Biomimetic Synthesis of Cyclic Peptides using Novel Thioester Surrogates

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ABSTRACT:

Acyl shifts involving N-S and S-S rearrangements are reactions central to the breaking of a peptide bond and forming of thioester intermediates in an intein-catalyzed protein splicing that ultimately leads to the formation of a new peptide bond by an uncatalyzed S-N acyl shift reaction. To mimic these three acyl shift reactions in forming thioesters and the subsequent peptide ligation, here we describe the development of two 9-fluorenylmethoxycarbonyl (Fmoc)-compatible thioester surrogates that can undergo uncatalyzed N-S, S-S and S-N acyl shifts for preparing thioesters and cyclic peptides. These surrogates were incorporated as a C-terminal amido moiety of a target peptide using Fmoc chemistry by solid-phase synthesis, and then transformed into a thioester or thiolactones via two acyl shift reactions with or without the presence of an external thiol under acidic conditions. The proposed intein-mimetic thioester surrogates were prepared using readily available starting materials including N-methyl cysteine or 2-thioethylbutylamide. A key functional moiety shared in their design is the thioethylamido moiety, which is essential to effect a proximity-driven N-S acyl shift under a favorable five-member ring transition in the breaking of a peptide bond. Thus, the tandem series of acyl shifts effected by a thioethylamido moiety in a thioester surrogate together with a thioethylamino moiety of an N-terminal Cys residue in a linear peptide precursor are chemical mimics of an intein, as they mediate both excision and ligation reactions in forming cyclic peptides including cyclic conotoxin and sunflower trypsin inhibitor described herein.

Keywords: thioester, thioethylamido, thioester surrogate, N-S acyl shift, intein mimetic, cyclic peptides, conotoxin, sunflower trypsin inhibitor

INTRODUCTION

Cyclic peptides, naturally occurring or synthetic, are generally referred to peptides with an end-to-end or head-to-tail linkage of the peptide backbone through an amide bond. Consequently, cyclic peptides without ends are resistant to degradation by exopeptidases and are more conformationally restricted than their linear counterparts. These advantages have long been recognized in drug design to increase the metabolic stability of a bioactive linear peptide.[1] For a synthetic cyclic peptide derived from a linear peptide, there is an additional advantage as a neo-epitope can be created by joining the amino acids of the N- and C-termini. Such an epitope can become a new bioactive fragment and can be exploited in the drug design of a peptide biologic.

For preparing cyclic peptides, a common approach is to use unprotected peptides containing a thioester.[2] Thioesters are key functional groups in various synthetic strategies for peptide cyclization and ligation.[3-8] Over the years, different methods for preparing thioesters or their precursors compatible with *t*-butoxycarbonyl (Boc) and fluorenylmethoxycarbony (Fmoc) chemistries have been developed for solid-phase synthesis of peptides and proteins. Thioesters are susceptible to base and intolerant to repetitive piperidine deprotection steps in Fmoc chemistry. Boc chemistry, which utilizes repetitive acid deprotection steps, is an effective approach for preparing peptide thioesters by assembling a peptide chain directly on a thioester linker functionalized on resin supports. However, the repetitive use of trifluoroacetic acid in deprotection steps may pose problems in preparing glyco- or phosphopeptides. In addition, the use of HF in the final cleavage steps requires a specialized apparatus and safety regulations, which limit the extensive application of Boc chemistry for preparing peptide thioesters. At present, Fmoc chemistry is the method of choice for solid-phase syntheses of many peptides. These needs have prompted strong interests in the development of Fmoc-compatible schemes for preparing thioesters.

Fmoc-compatible methods of preparing peptide thioesters include mild basic deprotection cocktails,[9] base-tolerant thioester linkers,[10] "safety-catch" auxiliaries,[11-15] use of a partially protected peptide,[16] and amide- or ester-based surrogates that can be transformed into thioesters via an N- or O-S acyl shift reaction, respectively. In recent years, the approach using amido-thioester surrogates has gained popularity and examples include N-4,5-dimethoxy-2-mercapto-benzyl (Dmmb),[17] N-alkylated Cys,[18] bis-(sulfanylehtylamino) (SEA) [19] or bis-(mercaptoethyl) amide (BMEA), [20] thio-proline,[21] and thio-thiazolidine.[22]

A particularly appealing aspect of the amide-based thioester surrogates is that they are generally stable to both Boc- and Fmoc-chemistries in solid-phase synthesis. A functional group essential in the amido thioester surrogate approach is the thioethylamido moiety (TEA) to effect a proximity-driven N-S acyl shift reaction via a five-member ring transition state to form the desired thioester. A similar functional group, the thioethyl amino moiety of an N-terminal cysteine is the key moiety to drive the reverse reaction on the cysteine-thioester ligation via an S-N acyl shift. Thus, the interconversion of the TEA and TE-amino moieties represents the reversible reactions of a X-Cys bond to form a thioester or an amide bond, a principle which has been exploited in the general design of amido thioester surrogate methods. However, current methods in preparing these TEA-based thioester surrogates are fairly complicated, and a simple and practical method or a new design of TEA-based thioester surrogates would be desirable.

As previously discussed, the amido-thioester formation via an N-S acyl shift is a reverse reaction of peptide ligation. It is thermodynamically unfavorable and requires a *cis* conformation of a tertiary X-Cys amide bond to undergo an N-S acyl shift, often under acidic conditions. Furthermore, the general scheme of a tertiary amide-based thioester surrogate to form a cyclic peptide bears a certain similarity to protein splicing, which undergoes a series of acyl shift reaction including an intramolecular N-S acyl shift to form a thioester (Figure 1). In particular, a C-terminal amido-thioester surrogate mimics the overall effect of an

intein-mediated splicing process to give an end-to-end cyclic peptide via a series of intramolecular acyl shift reactions, ending with the excision of the thioester surrogate.

Intein-mediated splicing involves four acyl shifts to excise an intein from its flanking exteins and rejoin the N- and C-exteins by a peptide bond (Figure 2a).[23-25] The first reaction is an N-S acyl shift to break the peptide bond at the N-splicing site of the N-extein/intein junction, forming a branched intermediate in which the N-extein is linked to the side chain of the N-terminal Cys of intein by a thioester bond. This is followed by an S-S acyl shift, a thioester exchange reaction, between the N-terminal Cys of C-extein and the newly formed thioester bond at the N-splicing site, resulting in the formation of the second branched intermediate. To excise the intein from this branched intermediate, an N-N acyl shift occurs at the C-terminal Asn residue of an intein to yield the third branched intermediate of N- and C-exteins linked by a thioester bond. The final S-N acyl between the N-terminal Cys of the C-extein and the newly formed thioester is uncatalyzed and results in a new peptide bond formation between the N- and C-exteins to complete the splicing process. Thus, the N-terminal Cys of both intein and C-extein, play a key role in the breaking and making of peptide bonds through the N-S, S-S and S-N acyl shift reactions, whereas the thioethylamido (TEA) moieties at the splicing sites play the essential role in these acyl shift reactions.

