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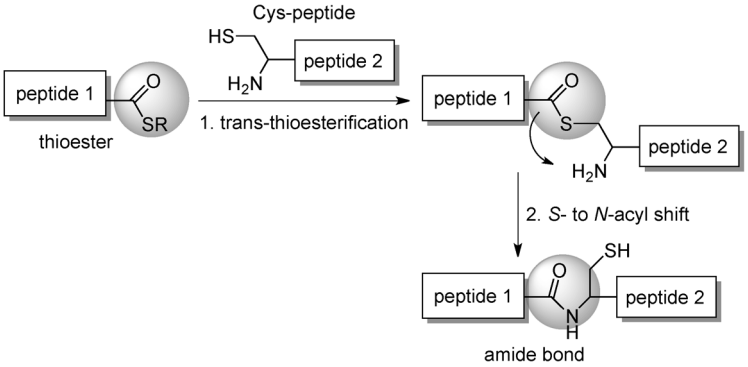
High-Throughput Synthesis of Peptide α-Thioesters: A Safety Catch Linker Approach Enabling Parallel Hydrogen Fluoride Cleavage

Andreas Brust,* Christina I. Schroeder, and Paul F. Alewood[a]

Peptide α-thioesters are fundamental building blocks in peptide and protein science, providing powerful tools for peptide medicinal chemistry. The application of peptide α-thioesters in native chemical ligation reactions has enabled synthetic access to cysteine-rich peptides and proteins, cyclic peptides as well as labeled and chemically modified biomolecules. An efficient high-throughput synthesis of peptide α-thioester building blocks would be beneficial for many medicinal chemical applications that require peptides and proteins. Herein we present a novel synthetic route to cysteine-rich peptide α-thioesters using a safety catch linker that enables a parallel synthetic strategy for chemical protein synthesis. ACP(68–75), bradykinin and dynorphin(1–13) were synthesized via Boc chemistry in their thioester form on a safety catch amide linker (SCAL), employing polystyrene- or poly(ethylene glycol)-based resins, compartmentalized in tea bags. This compartmentalized resin/linker strategy facilitated a parallel hydrogen fluoride cleavage in which each peptide thioester was subsequently cyclized by native chemical ligation, demonstrating the utility of this approach. A naturally occurring bioactive cyclic peptide, the sunflower trypsin inhibitor SFTI-1, was synthesized to demonstrate the viability of this method to access important peptide biomolecules.

Introduction

Native chemical ligation (NCL) has become the fundamental workhorse within modern protein chemistry for the study of biomolecular structure and function at the atomic level.[1] This technology has facilitated the total synthesis of cysteine-rich, small- and medium-sized proteins and peptides as well as cyclopeptides,[2] representing targets for peptide drug discovery. NCL is therefore a fundamentally important enabling technology for peptide-based medicinal chemistry. Fully synthetic access to bioactive peptides and proteins allows a variety of chemical probes and molecule modifications to be introduced, leading to a vastly improved information flow for the study of bioactive peptides.



Scheme 1. Native chemical ligation; α-thioesters form an amide bond with N-terminal-cysteine-containing peptides.

NCL (Scheme 1) relies on the availability of a fundamental building block: the peptide α-thioester. NCL is a combination of two reaction steps in which 1) a C-terminal peptide α-thioester reacts with an N-terminal cysteine-containing peptide, resulting in trans-thioesterification and formation of a peptide thioester bond to the cysteine sulfur atom, and 2) a rapid intramolecular S- to N-peptidyl shift, which yields the two peptide fragments linked together by a native amide bond. NCL is chemoselective and takes place in aqueous solutions at pH 7, usually in the absence of protecting groups, and does not require a large excess of reactants for completion. Furthermore, thiol catalysts can be used to enhance the reaction rate. Since the initial discovery[3] of this methodology, many variations have been introduced, and a wide variety of potential ligation sites have been employed.[1b,4] Apart from NCL, C-terminal peptide thioesters, selenoesters,[5] and to some extent thioacids[6] are also very useful synthons, due to their high reactivity. Peptide thioesters have been successfully used as versatile reactive building blocks for dendrimers,[7] the synthesis of protein microarrays, and selective peptide C-terminal and N-terminal modifications.[8] Peptide thioesters transform readily with nucleophiles to generate esters, amides, hydrazides, hydroxamic acids, or under reductive conditions to peptide alcohols.[9] This versatility makes peptide thioesters important reactive intermediates in peptide and protein bioscience and drug discovery, e.g., for the introduction of labels[10] or for anchoring on

[a] Dr. A. Brust, Dr. C. I. Schroeder, Prof. P. F. Alewood
Institute of Molecular Bioscience
The University of Queensland, Brisbane, 4072 (Australia)
E-mail: a.brust@imb.uq.edu.au

solid supports.[11] As the synthetic potential of peptide thioesters has grown, there has been an ongoing search for efficient synthetic methods, although a high-throughput approach has yet to be established.

Classic Fmoc chemistry protocols[12] are unsuitable when in concert with thioester linkers due to premature cleavage by the highly nucleophilic piperidine. However, in the past 10 years, several developments have emerged to enable Fmoc chemistry to generate peptide thioesters.[13] These include the use of less nucleophilic cleavage cocktails,[14] approaches using thiol-labile safety catch linkers,[15] backbone linkers, and post-synthetic thioesterification,[16] activation of protected peptides in solution,[17] the application of O- to S-[18] and N- to S-acyl transfer[19] and also on-resin linker transformation into activated esters reactive toward thiolysis.[20]

Despite these efforts, the most effective approach for the synthesis of peptide α-thioesters remains the in situ neutralization chain assembly protocol for *tert*-butoxycarbonyl solid-phase peptide synthesis (Boc-SPPS)[12,21] using thioester linkers.[9,22] Whereas this well-established methodology allows access to high-quality material following a straightforward reaction pathway, the throughput is limited due to the necessity of a final hydrogen fluoride (HF) cleavage usually performed in a sequential, time-limiting fashion. This limitation has so far only been addressed by Houghten and co-workers, employing Boc chemistry in combination with an aqueous HF volatizable support, suitable for the production of peptide thioester libraries.[23]

