringe equipped with a bottom 20 μm polyethylene filter (SPE-ed cartridge, Applied Separations). The resin was washed with *i*-Pr₂EtN (2 × 5 min) and *N,N'*-dimethylformamide (DMF, 5 × 2 min) until swelling was complete. Then 3-carboxypropanesulfonamide (0.95 mmol, Novabiochem), diisopropylcarbodiimide (DIPC, 0.95 mmol), and 1-hydroxybenzotriazole (HOBt, 0.95 mmol) were added in the minimum amount of DMF suitable to keep the resin covered with liquid. After stirring on a rotary plate for 24 h, the resin was extensively washed with DMF and used for the assembly of the peptide thioester or further washed with CH₂Cl₂ and dried in vacuo until further use. 3-Carboxypropanesulfonamide-PS resin is commercially available from NovaBiochem.

Attachment of the First Amino Acid. We followed the procedure of Backes & Ellman.²⁵ In a typical experiment to a 50 mL round-bottom flask were added 3-carboxypropanesulfonamide-resin (0.2 mmol), CH₂Cl₂ or CHCl₃ (5 mL), *i*-Pr₂EtN (342 μL, 2 mmol), and a Fmoc-amino acid (1 mmol). The reaction mixture was stirred for 20 min, followed by cooling to -20 °C. Then benzotriazole-1-yl-oxy-trispyrrolidino-phosphonium hexafluorophosphate (PyBOP, 520 mg, 1 mmol) was added as a solid, and the reaction mixture was stirred for 8 h at -20 °C, filtered, and washed with CH₂Cl₂ or CHCl₃ (5 × 5 mL). The extent of incorporation was quantitated by the Fmoc release UV assay.²⁸ Coupling was repeated when loading was <70%.

Alkylation of the Peptide-Acylsulfonamide Resin. In a typical experiment, *N*-acylsulfonamide resin (20 mg, 0.4 mmol/g or 42 mg,

(32) We have used the same thiol (HS-(CH₂)₂-COOC₂H₅) for all the peptides described in this work. This is only due to practical reasons: the chosen thiol is very effective as a displacer, it is essentially odorless, and the resulting thioester is very stable to workup and chromatographic manipulations. During native chemical ligation it is converted in situ to the more reactive phenyl-α-thioester by inclusion of thiophenol in the reaction medium, according to Dawson et al.³¹ α-benzyl thioesters have also been prepared with the same procedure.

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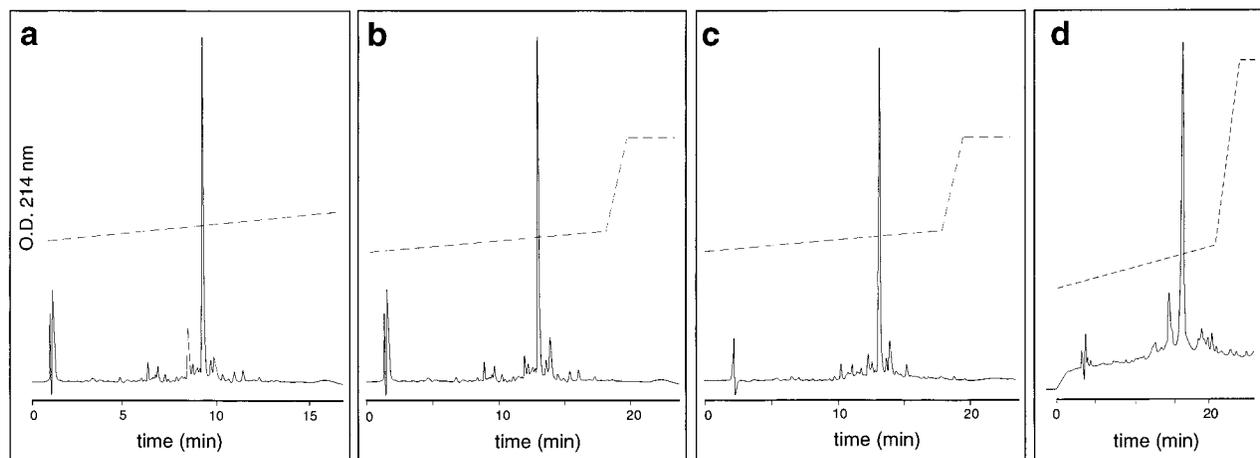