The presence of a thioethylamino moiety as an N-terminal Cys together with a C-terminal TEA-containing thioester surrogate in a cysteine-rich peptide makes it an ideal candidate for macrocyclization through a series of N-S, S-S and S-N acyl shift reactions (Figure 2). In addition, a cysteine-rich peptide precursor would provide a choice of multiple available cyclization sites. Moreover, the side-chain thiols of cysteine-rich peptides can replace the role of external thiols in the S-S acyl shift reaction to give a series of thiolactone intermediates instead of a C-terminal thioester. The N-terminal cysteine would eventually join the C-terminus of the peptide precursor by expansion of thiolactone rings through reversible S-S exchange reactions, leading to a S-N acyl shift reaction to afford a new N-to-C peptide bond to form an end-to-end macrocycle. This approach of entropy-driven thiolactone ring expansion accelerates peptide cyclization and has been known as the thia zip reaction in preparing cyclic peptides.[8,26-28] Thus we envisioned that, in the synthesis of end-to-end cyclic peptides, a C-terminal TEA thioester surrogate can mimic the role of intein in protein splicing to mediate N-S, S-S and S-N acyl shifts, leading to both thioester formation and ligation of the N- and C-termini as an end-to-end peptide macrocycle as well as their excision from the precursor sequence (Figure 1 and 2b).

Here we report the development of two TEA thioester surrogates that are compatible with Fmoc chemistry (Figure 3). Both surrogates were prepared using readily available starting materials from commercial sources, Fmoc-MeCys(Trt) and 2-thioethylbutylamine. They were readily attached to a highly acid-labile resin support functionalized by amine (Rink amide resin) or chlorotrityl chloride, to form MeCys and thioethylbutylamido (TEBA) resins, respectively. The MeCys or TEBA moieties on these resins served as the starting point for a solid-phase synthesis using Fmoc chemistry. They also served as the C-terminal residue of a peptide precursor that can be transformed into a peptide thioester (Figure 2b). N-alkyl cysteine derivatives have been previously reported by Hojo *et al.*[18] However, MeCys as a thioester surrogate has not been studied in detail or used for synthesis of cyclic peptides. Similarly, we reported the conformationally-constrained thiomethylthiazolidine (TMT) carboxylic acid as an amido-thioester surrogate.[22] Both MeCys and TMT resins have limitations, which led us to the development of the simplified TEBA thioester surrogates for preparing cyclic peptides.

RESULTS AND DISCUSSION

Two bioactive peptides were used to illustrate the efficacy of the proposed TEA-based thioester surrrogates. The family of ω-conotoxins is a potent *N*-type calcium channel inhibitor secreted by marine

cone snails.[29] They contain three conserved disulfide bonds in a cystine-knot motif. Synthetic ω-conotoxin MVIIA is 1000-fold more potent than morphine and a FDA-approved analgesic drug for the management of chronic and neuropathic pain.[30,31] However, ω-conotoxin MVIIA has low metabolic stability, which might be enhanced by backbone cyclization as an end-to-end macrocycle. Thus we designed a prototype of cyclic ω-conotoxin cCG29 based on the 25-residue ω-conotoxin MVIIA to improve its metabolic stability (Figure 4). The cyclic analog cCG29 (CKGKGAKCSRLMYDCCTGSCRSGKC-GGPG) was designed by adding a flexible four-residue linker GGPG at the C-terminus as a spacer to facilitate its end-to-end cyclization. The choice of a Gly-rich linker also allowed it to be used as a cyclization site. The commercially available Fmoc-MeCys(Trt) was used as a thioester surrogate in the solid-phase synthesis of cCG29 as a model peptide mimicking an intein for preparing cyclic peptides.

The second bioactive peptide selected in this study was sunflower trypsin inhibitor (SFTI-1). SFTI-1 is a naturally-occuring cyclic peptide and is one of the smallest and most potent trypsin inhibitor known. It contains 14 amino acids (GRCTKSIPPICFPD) that form two β-sheets stabilized by an intramolecular disulfide bond and an end-to-end cyclic backbone.[32] In its synthesis and end-to-end cyclization, we used our novel TEA-thioester surrogate, the TEBA linker. The Arg2-Cys3 junction was selected as the cyclization point. A linear precursor of SFTI with C-terminal TEBA moiety was prepared for thia zip cyclization to afford the reduced SFTI-1.

MeCys as a TEA Thioester Surrogate in the Synthesis of Cyclic ω-Conotoxin

The unprotected linear CG29 precursor with a C-terminal MeCys thioester surrogate was prepared using Fmoc chemistry (Figure 5). Fmoc-Gly was coupled to Rink amide resins to give Fmoc-Gly-NH-resin **1**. The commercially available Fmoc-MeCys(Trt) was then coupled to the Fmoc-deprotected glycine using DIC/HOBt to give Fmoc-MeCys(Trt)-Gly-Rink amide resin **2** as a functionalized resin. The C-terminal Gly of CG29 sequence was coupled to the hindered secondary amine of MeCys(Trt) on resin **2** using a more reactive coupling reagent HATU/DIEA with repeated couplings to afford Fmoc-Gly-MeCys(Trt)-Gly-Rink amide resin **3**. This coupling reaction was monitored by acetaldehyde/chloranil that reacted with a secondary amine to give a green-blue color. The remaining sequence of CG29 was coupled to resin **3** by a stepwise Fmoc solid-phase synthesis using PyBOP/DIEA in a microwave-assisted peptide synthesizer to give CG29-MeCys(Trt)-Gly-Rink amide resin **4**. The unprotected peptide CG29-MeCys-Gly-NH_2 **5** was removed from resin **4** by treating with TFA/TIS/H_2O (95/2.5/2.5, *v/v*) for 2 h to give a 21% isolated yield after HPLC purification and lyophilization.