Herein we present a more straightforward high-throughput approach for the synthesis of peptide α-thioesters that includes the inherent advantages of in situ neutralization Boc-SPPS[21c] on thioester linkers[9] combined with a safety catch amide linker (SCAL)[24] that is stable to HF cleavage. The SCAL[24] is highly stable in both TFA and HF, making it particularly useful for Boc chemistry applications. We have used this linker in previous work for the high-throughput production of conopeptide amide libraries employing Fmoc chemistry[25] as well as for the synthesis of bioisosteric diseleno analogues of α-conopeptides employing Boc chemistry.[26] Most recently, we used the SCAL to develop a high-throughput synthetic approach toward peptide turn mimetics.[27]

Results and Discussion

Peptide thioester synthesis on a SCAL

As the synthesis of peptide α-thioesters remains limited by low-throughput chemistry, we decided to remove this bottleneck by employing the SCAL. To achieve this we selected a standard aminomethylated polystyrene (PS) resin and loaded it with an Fmoc-SCAL using standard HBTU/DIEA activation. Fmoc deprotection with piperidine/DMF (50%) gave SCAL-AM-PS-resin **1**. Boc-glycine was then attached as a spacer on the SCAL followed by attachment of *S*-trityl-3-mercaptopropionic acid as the thiol linker (Scheme 2). The trityl group was subsequently removed with TFA/TIPS/water (90:5:5 *v/v/v*) resulting in resin **2** containing a free thiol moiety. This thiol was then used for the formation of the peptide thioester resin linkage of the synthesized peptide.

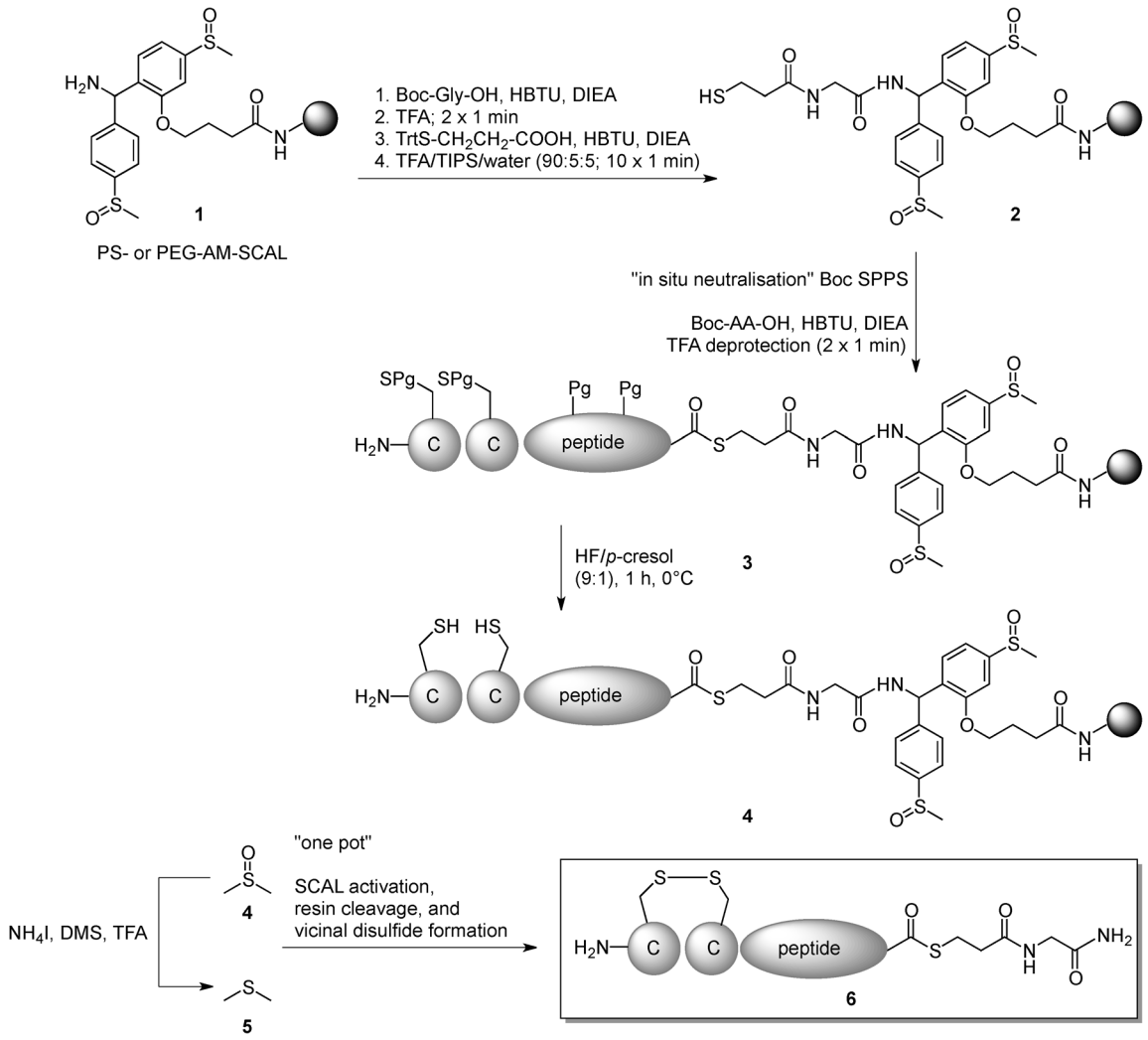
To evaluate the feasibility of the approach, test peptides were assembled (sequences: VQAAIDYING, **a**; RPPGFSPFR, **b**; and YGGFLRRIRPKLK, **c**; see Table 1), which were selected based on their difficulty of chain assembly as well as the diverse range of residues within their sequences, allowing potential amino acid side reactions to be observed. Peptide synthesis was performed on resin **2**, by employing in situ neutralization Boc-SPPS chemistry[12,21] with HBTU as activating reagent, throughout which led to the fully side-chain-protected peptidyl resins **3a**–**c**.