Figure 1. (a) Analytical HPLC of crude peptide KKKSTWVLVGGVLAALAAY-COOH assembled on hydroxymethylbenzoyl-PEG-PS. Column: Phenomenex C-4, 250 \times 4.6 mm, eluent A: water (0.1% TFA), eluent B: MeCN (0.095% TFA), linear gradient 30–70% B over 20 min, then 70–80% B over 3 min. (b) Analytical HPLC of crude analogue **3t** assembled with identical protocols on 3-carboxypropanesulfonamide-PS. Conditions as in (a). (c) Analytical HPLC of crude analogue **3b** cleaved from 3-carboxypropane sulfonamide-PS with benzylamine. Conditions are as in (a, b). (d) Analytical HPLC of crude peptide **2t**. Conditions as in (a–c) except linear gradient 25–35% B over 20 min, then 35–80% B over 3 min.

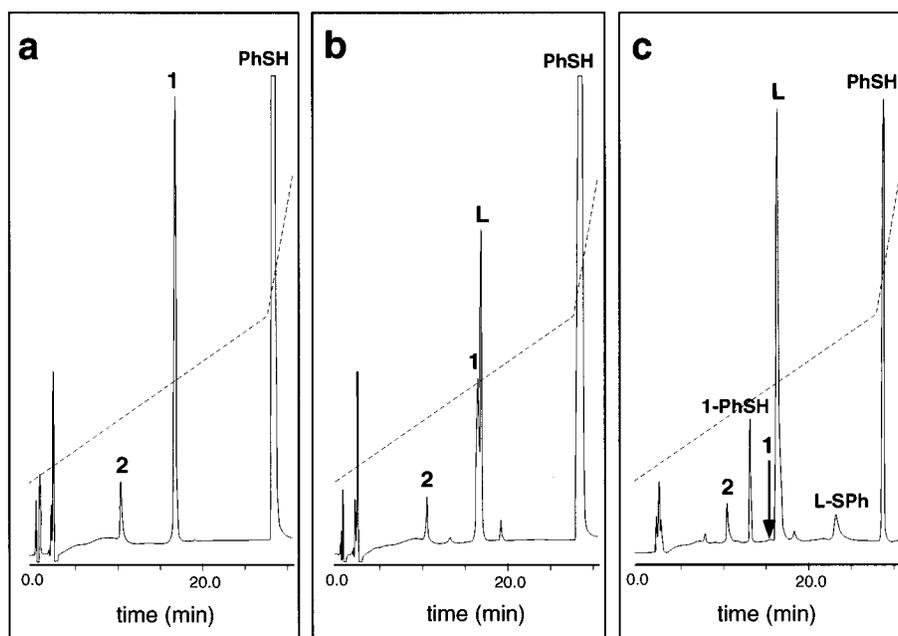


Figure 2. Native chemical ligation of peptides **1t–1c**: (a) time zero, (b) reaction after 90 min, and (c) reaction after 5 h. Peak **1** is peptide **1t**, peak **2** is peptide **1c**, the peak corresponding to the ligated product is labeled **L**. The peak labeled **1-PhSH** is the thiophenol thioester of peptide **1t**, while the peak labeled **L-SPh** is the disulfide formed by the cysteine of the ligated product with thiophenol. Chromatographic conditions: column, Ultrasphere C-18 (250 \times 4.6 mm, 5 μ m), eluent A, water (0.1% TFA), eluent B, MeCN (0.095% TFA), linear gradient 15–50% B over 28 min, then 50–80% B over 3 min.

0.19 mmol/g) was weighted into a 3 mL polypropylene syringe equipped with a bottom 20 μ m polyethylene filter (SPE-ed cartridge, Applied Separations). *Alkylation with ICH₂CN*. The resin was washed with *N*-methyl-2-pyrrolidone (NMP) until swelling was complete, and then 2.2 mL of NMP, 82 μ L of *i*-Pr₂EtN (0.48 mmol), and 174 μ L (2.4 mmol) of iodoacetonitrile, previously filtered through an alumina basic plug, were added to a final concentration of 1 M. After stirring on a rotary plate for 24 h, the resin was washed with NMP (5 \times 3 mL) and DMF (5 \times 3 mL) and used in the displacement reaction (see below) or further washed with CH₂Cl₂ (5 \times 3 mL) and dried in vacuo until further use. *Alkylation with TMS-CHN₂*. The resin was washed with tetrahydrofuran (THF) until swelling was complete, and then 2.4 mL of 1 M TMS-CH₂N₂ (50:50, v/v, hexane/THF) was added. After stirring on a rotary plate for 2 h, the resin was washed with THF (5 \times 3 mL) and DMF (5 \times 3 mL) and used in the displacement reaction

(see below) or further washed with CH₂Cl₂ (5 \times 2 mL) and dried in vacuo until further use.