The acid-catalyzed N-S acyl shift of CG29-MeCys-Gly-NH_2 **5** to give its thioester intermediate **6** was performed in an aqueous solution at pH 2. In the absence of an external thiol, the internal thiols of the cyclized CG29 spontaneously underwent a series of S-S acyl shift reactions to form CG29 thiolactones **8a-f**. For example, after treating the cyclized peptide CG29-MeCys-Gly-NH_2 **5** in a pH 2 solution at 40°C for 36 h, we isolated five out of six expected thiolactone isomers and labeled these peaks as **8a-d** and **8f** in the RP-HPLC traces (Figure 6c). The peaks **8a-d** and **8f** were characterized by MALDI-TOF mass spectrometry, and all gave the same m/z 2896.4 as the cyclic CG29 **9**, which suggested that they were thiolactone isomers. These thiolactones could be distinguished from the cyclic peptide **9** by monitoring at wavelength 260 nm in HPLC traces because thioesters and thiolactones are strongly absorbed at 260 nm whereas the absorbance of amide bonds is considerably weaker at this UV wavelength. Observation of thiolactone formation as a result of S-S acyl shift reactions indicated that side-chain thiols actively participated in transesterification. Thus we expected that cysteine-rich peptides, such as CG29 containing six cysteines, were able to cyclize without addition of external thiols. The series of thiolactones with different ring sizes were exchangable and collectively acted as the starting materials for the subsequent end-to-end backbone cyclization reaction. This cyclization reaction was performed at pH 7.5 buffered by 0.1 M phosphate buffer for 2 h at room temperature in 86% yield (Figure 6e). TCEP (10 mM) was added

in this cyclization buffer to prevent disulfide formation. The S-S exchange reactions were favored under pH 7.5 to permit the expansion of thiolactone rings as an end-to-end thiolactone formed by the N- and C-termini, and which led to a proximity-driven S-N acyl shift to form a backbone amide of the desired macrocycle. This thiolactone-assisted zipping mechanism was earlier descibed by our laboratory as the thia zip cyclization.[8,27] From a synthetic peptide precursor with thioester surrogate to end-to-end cyclized peptide product, the MeCys-mediated cyclization of cysteine-rich peptide ressembled the mechanism of intein-mediated protein splicing. Only a single purification step by RP-HPLC was required after completion of cyclization. Thus this method could afford a good yield in the preparation of a circular protein such as CG29.

In the presence of an external thiol, a stable thioester was obtained from the peptide precursor CG29-MeCys-Gly-NH_2 **5**. The reaction was performed in a pH 2 solution at 40°C using an excessive amount of an external thiol (50 eq. MESNa). After a 24-h treatment, two thioesters and one thiolactone were isolated by RP-HPLC (Figure 6d) as CG29-MES **7** (76%), MeCys thioester **6** (5%) and a thiolactone CG29 **8f** (4%), respectively. Compared to the N-S/S-S acyl shift reactions performed without an external thiol, the HPLC traces of thioesterification reaction were simplified by the addition of an excessive amount of external thiol, and CG29-MES **7** was the most abundent thioester product. The thiolactone **8f** with the retention time at 43.6 min was closest to the retention time of cyclic CG29 **9** at 43.9 min and we speculated that it could be the end-to-end thiolactone, based on the similarity in conformation with the end-to-end cyclized CG29 **9**. The thia zip cyclization was performed at pH 7.5 for 2 h at room temperature. The cyclic CG29 **9** was transformed from **6**, **7** and **8f** via S-S and S-N tandem acyl shift reactions to the desired cyclic CG29 (Figure 6e). The oxidative folding of cyclic CG29 was conducted using a highly diluted solution (10 μM) in 0.1 M ammonium phosphate buffer at pH 8 containing 2 M ammonium sulfonate and oxidized/reduced glutathionine (peptide:GSSG:GSH= 1:10:100 eq) at 4°C for 72 h to give ~15% folded cyclic conotoxin CG29. The native disulfide connectivity was characterized by partial reduction/S-alkylation and chymotrypsin digestion followed by MS/MS analysis.

Side Reactions and Rationale to Develop Simplified TEXA Types of TEA thioester surrogates

The use of MeCys(R) (R, the S-protecting group) thioester surrogate has several limitations. First is the slow coupling of a C-terminal amino acid derivative of a target peptide with the secondary amine of MeCys(R) on resin. The reactivity of this secondary amino group is low thus needs multiple couplings for completion. Hojo *et al.* reported a method to circumvent the difficulty of the coupling problem of the MeCys(R) on resin by a solution-phase preparation of different dipeptide units, which can be easily introduced on resin to give MeCys(R)-containing peptides.[33,34]

The second drawback of the MeCys(R) surrogate group was the formation of a MeAla(Pip) side product (+51 Da) during Fmoc-synthesis of a peptide containing a C-terminal MeCys(Trt) on resin. This side reaction was previously reported by Lukszo *et al.* [35] as a base-catalyzed β-elimination on a C-terminal Cys(Trt) or Cys(Acm) followed by a Michael addition of piperidine to form 3-(1-piperidinyl)alanine. MeCys(Trt) was also susceptible to this side reaction and the side product was accumulated during each piperidine deprotection step in Fmoc solid-phase synthesis. This synthetic problem was not ressolved by addition of a spacer residue Gly between MeCys(Trt) and the resin support. In the microwave-assisted peptide synthesis of CG29-MeCys(Trt)-Gly-NH_2 **5**, the side product CG29-MeAla(Pip)-Gly-NH_2 accumulated to 10% at the completion of the synthesis, suggesting that an average of 0.3% side reaction per deprotection cycle.

The third drawback of using MeCys(Trt) was that it was an expensive reagent, costing about 1,000 USD per gram.

These limitations led us to develop other amido types thioester surrogates, such as the 2-thiomethylthiazolidine (TMT)-carboxylic- acid thioester surrogate (Figure 3b), which we have reported

previously.[22] The thia-proline moiety of the TMT thioester surrogate facilitates an N-S acyl shift by enhancing a *cis* conformation and suppressing the β-elimination side reaction.

To completely eliminate the β-elimination side reaction, it would be desirable to remove the electron withdrawing α-carbonyl moiety of a MeCys(R) and the α-CH susceptible to abstraction by piperidine during the deprotection cycles. To achieve this objective, we have developed a novel family of TEA-thioester surrogates, which we designated as the TEXA thioester surrogates.

The TEXA thioester surrogates (where X=methyl, ethyl, propyl or butyl) were designed by removing the C-terminal reactive carbonyl functionality. Without the carboxylic group, the TEXA design could decrease the steric hindrance during the coupling reaction and eliminate the side reaction of β-elimination and the piperidine addition product. TEXA has an advantage in that it uses the resin support as a S-protecting group for the thioethyl moiety. Such a strategy further simplifies the synthesis of functionalization of the TEA thioester. An example of a TEXA thioester surrogate, TEBA (where X=butyl) is described below (Figure 3c).