Table 1. Yield and purity of peptide α-thioesters **6a**–**g** synthesized on mercaptopropionic acid SCAL resin **2** after parallel HF cleavage. Intramolecular NCL resulted in the cyclization products obtained (**7d**–**f**, **8d**–**f** and **9**).

Sequence[a]	Entry	Purity [%][b]	Yield [%][c]	M_r [Da] calcd	found
VQAAIDYING*	**6a**	80	15.6/83[b]	1207.3	1207.6
RPPGFSPFR*	**6b**	81	14/78[b]	1204.4	1204.8
YGGFLRRIRPKLK*	**6c**	91	26/88[b]	1748.1	1748.4
CCVQAAIDYING*	**6d**	72	2.6/75[b]	1413.5	1411.8[d]
CCRPPGFSPFR*	**6e**	55	2.5/69[b]	1410.6	1408.8[d]
CCYGGFLRRIRPKLK*	**6f**	65	3.0/74[b]	1954.3	1953.2[d]
CTKSIPPICFPDGR*	**6g**	67	17/55[b]	1677.8	1678.0
cyclo[CCVQAAIDYING]	**7d**	37	0.3/10[c]	1249.4	1249.8
cyclo[CCRPPGFSPFR]	**7e**	35	0.5/12[c]	1246.5	1246.8
cyclo[CCYGGFLRRIRPKLK]	**7f**	30	0.4/14[c]	1790.3	1790.2
cyclo[CCVQAAIDYING]	**8d**	39	0.5/15[c]	1251.4	1251.8
cyclo[CCRPPGFSPFR]	**8e**	36	0.7/1[c]	1248.5	1248.8
cyclo[CCYGGFLRRIRPKLK]	**8f**	39	0.6/21[c]	1792.3	1792.2
cyclo[CTKSIPPICFPDGR]	**9**	35	1.8/12[c]	1513.8	1514.2

[a] *Thioester = S-CH_2CH_2-CONH-Gly-NH_2. [b] Purity of crude products is determined by the peak area of HPLC at 214 nm. [c] Yields are based on the weight of the crude product and are relative to the substitution of the resin; yield after purification (>95%) is relative to the weight of crude product used for cyclization. [d] Vicinal-disulfide-containing peptides were obtained after cleavage; underlined 'C' represents disulfide-bound cysteine residues.

A fragment of the acyl carrier protein, ACP(65–74) has been known as a difficult peptide (VQAAIDYING, **a**) due to strong chain aggregation and has been used in multiple studies as a synthetically challenging target.[28] During the chain assembly of ACP(65–74) (**a**) on resin **2**, no difficult couplings were encountered. The coupling yields were monitored using the quantitative ninhydrin test[29] and were close to quantitative within 2 min coupling times. Further test peptides synthesized on resin **2** were the nonapeptide bradykinin (RPPGFSPFR, **b**) and dynorphin(1–13) (YGGFLRRIRPKLK, **c**), both of which exhibited very good coupling yields throughout the sequence. The amino acids used were side chain protected with protecting groups commonly used in Boc-SPPS: Asn(Xan), Arg(Tos), Asp(OcHxl), Gln(Xan), Lys(ClZ), Tyr(BrZ), Thr(Bzl), and Ser(Bzl). As we wished to cyclize peptides **a**–**c**, cysteine residues were intro-



Scheme 2. General procedure for parallel synthesis of peptide α-thioesters (peptide sequences **3a**–**g** to **6a**–**g**, see Table 1): Thiol linker attachment via an HF-stabile safety catch amide linker (SCAL, **1**) allows for Boc-SPPS (**2**→**3**) and HF cleavage of multiple peptides, compartmentalized in tea bags, at one time (**3**→**4**). Fully unprotected peptides **4** still attached to the solid support are released from the linker via NH_4I/DMS/TFA treatment (**4**→**6** via **5**) due to reduction of the linker sulfoxide group (**4**→**5**), rendering the linker TFA-labile. Peptides containing a vicinal cysteine pair form a vicinal disulfide bond during SCAL cleavage; Pg = protecting group.

duced to allow intramolecular NCL. This was achieved by N-terminal extension of the test peptides ACP(68–75) (**a**), bradykinin (**b**) as well as dynorphin(1–13) (**c**) by coupling of two cysteine residues (Cys(4-MeBzl)) to the N terminus. Again, good coupling yields were observed during chain assembly employing in situ neutralization Boc-SPPS, resulting in fully protected peptidyl resins **3d**–**f** (Sequence: CCVQAAIDYING, **d**; CCRPPGFSPFR, **e**; and CCYGGFLRRIRPKLK, **f**).

Parallel HF side chain cleavage of peptide thioester resins

To achieve simultaneous parallel HF cleavage of the peptide thioester resins **3a**–**f**, it was necessary to ensure a suitable method of compartmentalization prior to selecting cleavage conditions, which would only remove side chain protecting groups, while avoiding premature peptide cleavage from the solid support. It was also essential to ensure that the products obtained are not contaminated by other peptides cleaved under the same conditions.

The assembled peptide-thioester resins **3a**–**f** obtained after N-terminal Boc-group removal and thorough washing with DMF and dichloromethane were dried in vacuum and weighed. The dried peptide resins were then compartmentalized employing tea bags.[30] These were made from polypropylene mesh sheets (Cole–Parmer, 105 μm pore size), as they can be heat welded to size as required. The optimum HF cleavage conditions were elaborated by performing multiple cleavage experiments on small (10 mg) peptidyl-resin samples (**d**–**f**) using *p*-cresol and *p*-thiocresol in 2–10% overall quantity and variable ratios. These experiments have shown that *p*-cresol[31] is the most suitable scavenger, avoiding premature cleavage from the resin. Inclusion of *p*-thiocresol led to a partial reduction of the SCAL, resulting in some release of peptide thioester (**6a**–**f**) during HF cleavage. The optimum cleavage conditions were found to be 5% vol *p*-cresol in HF with a cleavage time

of 1 h at 0 °C. For the final large-scale cleavage, all tea bags containing peptide (**3a–f**) resin were transferred into a single HF cleavage reactor. The cleavage was undertaken with 2.5 mL of *p*-cresol,[31] in 50 mL HF at 0 °C for 1 h. After evaporation of the excess HF under vacuum, the tea bags were washed with cold diethyl ether (2×) and dichloromethane (2×) to remove residual *p*-cresol and protecting group quencher adducts. The organic wash fractions were collected, concentrated, and investigated by LC–ESMS. No peptide thioester (**6a–f**) were detected, indicating that the SCAL as well as the thioester linkage is sufficiently stable under the selected reaction conditions.