Displacement with HS-(CH₂)₂-COOC₂H₅. The activated *N*-acylsulfonamide resin was swollen in DMF and drained. DMF or CH₂Cl₂ (350 μ L) and ethyl-3-mercaptopropionate (52 μ L, 400 μ mol, 50 equiv) were then added to a final concentration of 1 M. The mixture was stirred on a rotary plate for 24 h, and the resin was filtered and washed with DMF (3 \times 1 mL). The combined filtrate and washes were collected in a 25 mL round-bottom flask and rotary evaporated. To completely dry the protected peptide before cleavage (traces of DMF would interfere with the action of TFA)²⁸ and to remove as much as possible ethyl-3-mercaptopropionate the residue was triturated and washed with cold Et₂O (5 \times 4 mL).

Displacement with NaSPh/HS-(CH₂)₂-COOC₂H₅. The experiment was carried out exactly as described in the previous section, except

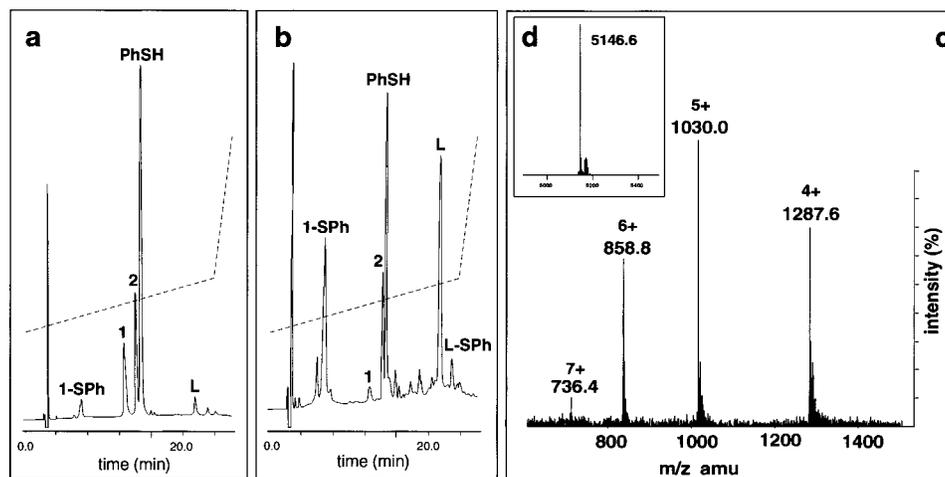


Figure 3. Native chemical ligation of peptides **2t–2c**. (a) Reaction between **2t** (peak **1**) and **2c** (peak **2**) after 5 min. The peak corresponding to the ligated product is labeled **L**, while the peak labeled **1-Sph** is the thiophenol thioester of peptide **2t**. Chromatographic conditions: column, Phenomenex C-4, 250 × 4.6 mm, eluent A: water (0.1% TFA), eluent B: MeCN (0.095% TFA), linear gradient 25–35% B over 20 min, then 35–80% B over 3 min. (b) **2t–2c** ligation after 4 h. The peak labeled **L-Sph** is the disulfide formed by the cysteine of the ligated product with thiophenol. Chromatographic conditions as in (a). (c) Ion-spray mass spectrometry of HPLC-purified peak **L** acquired on an API-365 (PE-SCIEX) triple-quadrupole mass spectrometer; the multiple charge states arise from protonation of a single species of MW 5146. (d) Hypermass reconstruction of the spectrum shown in (c) [calculated (average isotopic composition) 5146.97 daltons, found 5146.6].

for the addition of a catalytic amount of sodium thiophenolate (0.5 mg, 4 μ mol, 0.5 equiv).

Removal of N-Terminal and Side-Chain Protecting Groups. In a typical experiment, 12 mg of cleaved, protected peptide were treated with 3 mL of TFA 88%, phenol 5%, triisopropylsilane 2%, water 5% (Reagent B)²⁹ for 2 h at 21 °C. The TFA solution was added dropwise to screw cap centrifuge tubes containing MTBE with a TFA/MTBE ratio of 1/10; after centrifugation at 3200g (30 min), the ether was removed and the peptide precipitate resuspended in 50 mL of MTBE: the process was repeated twice. The final precipitate was dissolved in 50% MeCN/water and lyophilized.