Thioethylbutylamide (TEBA) as a TEA Thioester Surrogate in the Syntheis of SFTI

The preparation of SFTI using the TEBA linker commenced with the attachment of the commercially available (2-butylamino)-ethanethiol **10** to Cl-Trt(2-Cl) resin to give TEBA-Trt(2-Cl) resin **11** (Figure 7). The C-terminal Fmoc-Arg(Pbf) (4 eq) was coupled to the secondary amine **11** using HATU/DIEA for 1 h to give Fmoc-Arg(Pbf)-TEBA resin **12**. The amino group on **11** was found to be more reactive than that on MeCys due to the absence of the carbonyl group. Acetaldehyde/chloranil method for detection of a secondary amino group confirmed that the condensation reaction was complete in a single coupling reaction. Peptide elongation of the SFTI linear precursor was carried out by a manually stepwise coupling protocol using Fmoc amino acid derivative/HBTU/HOBt/DIEA in DMF for 30 min for each coupling reaction. As for Fmoc deprotection, 20% morpholine/DMF [36] (5 min x 4) was used, rather than piperidine, for prevention of aspartimide formation at Asp^{14}-Gly^{1}. The protected SFTI-TEBA resin **13** was treated with TFA/TIS/H_2O (95/2.5/2.5) to remove all protecting groups to give SFTI-TEBA **14** without any aspartimide formation in 17% yield from Cl-Trt(2-Cl) resin after purification by RP-HPLC.

After investigating a suitable pH as an optimal condition for a tandem N-S and S-S acyl shift reaction (data not shown), we selected pH 3 with MESNa as an external thiol incubated at 40^{o}C. When **14** was treated with MESNa in an aqueous solution buffered at pH 3 and 40^{o}C for 15 h, the thioester **15** derived from the N-S acyl shift of **14** was observed first and then converted to the MES thioester **16**, and thiolactone **17** (24%) was also observed on HPLC traces. Both forms underwent a thia zip cyclization smoothly to obtain **18** in 52% yield after adjusting the pH to 7 in the presence of TCEP. The disulfide formation using DMSO as the oxidant was successfully carried out under a neutral condition to afford SFTI-1 in 82% yield (Figure 8).

CONCLUSIONS

The TEA thioester surrogates are chemical mimics of inteins as they fulfill their roles, just as inteins do in protein splicing, to enable both excision and ligation reactions in forming end-to-end cysteine-rich cyclic peptides, examples of which include cyclized conotoxin and SFTI. Under acid conditions the thioester surrogate, such as MeCys in a cysteine-rich peptide, undergoes an N-S acyl shift to form a thioester, after which it undergoes a series of S-S acyl shifts to form a thiolactone with the excision of the MeCys moiety. These thiolactones eventually undergo an S-N acyl shift via a thia zip cyclization at basic pH to form the peptide backbone of an end-to-end macrocycle. A simplified version of MeCys is the TEBA thioester

surrogate, which eliminates the side reactions associated with the MeCys thioester surrogate in peptide synthesis.

TEA thioester surrogates may have additional applications in sequential peptide ligation to prepare large proteins as the difference in suitable pH range for N-S, S-S and S-N acyl shift reaction may allow selective activation of TEA thioester surrogates attached on C-termini of peptide segments.

MATERIALS AND METHODS

General

Fmoc amino acid derivatives were purchased from Impex-Chem including Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, , Fmoc-Cys(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Phe-OH, Fmoc-Thr(tBu)-OH, Fmoc-Val-OH and Fmoc-Trp(Boc)-OH. Fmoc-MeCys(Trt)-OH was purchased from Advamac. Wang resin (0.9 mmol/g), Chlorotrityl chloride resin (1.2 mmol/g), Rink amide resin (0.4 mmol/g), HOBt and BOP were purchased from Novabiochem. DIEA, 2-mercaptoethanol, triphenylmethyl alcohol, pyridinium chlorochromate, thiocresol (TC), DIC, and L-Cysteine hydrochloride were purchased from Aldrich. TFA was purchased from Alfa Aesar. PyBOP, HOAt, HATU and HBTU were purchased from GL Biochem. Solvents DMF, DCM, piperidine, morpholine and diethyl ether were purchased from Merck.

The C-terminal Fmoc-Xaa was coupled manually in all peptides. The rest of peptide sequences were synthesized by automated microwave peptide synthesizer (CEM, Liberty1) using 5 eq. of PyBOP and 10 eq. of DIEA for coupling and 20% piperidine (0.1 M HOBt) in DMF for deprotection.

Synthesis of CG29-MeCys-Gly-NH_2 on Rink amide resin

The Rink amide resin (1 g, 0. 34 mmol) was swollen for 10 min in DCM (10 mL) and the resin was filtered and washed with DCM (1 min x 2). The spacer amino acid Fmoc-Gly (1.36 mmol, 404 mg) was manually coupled with 4 eq. of DIC (1.36 mmol, 143 µl) and 4 eq. of HOBt (1.36 mmol, 184 mg) in DMF (10 ml) (shaking for 1 h at room temperature) to give Fmoc-Gly-NH resin **1**. The resin was washed by DMF and DCM respectively followed by deprotection using 20% piperidine solution in DMF (5 min x 2). A mixture of 2 eq. Fmoc-MeCys(Trt)-OH (0.68 mmol, 408 mg), 4 eq. DIC (1.36 mmol, 143 µl) and 4 eq. HOAt (1.36 mmol, 185 mg) in DMF (10 ml) was added into resin-containing reaction vessel. The reaction was performed by shaking for 2 h at room temperature. Fmoc was deprotected by 20% piperidine in DMF (5 min x 2 at room temperature) and resin was washed (DCM x 2, DMF x 2) to give MeCys(Trt)-Gly-amide resin **2**. Fmoc-Gly (1.36 mmol, 404 mg) was coupled manually to the secondary amine on MeCys(Trt) of resin **2** by 4 eq. HATU (1.36 mmol, 517 mg) and 6 eq. DIEA (2.04 mmol, 341 µl) to obtain resin **3** after triple coupling. The completion of reaction was examed by acetaldehyde /chloranil test (2% acetaldehyde, 2% chloranil in DMF, 5 min at room temperature). The substitution of Fmoc-Gly-MeCys(Trt)-Gly-amide resin **3** (1.2 g) was ~0.28 mmol/g. 350 mg resin **3** (0.1 mmol) was used for synthesis of the full-length CG29 using microwave-assisted peptide synthesizer using PyBOP/DIEA (5/10 eq) to give CG29-MeCys-Gly-amide resin **4** (783 mg). A mixture of 5 ml TFA/TIS/H2O (95/2.5/2.5, *v/v*) was added to 200 mg resin **4** to remove side chain deprotection group and cleave peptide from solid support. The reaction mixture was shaken for 2 h at room temperature and then added to chilled diethyl ether for peptide precipitation. The precipitate was dried *in vaccuo* and purified by RP-HPLC to give CG29- MeCys-Gly-NH_2 **5** (17.6 mg, 21% isolated yield).