After removal of excess solvent under vacuum, the tea bags containing the dried unprotected peptide resins (**4a–f**) were transferred into separate glass reactors. SCAL activation by reduction of the sulfoxide group was performed as previously described using NH_4I/DMS in TFA.[25] The resulting peptidyl resins (**5a–f**) are labile in TFA, and the peptides were released from the resin into solution over a period of 18 h. The TFA solution was transferred into cold diethyl ether resulting in precipitation of the desired peptide thioesters **6a–f**. Repeated centrifugation and washing steps with cold diethyl ether resulted in crude peptide, which was readily dissolved in acetonitrile/water/0.1% TFA (50%) freeze-dried, weighed, and analyzed by ESI LC–MS (Table 1). No cross-contamination of the crude peptides with other peptide thioesters was observed, demonstrating that the methodology is well suited for parallel peptide thioester library production. All peptides synthesized were obtained in good yield and purity. The HPLC traces of the obtained crude peptide thioester **6a–f** are shown in Figure 1,

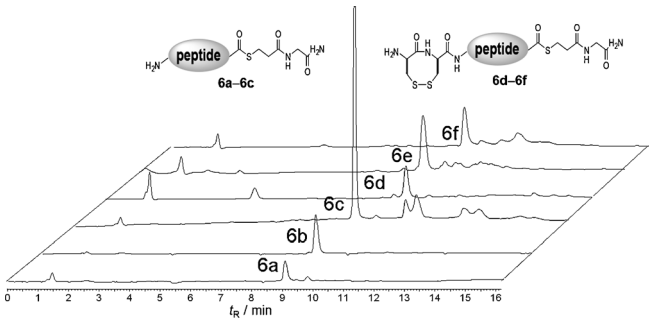


Figure 1. HPLC traces of obtained crude peptide α-thioesters **6a–f**.

and results are summarized in Table 1. The SCAL cleavage yields, obtained for peptide thioesters **6a–f**, were strongly dependent on cleavage time. Crude peptide workup after 2, 6, and 18 h showed that the highest yields are obtained after overnight cleavage. This long cleavage time did not influence the quality of the obtained peptide thioester. No side products were formed due to extended NH_4I/DMS/TFA treatment which is consistent with previous observations that peptide thioesters are stable under the selected SCAL cleavage conditions.[32]

Cysteine oxidation during NH_4I/TFA treatment has been described previously.[25,33] Vileseca et al.[33] obtained disulfide folded peptides as main products, whereas we observed oxidation during SCAL cleavage, in particular with peptides containing vicinal cysteine residues.[25,27] This was also observed for peptides **6d–f** (Figure 1). In all cases, the formation of a vicinal disulfide bond was observed, and the peptide thioester products **6d–f** were formed preferentially in the disulfide form, as was confirmed by mass spectrometry (Table 1). This oxidation is attributed to the formation of an iodosulfonium cation intermediate during NH_4I sulfoxide reduction.[25,33] Further disulfide formation promoters are likely trace amounts of iodine formed during the cleavage step.[25,33] Iodine in the presence of acid is an excellent reagent for disulfide bond formation. Furthermore, DMSO is also formed during the NH_4I reduction from the DMS scavenger used,[25,33] and DMSO in neat TFA is also an excellent reagent for disulfide formation.[25]

Intramolecular NCL of peptide thioesters 6d–f

To confirm that peptide thioesters **6d–f** were useful as building blocks for NCL chemistry, cyclization experiments were performed. To evaluate if the impurities resulting from the SCAL cleavage cocktail may influence the ability of the obtained thioester to be used in ligation reactions, we performed cyclization using crude peptide thioesters **6d–f**. Peptide thioesters **6d–f** were dissolved in NH_4HCO_3 buffer (pH 7.4), and the progress of the cyclization was monitored by LC–MS analysis. A single reaction product was formed with a molecular mass of 162 ± 0.5 amu less than the starting thioester, indicating intramolecular cyclization with loss of a S-$(CH_2)_2$-CO-Gly-NH_2 moiety. In all experiments, cyclization was complete within 3 h. No residual starting thioester was observed, suggesting that the vicinal disulfide bond must undergo partial reduction to consequently enable intramolecular ligation. In addition, the release of the thiol linker autocatalyzes disulfide–thiol exchange, thus enabling intramolecular "thia zip"[34] NCL, driving the equilibrium toward the cyclized products (Figure 2). The final products obtained are cyclic peptides containing a backbone amide linkage and a disulfide bond formed between the vicinal cysteine residues (**7d–f**). Alternatively, peptides **6d–f** were also cyclized in the presence of an additional thiol catalyst, thought to help vicinal disulfide–thiol exchange as well as trans-thioesterification, which may be required to achieve more efficient cyclization. Under these conditions, (MESNA, 5 equiv NH_4HCO_3 solution, pH 7.4) cyclization was accelerated, and cyclized peptide was obtained with the difference that the disulfide bond was not formed due to the excess thiol used. The cyclic reduced peptides **8d–f** were obtained as main products under these conditions. Overall, a loss of 160 ± 0.5 amu was observed, consistent with the loss of the thioester moiety (S-$(CH_2)_2$-CO-Gly-NH_2) and the gain of two protons due to disulfide bond reduction. For all cyclic peptides (**7d–f** and **8d–f**), hydrolysis experiments with 0.1 M NaOH solution were performed and monitored by LC–MS. In none of the investigated cyclic products was hydrolysis ($\Delta m = +18$) observed. This lead us to the conclusion that all products were backbone cyclized through an amide bond with the intramolecular thioester intermediates absent.