Synthesis of Peptide Thioesters 1t–7t. Peptides **1t–7t** were assembled by machine-assisted Fmoc/*t*-Bu chemistry²⁸ on the alkyl-sulfonamide resins: polystyrene for **3t**, **4t**, and **5t**, Tentagel for **7t**, and PEG-PS for **1t**, **2t**, **6t**. The protecting groups, synthetic protocols, and workup were the same as reported for peptides **1c–3c** (see below) except that the N-terminal residues were incorporated as N^α-Boc derivatives: Boc-Lys(*tert*-butoxycarbonyl)-OH, Boc-Met-OH, and Boc-Ala-OH. Coupling times were as follows: **1t**) all residues, 60 min; **2t**) residues Ala1-Asn14, 90 min, Cys15-Pro23, 60 min; **3t**) residues Ala18-Ala15, 60 min, Ala14-Gly10, 90 min, Val9-Lys1, 120 min; and **4t–7t**), all residues, 30 min. **Peptide 1t.** The crude material was 6.35 mg (yield, 85%). It contained approximately 88% of the target material as judged by analytical HPLC (Ultrasphere C-18 column, 250 × 4.6 mm, 5 μ m, gradient 20–40% B over 20 min and then 40–80% B over 3 min, t_R of the target peptide 14.3 min). The peptide was purified on a Waters Delta-Pak C-18 column. In a typical run, the peptide (10 mg) was dissolved in 5 mL of MeCN/H₂O/TFA (10:89.9:0.1 v/v) and immediately loaded onto the preparative column at a flow rate of 5 mL min⁻¹. The flow was raised to 30 mL min⁻¹, and the peptide was eluted with a linear gradient between 20 and 35% B over 20 min. The fractions containing the desired peptide (>98% purity) were pooled and lyophilized: yield, 5.8 mg, 58%. Ion-spray mass spectrometry: calculated (average isotopic composition) 934.1 daltons, found 934.6. **Peptide 2t.** The crude material was 9.8 mg (yield, 75%) containing 59% of the target material (Figure 1d). The peptide (5 mg) dissolved in 5 mL of MeCN/H₂O/TFA (10:89.9:0.1 v/v) was loaded onto a semipreparative Phenomenex C-4 column at a flow rate of 1 mL min⁻¹. The flow was raised to 5 mL min⁻¹, and the peptide was eluted with a linear gradient between 25 and 30% B over 20 min. The fractions containing the desired peptide (>98%) were pooled and lyophilized: yield 2 mg, 40%. Ion-spray mass spectrometry: calculated (average isotopic composition) 2698.1 daltons, found 2697.4. **Peptide 3t.** The crude material was 15.5 mg (yield, 83%) containing 61% of the target material (Figure 1b). The peptide (10 mg) dissolved in 6 mL of MeCN/