Synthesis of SFTI on thiolethylbutylamido (TEBA) resin using Cl-Trt(2-Cl) resin

Cl-Trt(2-Cl) resin (0.5 g, 0.6 mmol) was swollen for 30 min in DCM (10 mL) and the resin was filtered and washed with DCM (1 min x 2). (2-butylamino)ethanethiol **10** (0.3 mmol, 44 µl) in DCM (20 ml) was

added, and the suspension was shaken for 1 h at room temperature**.** 3.6 mmol DIEA (3.6 mmol, 627 µl) in MeOH was added, and the reaction mixture shaken for 10 min to quench the unreacted resin. The resin was washed with DCM and DMF, respectively, to give TEA resin **11**. A mixture of Fmoc-Arg(Pbf) (1.2 mmol, 779 mg), HATU (1.2 mmol, 456 mg) in DMF and DIEA (1.8 mmol, 314 µl) was added to resin **11**. The suspension was shaken for 1 h and coupling procedure was duplicated to give Fmoc-Arg(Pbf)-TEBA resin **12**. The rest of peptide sequence was synthesized manually to give SFTI-TEBA resin **13**. A mixture of TFA/TIS/H_2O (95/2.5/2.5, v/v) was added to peptide resin **13** and the reaction mixture was shaken for 2 h. After dropwise addition of the reaction mixture to chilled diethyl ether for precipitation, the precipitate was dried *in vaccuo* and purified by RP-HPLC to give SFTI-TEBA **14** (27.4 mg, 17% yield).

Cyclization of cysteine-rich peptide conotoxin CG29

CG29-MeCys-Gly-NH_2 **5** (1 µmol, 3.1 mg) was dissolved in 5 mM H_2SO_4 solution (pH 2, 1 mL) at a concentration of 1 mM with 50 eq. MESNa (50 µmol, 8.2 mg) and incubated at 40°C for 24 h. The formation of thioester-form **6**, MES thioester **7** and thiolactones **8a-f** from **5** was monitored by RP-HPLC. TCEP (10 µmol, 2.8 mg) were added to the reaction mixture to avoid disulfide formation. The pH of reaction mixture remained about pH 2 after addition of TCEP. After adjusting the pH to 7.5, thia zip cyclization was performed at room temperature with gentle stirring for 2 h to give cyclic reduced CG29 **9** (comparative yield 73%). In the second cyclization reaction, CG29-MeCys-Gly-NH_2 **5** (1 µmol, 3.1 mg) was dissolved in 5 mM H_2SO_4 solution (pH 2, 1 mL) at a concentration of 1 mM without addition of MESNa. The reaction was conducted at 40°C for 36 h when **5** was not observed in HPLC monitoring. Thioester-form **6** and five out of six thiolactone **8a-f** was isolated from HPLC and identified by MALDI-TOP mass spectrometry. After adjusting the pH to 7.5, thia zip cyclization was performed at room temperature with gentle stirring for 2 h to give cyclic reduced CG29 **9** (comparative yield 86%). To a solution of cyclic CG29 **9** (1 µmol, 2.9 mg) in 0.1 M ammonium phosphate buffer (pH 8, 100 ml) that contained 2 M ammonium sulfonate, a redox reagent including oxidized/reduced glutathionine (molar ratio of peptide:GSSG:GSH= 1:10:100) was added to catalyze disulfide formation at 4°C for 72 h to give 14% folded cyclic conotoxin CG29 with native disulfide connectivity. The disulfide connectivity was analyzed by partial reduction/alkylation method. 300 µg folded cCG29 was reduced by TCEP (10 mM) for 30 min and S-alkylated by 50 mM N-ethylmaleimide (NEM) for another 30 min to obtain intermediates with one or two pairs of intact disulfide bonds. The 1SS and 2SS species were isolated by RP-HPLC and reduced completely by 20 mM DTT followed by 50 mM iodoacetamide (IAM) alkylation. Chymotrypsin treatment opened the cyclic backbone at Tyr13-Asp14 site to give linear alkylated products for MS/MS analysis.

Cyclization of cysteine-rich peptide trypsin inhibitor SFTI

SFTI-TEBA **14** (5.85 µmol, 9.64 mg) was dissolved in 0.1 M sodium phosphate buffer (pH 3, 1.95 mL) at a concentration of 5 mM with MESNa (292 µmol, 48.0 mg) and incubated at 40°C for 18 h. The formation of S form **15**, MES thioester **16** and thiolactones **17a** and **17b** from **16** was monitored by RP-HPLC. TCEP (58.5 µmol, 16.8 mg) and 0.1% sodium phosphate (pH 3, 3.9 mL) were added to the reaction mixture to avoid disulfide formation and dilute the reaction mixture, respectively. After adjusting the pH to 7 by addition of 2 N NaOH, thia zip cyclization was performed at room temperature with gentle stirring for 4 h to give cyclic reduced SFTI **18** (4.6 mg, 52%). To a solution of **18** (16.2 µmol, 24.5 mg) in 0.1 M sodium phosphate (pH 7.5, 15.2 mL), DMSO (800 □L, 5%, v/v) were added and the solution was allowed to stand for 15 h at room temperature. The reaction mixture was quenched with 1N HCl for a final pH of 2 and purified by using RP-HPLC to give SFTI-1 (20.2 mg, 82%).

RP-HPLC and mass spectrometry

Peptides with different C-terminal thioester surrogates and their intermediates were monitored by analytical RP-HPLC (high-pressure analytical column Aeris Peptide 3.6u XB-C18, 250 x 4.6 mm, HPLC

gradient 5-35% acetonitrile in 0.1% TFA for 60 min) and MALDI-TOF mass spectrometry (reflectron-mode) or ESI. The sequence analysis was performed by MS/MS.