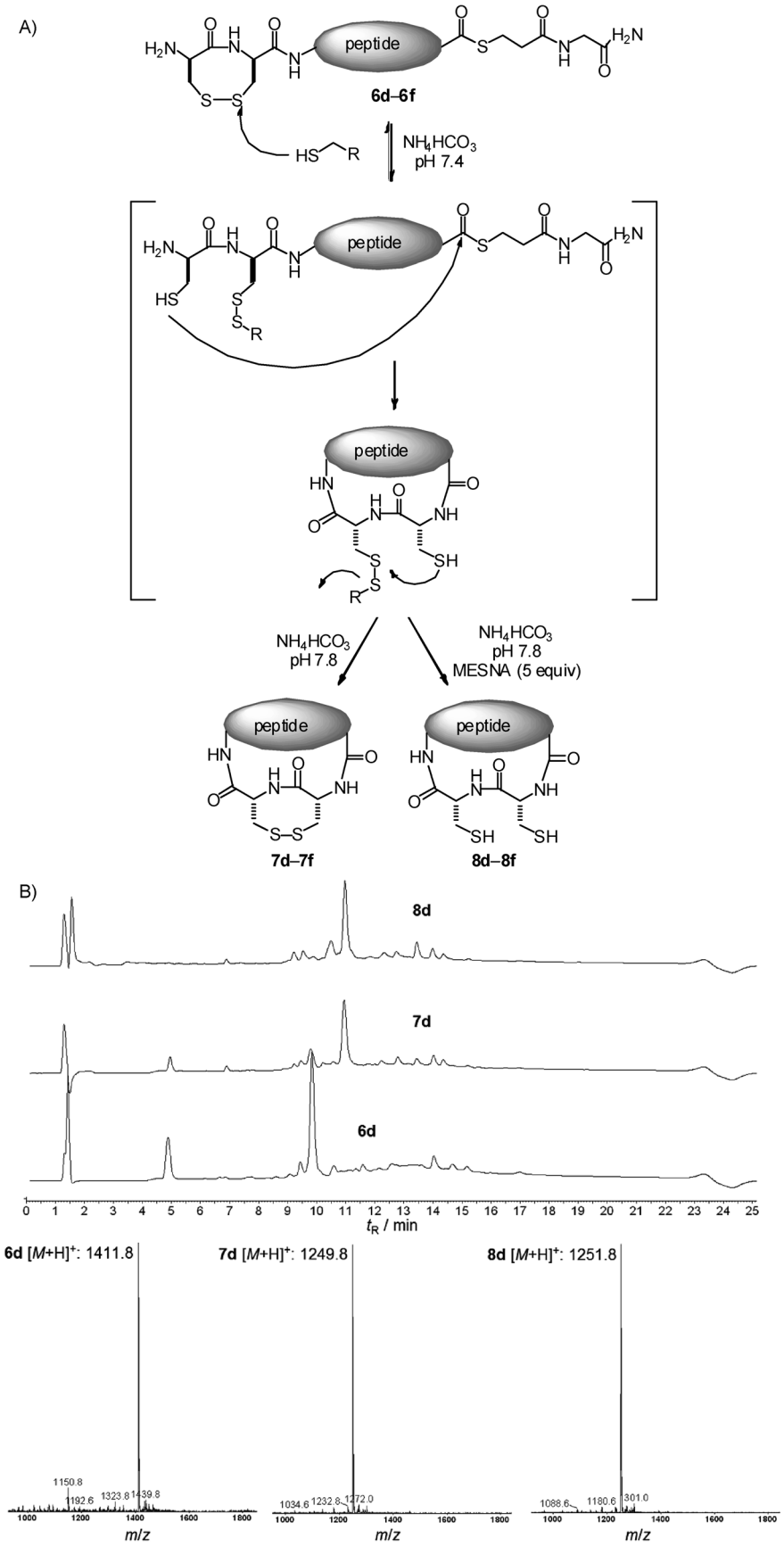


Figure 2. A) Cyclization of peptide α-thioesters **6d–f** via intramolecular native ligation: free thiol from thiol linker or added as MESNA catalyzes vicinal disulfide thiol exchange, thereby facilitating cyclization. Cyclized and vicinal disulfide-containing peptides **7d–f** are formed in aqueous buffer at pH 7.4, versus formation of cyclized and reduced cysteine-containing peptides **8d–f** with the thiol MESNA in excess. B) HPLC–MS monitoring of cyclization of peptide α-thioester **6d** ($[M+H]^+$ 1411.8), yielding simultaneous cyclized and oxidized peptide **7d** ($[M+H]^+$ 1249.8) if reacted in a 0.1 M NH_4HCO_3 buffer at pH 7.4. In contrast, in the presence of excess MESNA (5 equiv), cyclic reduced peptide **8d** ($[M+H]^+$ 1251.8) is formed.

Synthesis of SFTI-1 (9)

The sunflower trypsin inhibitor SFTI-1 (**9**) is a 14-residue cyclic peptide cross-braced with one disulfide bond and a network of hydrogen bonds, giving it a well-defined structure.[35] SFTI-1, originally isolated from sunflower seeds, is a very potent inhibitor of trypsin and other proteases. The inhibition of trypsin-like enzymes, in particular serine proteases, is of great interest in medicinal science, due to implications in cancer propagation.[36] These findings have initiated the search for novel serine protease inhibitors as potential cancer therapeutics. This has prompted the need for rapid access to combinatorial libraries of SFTI-1 analogues. Multiple approaches have been used to synthesize such libraries.[35,37] The novel technology presented in this work enables improved access to libraries of cysteine-rich cyclic peptides and demonstrates the potential of the approach for combinatorial-chemistry-based drug discovery.