H₂O/TFA (10:89.9:0.1 v/v) was purified on the semipreparative Phenomenex C-4 column using a linear gradient between 30 and 70% B over 20 min. Yield (>98% purity) 5.2 mg, 52%. Ion-spray mass spectrometry: calculated (average isotopic composition) 2091.6 daltons, found 2091.41. **Peptide 4t.** The crude material was 3.5 mg (yield, 85%) containing 89% of the target material (column, Ultrasphere C-18). Ion-spray mass spectrometry: calculated (average isotopic composition) 556.4 daltons, found 556.0. **Peptide 5t.** The crude material was 3.3 mg (yield, 75%) containing 87% of the target material (column, Ultrasphere C-18). Ion-spray mass spectrometry: calculated (average isotopic composition) 506.4 daltons, found 506.0. **Peptide 6t.** The crude material was 2.6 mg (yield, 82%) containing 96% of the target material (column, Ultrasphere C-18). Ion-spray mass spectrometry: calculated (average isotopic composition) 464.3 daltons, found 464.0. **Peptide 7t.** The crude material was 2.2 mg (yield, 80%) containing 95% of the target material (column, Ultrasphere C-18). Ion-spray mass spectrometry: calculated (average isotopic composition) 431.3 daltons, found 431.0. For this peptide, assignment of ¹H and ¹³C resonances in DMSO with an HSQC experiment are reported below: Lys, α CH₂ (4.136; 50.490), β CH₂ (1.706; 29.296), γ CH₂ (1.429; 20.444), δ CH₂ (1.543; 26.401), ϵ CH₂ (2.749; 38.368); Pro, α CH (4.459; 59.098), β CH₂ (2.158–1.943; 28.395), δ CH₂ (1.912; 24.457), ϵ CH₂ (3.691–3.470; 46.774); Ala, α CH (4.357; 54.642), β CH₃ (1.281; 17.140); ethyl-3-mercaptopropionate, α CH₂ (2.550; 33.546), β CH₂ (2.981; 23.093), ethyl-CH₂ (4.063; 60.093), ethyl-CH₃ (1.179; 13.944). Analysis of HMBSC experiment for carbon-proton connectivities: AlaNH–ProC (170.900), Ala NH– α C (54.642), Ala NH– β C (17.140), Ala α CH–C (201.675), Ala α CH–ProC (170.900), Ala α CH– γ C (17.140), Ala β CH₃–C (201.675), Ala β CH₃– α C (54.642), Ala β CH₃– β C (17.140), ethyl-3-mercaptopropionate β CH₂–AlaC (201.675), ethyl-3-mercaptopropionate β CH₂–C (170.970), ethyl-3-mercaptopropionate β CH₂– α C (33.546), ethyl-3-mercaptopropionate β CH₂– β C (23.093), ethyl-3-mercaptopropionate α CH₂–C (170.970), ethyl-3-mercaptopropionate α CH₂– α C (33.546), ethyl-3-mercaptopropionate α CH₂– β C (23.093), ethylCH₂-propionate C (170.970), ethylCH₂-ethyl CH₃ (13.944), ethylCH₃-ethyl CH₂ (60.093), ethylCH₃-ethyl CH₃ (13.944), Pro α CH–C (170.900), Pro α CH– β C (28.395), Pro α CH– γ C (24.457), Pro β CH₂–C (170.900), Pro β CH₂– γ C (24.457), Pro γ CH₂– β C (28.395), Lys β CH₂–C (166.807).

Synthesis of Peptide Benzylamides 1b–6b. The procedure was the same as for **1t–6t** except for the use of benzylamine in the nucleophilic displacement step. The activated *N*-acylsulfonamide resin was swollen in DMF and drained. DMF or CH₂Cl₂ (360 μ L) and benzylamine (44 μ L, 400 μ mol, 50 equiv) were then added to a final concentration of 1 M. The HPLC chromatogram of crude peptide **3b** is shown in Figure 1c.

Synthesis of N-Terminal Cysteine Peptide Fragments 1c–3c. The peptides were assembled by machine-assisted Fmoc/*t*-Bu chemistry²⁸ on 1 g of PEG-PS resin (0.16 mmol g⁻¹). Side-chain protection was as follows: Fmoc-Arg(2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl), Fmoc-Asp(*O*-*tert*-butyl), Fmoc-Asn(trityl), Fmoc-Cys(trityl), Fmoc-Glu(*O*-*tert*-butyl), Fmoc-Gln(trityl), Fmoc-His(trityl), Fmoc-Lys(*tert*-butoxycarbonyl), Fmoc-Ser(*tert*-butyl), Fmoc-Thr(*tert*-butyl), Fmoc-Trp(*tert*-butoxycarbonyl), and Fmoc-Tyr(*tert*-butyl). The Fmoc-amino acid (1 equiv) was preactivated (5 min) with *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU, 1 equiv) and *i*-Pr₂EtN (2 equiv); 5-fold excess of acylating agent was used over the resin amino groups. Coupling times were as follows: **1c** residues Arg8-Cys1, 60 min; **2c** residues Arg20-Gly18, 60 min; residues Asn17-Cys1, 90 min; **3c** see ref 33. After the last piperidine cycle, the resin was washed with DMF, MeOH, and CH₂Cl₂ and dried to constant weight in vacuo. **Cleavage.** In a typical experiment, 500 mg of peptide-resin were treated with 15 mL of TFA 88%, phenol 5%, triisopropylsilane 2%, and water 5% (Reagent B)²⁹ for 3 h at 21 °C. The resin was filtered and rinsed with TFA. The remaining workup was as previously described (“Removal of N-terminal and side-chain protecting groups”). **Peptide 1c.** The crude material (115 mg, 78% of theoretical yield, based on starting functionalization of the resin) contained 95% of the target peptide as judged by analytical HPLC (column Ultrasphere C-18, linear gradient 15–30% B over 20 min and then 30–80% B over 3 min, *t*_R of the target peptide 12.4 min). The peptide (7.5 mg) dissolved in 6 mL of MeCN/H₂O/TFA (10:89.9:0.1 v/v) was purified on the Waters Delta-Pak C-18 column with a linear gradient between 10 and 30% B over 30 min. Yield (>98% purity) 6.4 mg, 85%. Ion-spray mass spectrometry: calculated (average isotopic composition) 920.15 daltons, found 920.8. **Peptide 2c.** The crude material (269 mg, 68% synthetic yield) contained approximately 22.3% of the target peptide as judged by analytical HPLC (column, Phenomenex C-4, linear gradient 25–40% B over 20 min and then 40–80% B over 3 min, *t*_R 15.5 min). The peptide (20 mg) dissolved in 6 mL of MeCN/H₂O/TFA (10:89.9:0.1 v/v) was purified on a Waters Delta-Pak C-4 column, using a linear gradient between 20 and 30% B over 30 min. Yield (>98% purity) 2.6 mg, 13%. Ion-spray mass spectrometry: calculated (average isotopic composition) 2583.1 daltons, found 2583.7. **Peptide 3c.** The synthesis and purification have already been described.³³