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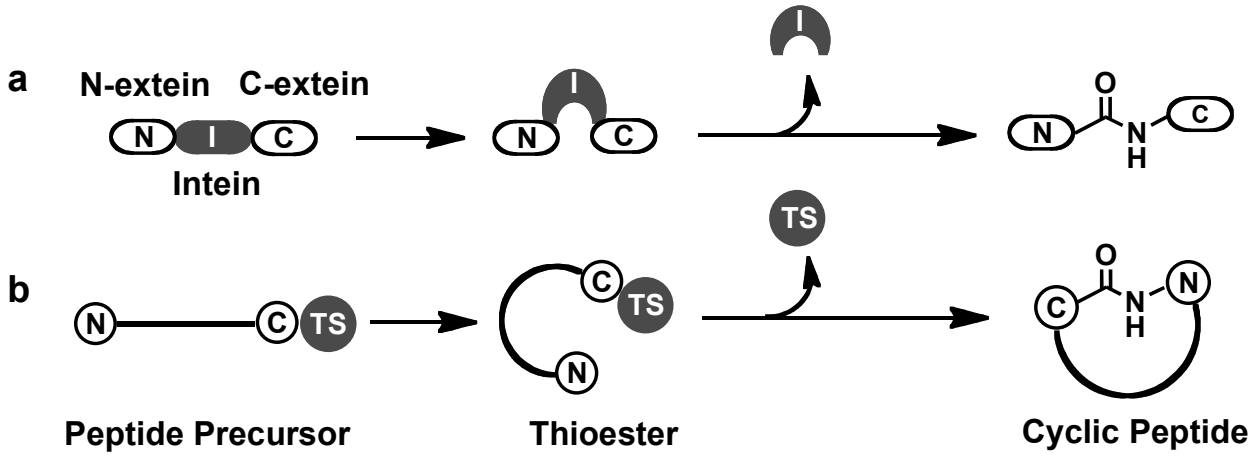

FIGURE 1 Comparison of (a) intein-catalyzed protein splicing (I, intein; N, N-extein; C, C-extein) and (b) thioethylamide (TEA)-mediated peptide cyclization using thioester surrogate (TS).

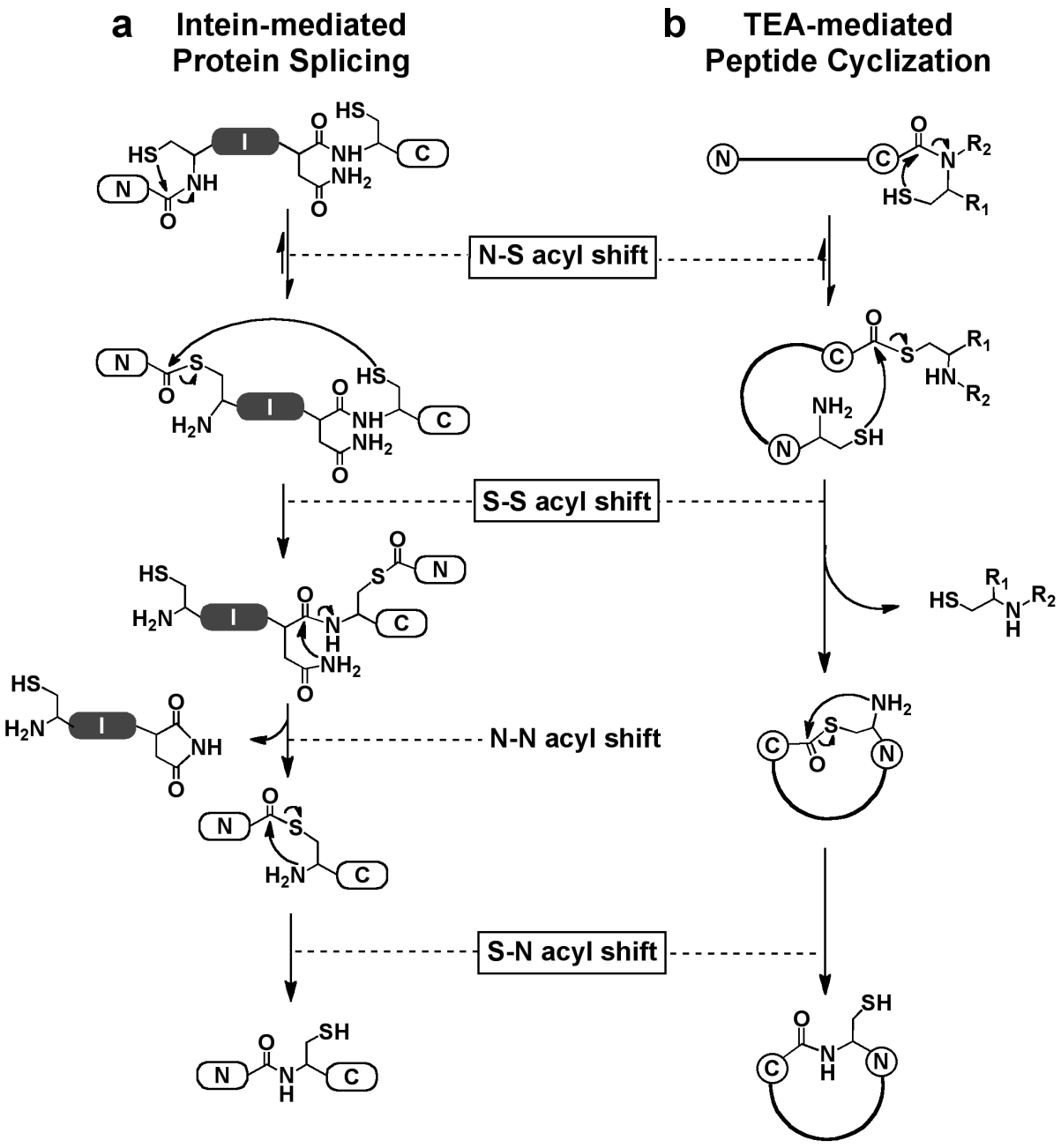

FIGURE 2 Acyl shift reactions common to intein-mediated protein splicing and TEA-mediated peptide cyclization using thioester surrogates in chemical synthesis. (a) Four acyl shift reactions found in intein-mediated protein splicing. I, intein; N, N-extein; C, C-extein. (b) Three acyl shift reactions found in peptide cyclization mediated by TEA thioester-surrogate. N, N-terminus; C, C-terminus.