The synthesis of SFTI-1 (**9**) was performed on the aminomethyl-PEG based resin (**1-ChemMatrix**) resulting in superior product quality. This resin has been used in our hands very successfully for difficult sequences[28d] as well as for the synthesis of selenocys-

teine-containing α-conopeptides.[26] The ChemMatrix resin was pretreated in DMF overnight, and a sequence of three glycine residues was attached to improve the handling of the resin. After attachment of the SCAL followed by a further glycine residue, the mercaptopropionic acid linker was attached. The synthesis of SFTI-1-thioester **3g** was performed using in situ neutralization Boc-SPPS[12] employing HBTU as the coupling reagent with good yields throughout. The sequence was modified to allow for NCL with an N-terminal cysteine and an arginine residue adjacent to the thioester (sequence: CTKSIPPICFPDGR). After completion of chain assembly, the resin was transferred into a tea bag, and HF cleavage was performed (**3g**→**4g**). The SFTI-1 resin **4g** was then treated with NH_4I/DMS/TFA for 4 h to cleave the SCAL and generate the SFTI-1 thioester **6g** in a moderate yield of 55%. In contrast to the PS-based resin used for the synthesis of **3a**–**f**, extended cleavage times did not improve the yield. The cyclization of crude SFTI-1-thioester **6g** was performed in NH_4HCO_3 solution (pH 7.6) and did not require a thiol catalyst (Figure 3), giving the fully oxidized SFTI-1 **9**, which co-eluted with an authentic sample.[35] To further confirm the identity of the product **9**, NMR was used to compare the secondary Hα chemical shifts with wild-type SFTI-1 and demonstrated the structural identity of both molecules (Figure 4). Furthermore, the activity of product **9** as a protease inhibitor was confirmed by using a colorimetric assay[38] with the substrate L-BAPNA, in which the product **9** produced the same protease inhibition as did wild-type SFTI-1.[38b]

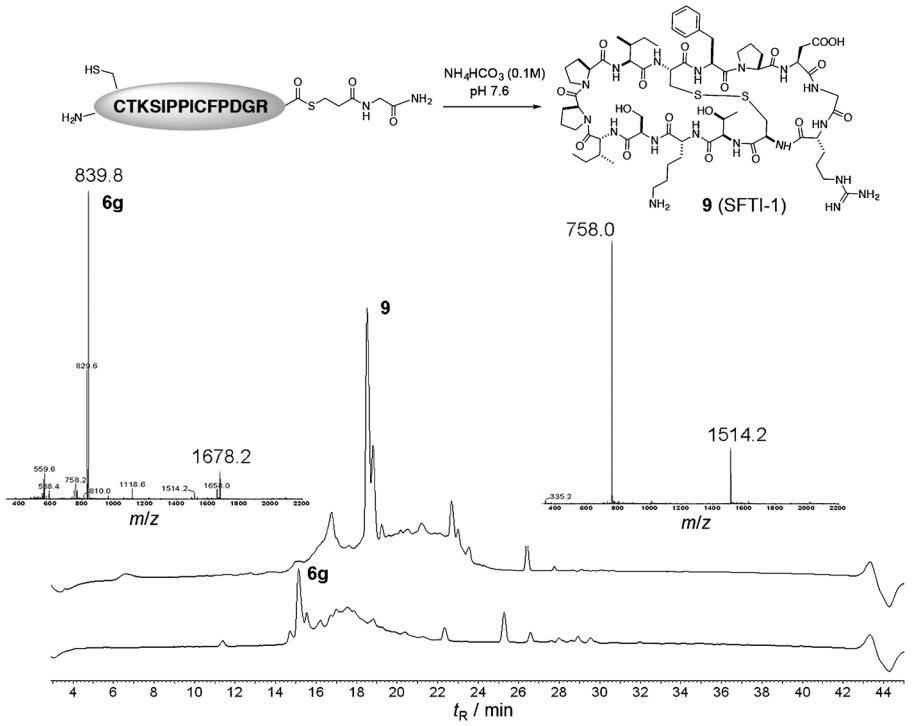


Figure 3. Cyclization of crude peptide α-thioester **6g** ($[M+H]^+$ 1678.2) in 0.1 M NH_4HCO_3 buffer at pH 7.6 yielding simultaneous cyclized and oxidized peptide **9** (SFTI-1, $[M+H]^+$ 1514.2).

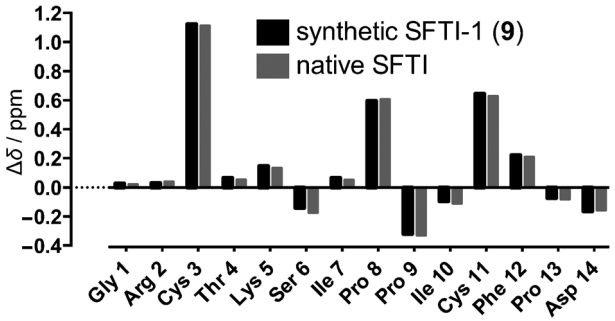


Figure 4. Comparison of the secondary α-shift NMR data of synthesized SFTI-1 (**9**) with wild-type SFTI-1.

Conclusions

A general method for the synthesis of peptide α-thioester libraries has been established by employing a safety catch amide linker (SCAL)[24] and compartmentalizing the peptide resin during HF cleavage. The peptide thioesters synthesized containing additional cysteine residues were subsequently readily cyclized employing intramolecular NCL. The synthetic technology was then applied to the synthesis of the biologically important sunflower trypsin inhibitor 1 (SFTI-1, **9**), a lead molecule for the development of serine protease inhibitors with potential for cancer therapy. In summary, we have successfully developed a technology to remove the bottleneck of peptide thioester synthesis, making access to cyclic peptide libraries highly feasible.