Native Chemical Ligation of Peptide Thioesters. 1t–1c. In a 1.5-mL polypropylene eppendorf vial, peptide **1t** (1.26 mg, 1.35 μmol) and peptide **1c** (1.25 mg, 1.35 μmol) were dissolved in 360 μL of 0.1 M NaPi, pH 7.5, and 180 μL of dioxane to a final concentration of 2.5 mM (clear solution). The ligation was started by addition of 1%

thiophenol (5.4 μL) to promote conversion of the less reactive 2-mercaptopropionate thioester to the more reactive phenyl α-thioester.³¹ The vial was vortexed at 27 °C in an Eppendorf 5436 Thermomixer, and progress of the reaction was monitored by analytical HPLC (column, Ultrasphere C-18, linear gradient 15–50% B over 28 min and then 50–80% B over 3 min, *t*_R of the target peptide 16.4 min). The reaction was fast, being complete in 5 h (Figure 2). The reaction mixture was acidified with 0.1% TFA and lyophilized. The lyophilized material was taken up in neat TFA (0.5 mL) and precipitated in 10 mL of MTBE; after centrifugation at 3200g (30 min), the ether was removed, the peptide pellet was resuspended in MTBE, and this process was repeated three times. The final pellet was dissolved in MeCN/H₂O/TFA (25:74.9:0.1 v/v) and lyophilized; this material was purified by preparative HPLC. The peptide was dissolved in MeCN/H₂O/TFA (10:89.9:0.1 v/v) and purified on a Waters Delta-Pak C-18 column using a linear gradient between 15 and 45% B over 30 min. Ion-spray mass spectrometry of the HPLC-purified ligated peptide gave the expected molecular weight: calculated (average isotopic composition) 1720.1 daltons, found 1720.0. **Peptides 2t–2c.** In a 1.5-mL polypropylene eppendorf vial, peptide **2t** (0.54 mg, 2.0 mmol) and peptide **2c** (0.52 mg, 2.0 mmol) were dissolved in 66 μL of 0.1 M NaPi, pH 7.5, and 33 μL of dioxane to a final concentration of 2.0 mM (clear solution). The ligation was started by addition of 1 μL of thiophenol (1% v/v). The vial was vortexed at 27 °C in an eppendorf 5436 Thermomixer. The reaction was monitored by analytical HPLC, through repeated injections of an aliquot (10 μL) of the reaction mixture diluted 1:10 with MeCN/H₂O/TFA (50:49.9:0.1 v/v) to stop the reaction. Column: Phenomenex C-4, linear gradient 25–40% B over 30 min and then 40–80% B over 3 min, *t*_R of the target peptide 27.06 min (the assignment was done a posteriori by LC/MS). The reaction was again fast, being essentially complete in 4 h (Figure 3). Workup was done as described for the previous ligation. The peptide was purified on a Phenomenex C-4 column using a linear gradient between 25 and 40% B over 30 min. Ion-spray mass spectrometry: calculated (average isotopic composition) 5146.97 daltons, found 5146.6. **Peptides 3t–3c.** This ligation was carried out as previously described.³³

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