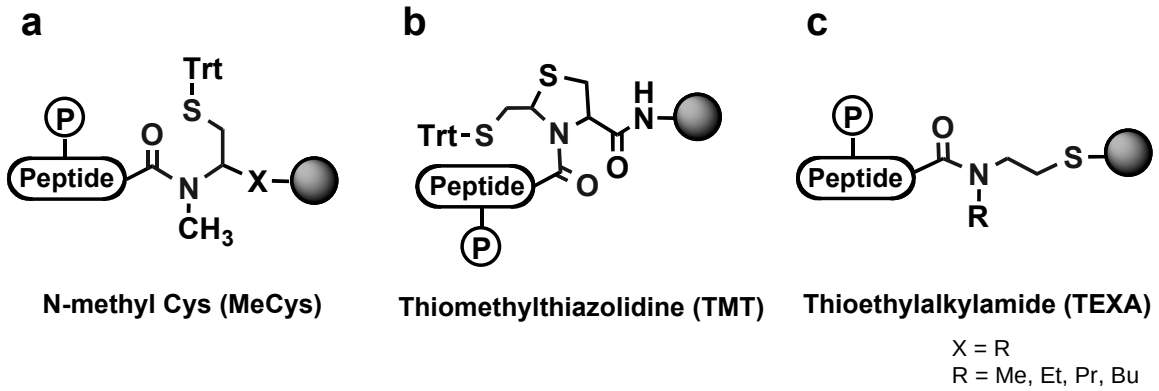

FIGURE 3 The design of thioethylamide (TEA) thioester surrogates including (a) N-methyl Cys (MeCys), (b) thiomethyl-thiazolidine (TMT) carboxylic acid and (c) thioethylalkylamide (TEXA) (where X = M for methyl, E for ethyl, P for propyl, B for butyl).

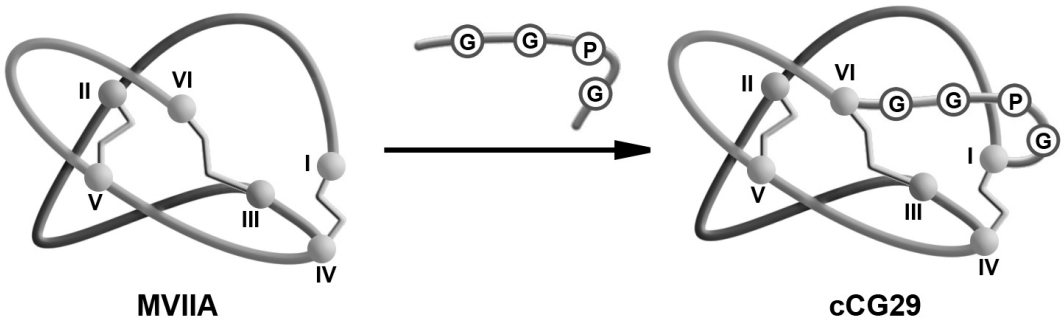

FIGURE 4 Strategy for the cyclization of ω-conotoxin MVIIA by a short linker sequence.

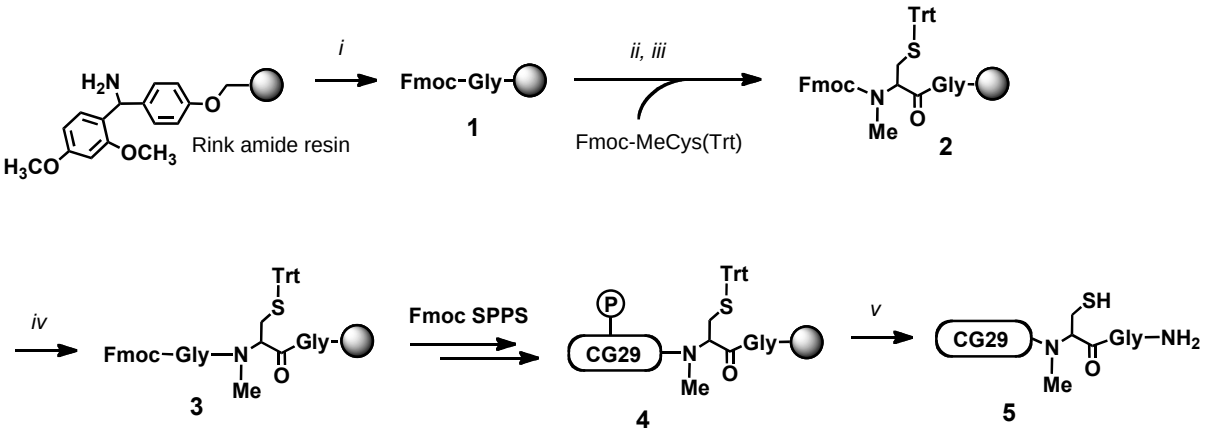

FIGURE 5 Preparation of peptide-MeCys-Gly-NH_2 on Rink amide resins using Fmoc synthesis. *i* = Fmoc-Gly, DIC,HOBt. *ii* = 20% piperidine deprotection, *iii* = Fmoc-MeCys(Trt), DIC, HOBt. *iv* = Fmoc-Arg(Pbf), HATU, DIEA, DMF. *v* = TFA/TIS/H2O (95/2.5/2.5, v/v), 2 h.