Experimental Section

Materials and methods

All solvents and reagents were obtained commercially and were used without further purification. N^{α}-Boc-L-amino acids were purchased from NovaBiochem (Merck Pty., Kilsyth, Vic., Australia). The following side chain protected Boc-amino acids were used: Arg(Tos), Asn(Xan), Asp(Chxl), Cys(4-MeBzl), Gln(Xan), Tyr(2-BrZ), Thr(Bzl), Trp(For), Ser(Bzl), Lys(2-ClZ). *N*,*N*-Dimethylformamide (DMF), diisopropylethylamine (DIEA), and trifluoroacetic acid (TFA) were all peptide synthesis grade supplied by Auspep P/L (Melbourne, Australia). 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), ammonium iodide, triisopropylsilane (TIPS), dimethyl sulfide (DMS), HPLC-grade acetonitrile, diethyl ether, methanol, bovine trypsin, *N*-α-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) and scavengers *p*-cresol and *p*-thiocresol were supplied by Sigma–Aldrich (Australia).

Anhydrous HF gas was purchased from BOC Gases (Sydney, NSW, Australia). Aminomethyl polystyrene hydrochloride resin (loading = 0.17 mmol g^{-1}, 100–200 mesh, 1% DVB) was purchased from Nova-Biochem (Merck Pty., Kilsyth, Vic., Australia). Aminomethyl ChemMatrix hydrochloride resin (loading = 0.74 mmol g^{-1}, 35–100 mesh) was purchased from Matrix Innovation (Montreal, QC, Canada). Fmoc-SCAL was purchased from CSPS Pharmaceutical (San Diego, CA, USA). Polypropylene mesh sheets for the assembly of tea bags were purchased from Cole–Parmer (Vernon Hills, IL, USA).

HS-CH_2CH_2-CO-Gly-SCAL-AM-PS resin (2-PS)

Aminomethyl polystyrene hydrochloride resin (5 g, 0.17 mmol g^{-1}) was conditioned overnight in DMF. After washing with DMF, the resin was neutralized (1 min) with a 10% solution of DIEA in DMF (10 mL, 2×5 min). Fmoc-SCAL (1.74 g, 2.7 mmol) was dissolved in 5.4 mL of a solution of HBTU in DMF (0.5 M, 2.7 mmol). After activation with 470 μL (2.7 mmol) DIEA, the linker mixture was added to the resin and coupled overnight. After Fmoc removal with piperidine (50% in DMF, 2×2 min), Boc-glycine (2.7 mmol) was coupled using HBTU/DIEA activation. After Boc deprotection with neat TFA, the 3-trityl-mercaptopropionic acid linker (2.7 mmol) was attached by HBTU (2.7 mmol)/DIEA (3.0 mmol) activation, followed by the removal of trityl protection by treatment with a solution of 5% TIPS and 5% water in TFA (10×1 min). The HS-$(CH_2)_2$-CO-Gly-SCAL-AM-PS resin (**2-PS**) with a substitution value (SV) of 0.1 mmol g^{-1} was used for the synthesis of peptide thioester **6a–f**. The obtained SV was determined by using the quantitative ninhydrin test.[29]

HS-CH_2CH_2-CO-Gly-SCAL-(Gly)$_3$AM-ChemMatrix resin (2-ChemMatrix)

Aminomethyl-ChemMatrix hydrochloride resin (1 g, 0.74 mmol g^{-1}) was conditioned overnight in DMF. By employing in situ neutralization Boc chemistry,[12] a triple glycine sequence (3×2.7 mmol) was attached consecutively to achieve a less sticky resin that allowed for better handling during ninhydrin testing.[29] Identical conditions were employed as for the assembly of resin **2-PS** to attach the SCAL (2.7 mmol) as well as the thiol linker (2.7 mmol, see above). The HS-$(CH_2)_2$-CO-Gly-SCAL-Gly$_3$AM-ChemMatrix resin (**2-ChemMatrix**, SV = 0.47 mmol g^{-1}) was used for the synthesis of peptide thioester **6g**.

Peptide synthesis

The chain assembly of the peptides (0.1 mmol resin used) was performed on a manual shaker system using Boc-amino acid (0.5 mmol)/HBTU (0.5 mmol)/DIEA (0.6 mmol) activation and in situ neutralization protocols[12] to couple the Boc-protected amino acid to the resins (**2**) prepared above. The Boc protecting group was removed using 100% TFA (2×), and DMF was used as both the coupling solvent and for flow washes throughout the cycle. The progress of the assembly was monitored by quantitative ninhydrin assay.[29]

Parallel HF side chain deprotection

After completion of assembly of peptides **3a–g**, subsequent removal of the N^{α}-Boc group, using TFA (2×1 min) and flow-washes with DMF, CH_2Cl_2, and drying under nitrogen, the peptide resin was transferred into individual tea bags. All tea bags were transferred into one HF reactor tube. The HF cleavage, which removes the side chain protecting groups, but does not cleave the peptide from the SCAL, was carried out in apparatus supplied by the Peptide Institute (Osaka, Japan) using 50 mL HF covering all tea bags and scavenger (*p*-cresol, 5% vol) for 1 h at 0 °C. After removal of excess HF, the peptide resin tea bags were washed with diethyl ether and CH_2Cl_2 and dried under vacuum.

SCAL activation/cleavage

The washed and dried peptidyl-CO-S-$(CH_2)_2$-CO-Gly-SCAL-AM-PS resin or peptidyl-CO-S-$(CH_2)_2$-CO-Gly-SCAL-Gly$_3$-AM-ChemMatrix resins (**4a–g**), still in the tea bags, were separated into individual cleavage vessels. To achieve safety catch linker activation 50 mg of NH_4I, 100 μL DMS and 2.5 mL neat TFA were added. The cleavage was performed, while shaking, for 18 h (PS-based resin) or 4 h (ChemMatrix resin) at RT. After SCAL activation/cleavage the peptide solution was filtered, and cold diethyl ether (30 mL) was added to the cleavage mixtures resulting in precipitation of the respective peptide thioester (**6a–g**). The precipitate was collected by centrifugation and subsequently washed with further cold diethyl ether (2×20 mL). The final product was dissolved in 50% aqueous acetonitrile/0.1% TFA (30 mL) and lyophilized to yield a white solid. The crude peptide thioesters (**6a–g**) were examined by reversed-phase HPLC for purity, and the correct molecular weight confirmed by electrospray ionization mass spectrometry (ESIMS). For obtained crude yields and LC–MS data, see Table 1.