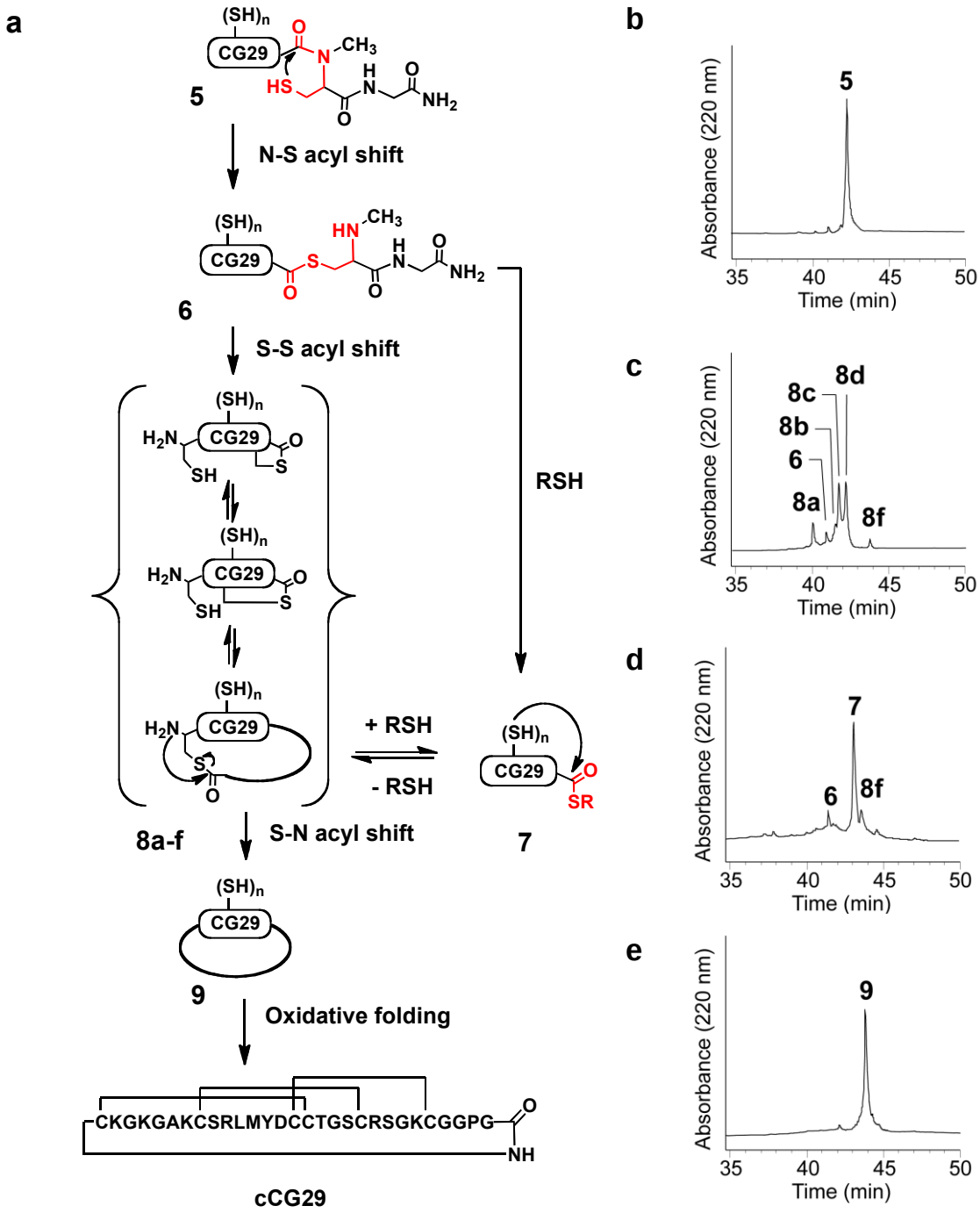

FIGURE 6 Schema and HPLC analysis of CG29-MeCys-NH_2 **5** cyclization via tandem thiol switch reactions mediated by intramolecular thiols or external thiols. The first N-S acyl shift of CG29-MeCys-Gly-NH **5** in pH 2 solution resulted in the formation of the peptide thioester **6** that subsequently was trapped by internal thiols to give intramolecular thiolactones **8a-f** (c), or transformed into stable thioester CG29-MES **7** in the presence of external thiol MESNa (d). Thia-zip cyclization was performed at pH 7.5 at room temperature for 2 h to give cyclic CG29 **9** (e).

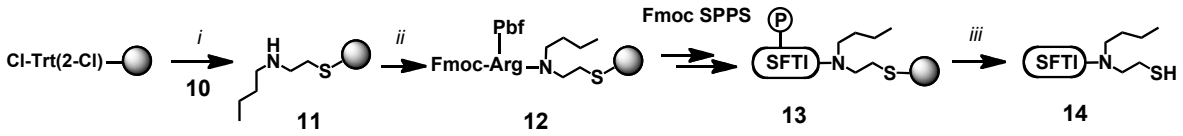

FIGURE 7 The synthesis scheme of SFTI-TEAB on Cl-Trt(2-Cl) resin. *i* = compound **10** (2-butylamino)ethanethiol**,** CH_2Cl_2**,** 30 min. *ii* = Fmoc-Arg(Pbf), HATU, DIEA, 1 h. *iii* = TFA/TIS/H_2O (95/2.5/2.5, v/v), 2 h.

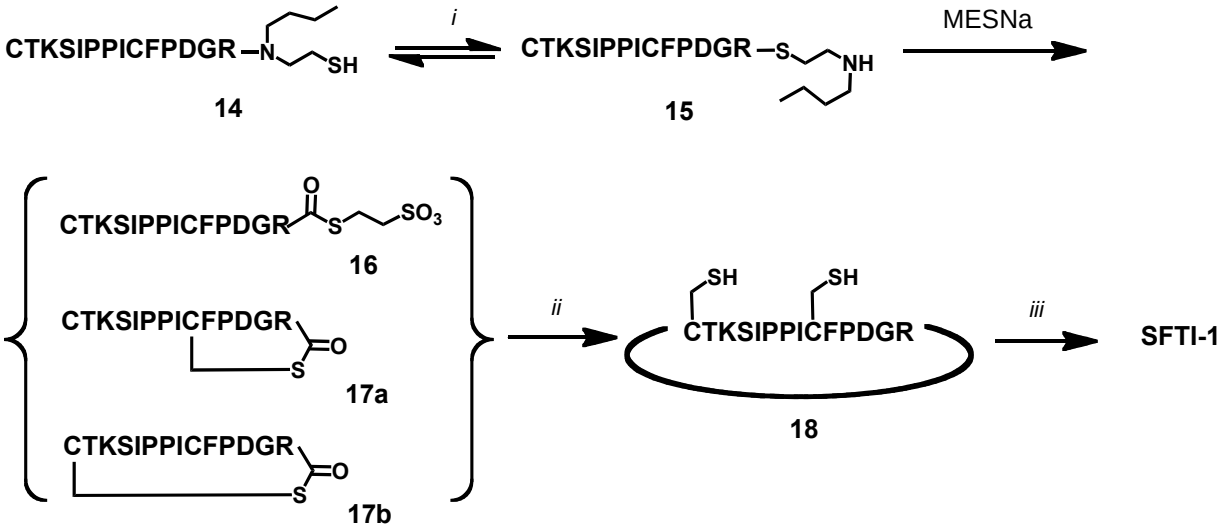

FIGURE 8 One-pot tandem thiol switch and cyclization of SFTI-TEAB. *i* = MESNa, 0.1 M sodium phosphate (pH 3), 40°C, 17 h, *ii* = TCEP, pH 7 (addition of 2 N NaOH), rt, 3 h, *iii* = DMSO, 0.1 M sodium phosphate (pH 7.5), 15 h.