Cyclisation of peptide thioester 6d–f

The crude peptide thioesters **6d–f** were cyclized employing intramolecular native chemical ligation under two different conditions:

A: cyclization in 0.1 M NH_4HCO_3 (pH 7.6) was performed at a peptide concentration of 1 mg mL^{-1}. The progress of the cyclization was monitored by LC–MS. The cyclization resulting in a loss of an S-$(CH_2)_2$-CO-Gly-NH_2 moiety ($\Delta m = -162$ amu) was completed within 3 h, yielding a single main product (**7d–f**), the backbone amide cyclized with a disulfide bond formed as well.

B: cyclization in 0.1 M NH_4HCO_3 in the presence of MESNA (5 equiv) with a peptide concentration of 1 mg mL^{-1}. The progress of the cyclization was monitored by LC–MS. ($\Delta m = -160$ amu). The cyclization was completed within 2 h, yielding a single main product identified as backbone cyclized peptide with reduced cysteine (**8d–f**). For obtained crude yields and MS data, see Table 1.

Cyclization of peptide thioester (6 g) to SFTI-1 (9)

Crude peptide thioester (**6g**, 17 mg) was dissolved in 20 mL 0.1 M NH_4HCO_3 solution (pH 7.6) and stirred at room temperature. Monitoring by LC–MS indicated a straightforward cyclization and oxidation to SFTI-1 (**9**) within 3 h ($\Delta m = -164$ amu). For obtained yields and MS data, see Table 1.

HPLC analysis and purification

Analytical HPLC was performed using a Shimadzu HPLC system LC10A with a dual-wavelength UV detector set at 214 and 254 nm. A reversed-phase C_{18} column (Hypersil Gold C_{18}, 3 μm, 100 mm × 2.1 mm) was used at a flow rate of 0.3 mL min^{-1}. Gradient elution was performed (40 °C) with the following buffer systems: **A**, 0.05% TFA in water and **B**, 0.043% TFA in 90% acetonitrile in water, from

0% **B** to 80% **B** in 20 min. Absorbance was monitored at 214 and 254 nm, and crude purities are given by peak areas at 214 nm.

Peptides were purified by preparative HPLC on a Shimadzu HPLC system associated with a reversed-phase C_{18} column (Vydac C_{18}, 25 cm × 25 mm) at a flow rate of 15 mL min^{-1} with a 0.5% per min increase in buffer **B** concentration from 10–60% **B**. The purity of the final products was evaluated by analytical HPLC (Hypersil C_{18}, 130 Å, 5 μm, 250 mm × 4.6 mm, 1 mL min^{-1} flow rate, gradient 10% **B** to 60% **B** in 50 min).

ESIMS

Electrospray ionization mass spectra were collected inline during analytical HPLC runs on an Applied Biosystems, quadrupole spectrometer (API-150) operating in the positive ion mode with a declustering potential (DP) of 20 V, a focusing potential (FP) of 220 V, and a turbospray heater temperature of 350 °C. Masses between 300 and 1800 amu were detected (Step 0.2 amu, Dwell 0.3 ms).

NMR spectroscopy

NMR is a sensitive technique that detects minor local variations in the chemical environment for particular parts of molecules. For peptides, this is routinely done by comparing the Hα chemical shifts with chemical shifts derived from a random coil.[39] The resulting secondary Hα chemical shifts for SFTI synthesized in this work are subsequently compared with those derived from native SFTI isolated from sunflower using 1D and 2D NMR. This provides a sensitive method to determine if the backbone Hα protons are experiencing the same chemical environment, that is, if their structures are the same without having to carry out a full 3D solution structure determination. NMR measurements were carried out on peptide samples prepared in 90% H_2O/10% D_2O (Cambridge Isotope Laboratories, Andover, MA, USA) at ~1 mM and pH 4 on a 600 MHz Bruker Avance NMR spectrometer at 298 K. Spectra recorded were 1D ^{1}H and 2D TOCSY[40] spectra; 2D spectra were recorded in phase-sensitive mode using time-proportional incrementation for quadrature detection in the t_1 dimension.[41] Water suppression was achieved using excitation sculpting gradients.[42] TOCSY spectra were acquired with 4096 data points in the F_2 dimension and 512 data points in the F_1 dimension. The t_1 dimension was zero-filled to 1024 real data points. Spectra were processed using TopSpin (Bruker) and assigned in Xeasy[43] using the sequential assignment protocol.

Bioassays

N-α-Benzoyl-L-arginine 4-nitroanilide hydrochloride,[38] (BAPNA) is a colorless chromogenic trypsin substrate that turns yellow when hydrolyzed, and light absorption can be determined at 410 nm. BAPNA is commonly used as a measurement of trypsin inhibition activity, and the readout is the percent inhibition of trypsin activity displayed by the inhibitor, in this case SFTI, compared with a control, such as buffer. The inhibitory activity of SFTI-1 was assayed in a 96-well plate against bovine trypsin using the colorimetric trypsin substrate L-BAPNA.[38] Briefly, SFTI-1 (**9**) was diluted from a stock of 0.1 mM in 0.05 M Tris·HCl (pH 8.2) with 0.02 M $CaCl_2$ (assay buffer). Trypsin was dissolved in 1 mM HCl to a concentration of 4.5 mg mL^{-1}. Immediately prior to use, the trypsin was diluted 1:10 in the assay buffer. L-BAPNA (0.435 mg mL^{-1}) was dissolved in assay buffer with 1% DMSO. Peptide (15 μL, at various concentrations), and trypsin (5 μL) were added to the plate followed by 125 μL L-BAPNA. The plate was incubated in the dark for 10 min, the reaction stopped by addition of 25 μL 30% acetic acid, and the plate was read at 410 nm using a fluorescence plate reader.

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