A Thioethylalkylamido (TEA) Thioester Surrogate in the Synthesis of a Cyclic Peptide via a Tandem Acyl Shift
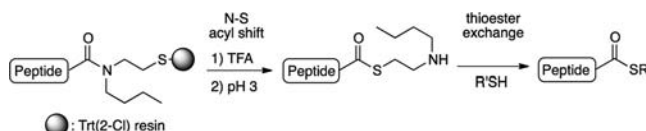
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ABSTRACT

The cyclic cystine-knot peptide, kalata B1, was synthesized by employing a novel Fmoc-compatible thioethylalkylamido (TEA) thioester surrogate via an N−S acyl shift followed by a thiol-thioester exchange reaction. TEA thioester surrogate is cost-effective, conveniently prepared in one-step with starting materials, readily available from commercial sources, and highly efficient in preparing peptide thioesters.

Peptide thioesters are key intermediates for the chemical ligation of peptides and proteins.[1] Although peptide thioesters can be directly prepared using Boc chemistry,[2] it is generally not suitable for the synthesis of phospho- or glycopeptides, which are sensitive to the strongly acidic conditions of anhydrous HF. In Fmoc chemistry, a thioester is susceptible to basic conditions and intolerant to the repetitive piperidine deprotection steps. To overcome this problem, methods for the direct or indirect preparation of peptide thioesters by Fmoc chemistry have been reported. Direct methods include the use of a less basic Fmoc deprotection cocktail[3] and conversion to a thioester from a C-terminal acid.[4] Indirect methods use thioester surrogates which require activation followed by thiolysis after peptide elongation.[5] Examples include the safety-catch categories of sulfonamide,[6] N-acylurea,[7] acid hydrazide,[8] and O/N−S acyl shift methods.[9]

Among the thioester-surrogate approach, one of the promising methods is employing the N−S acyl shift reactions. Examples include N-4,5-dimethoxy-2-mercaptobenzyl (Dmmb),[10] N-alkylated cysteine,[11] bis(sulfanylethylamino) (SEA)[12] or bis(mercaptoethyl)

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amide (BMEA),[13] SEA-anilide,[14] thioproline,[15] and thiazolidine.[16] A common structure of these thioester surrogates is the thioethylalkylamido (TEA) moiety which facilitates a proximity-driven N−S acyl shift reaction via a five-member ring to afford a thioester after a thiol-thioester exchange reaction with an external thiol (Figure 1). TEA thioester surrogates include *N*-alkylated cysteine-containing linkers. An example is the MeCys linker which can be conveniently prepared by a commercially available Fmoc-MeCys(Trt), and several applications of this method have been reported.[17] The MeCys-thioester surrogate has several limitations. The coupling reactions of a C-terminal amino acid onto the MeCys-attached resin is slow due to the low reactivity of the secondary amine on the MeCys residue, and multiple couplings are often required. Hojo et al. reported a method in overcoming this problem by a solution-phase assembly of a series of EtCys-containing dipeptide units which can be then introduced on resin.[18] The other drawback is the side reaction associated with the *β*-elimination of a protected MeCys residue followed by the conjugate addition of piperidine during peptide elongation to give MeAla(Pip).[19]
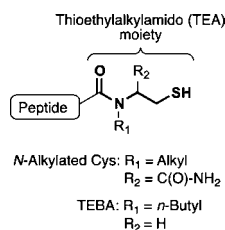

Figure 1. Structure of *N*-alkylated cysteine and TEBA thioester surrogates containing the thioethylalkylamido (TEA) moiety.

These limitations prompted us to develop a new series of thioester surrogates via an N−S acyl shift. Previously we reported the 2-thiomethylthiazolidine (TMT)-carboxylic acid thioester surrogate to enhance a *cis*-conformation which facilitates the N−S acyl shift and to suppress the *β*-elimination side reaction.[16] To avoid this side reaction completely, it would be desirable to remove an electron-withdrawing carbonyl group of the MeCys residue.

Toward this end, we have developed a novel TEA-thioester surrogate using the thioethylbutylamido (TEBA) moiety, which is simplified from a MeCys residue by retaining the TEA group but eliminating the carbonyl group (Figure 1). As such, the TEBA-group would minimize the side reaction of the *β*-elimination by a base abstraction of the α-CH found in MeCys during the Fmoc deprotection steps. An additional advantage of the TEBA-thioester surrogate is that the thiol group of the TEBA-moiety can be directly attached onto the solid support which acts as its protecting group in solid-phase peptide synthesis (SPPS). This would greatly simplify its preparation as a thioester surrogate.

Here, we report the development of a novel thioester surrogate using the thioethylbutylamido (TEBA) group via a tandem acyl shift, a comparison of the TEBA-group with the MeCys-thioester surrogate in the N−S acyl shift and thiol-thioester exchange reactions under acidic conditions, and its application in the synthesis of the prototypic cyclotide, kalata B1.

Synthesis of a peptide with the TEBA-thioester surrogate started with the preparation of the TEBA-resin **2** which was readily obtained from a commercially available 2-(butylamino)ethanethiol **1** and Cl-Trt(2-Cl) resin (Scheme 1A). We first synthesized a model peptide, Thr-Ile-Gly-Gly-Ile-Arg (TIGGIR), using the TEBA-resin or the MeCys(Trt)-Gly-Rink resin. A single coupling reaction of the C-terminal Fmoc-Arg(Pbf) with the secondary amine **2** using HATU/DIEA was completed in 1 h, as determined by the colorimetric assay with the acetaldehyde/chloranil method for detection of a secondary amine. Stepwise peptide elongation was carried out manually using standard Fmoc chemistry. Treatment of the protected peptide resin with TFA gave the desired crude peptide in excellent yield, and no side product (piperidine adduct) associated with the *β*-elimination side reaction was observed as determined by RP-HPLC trace and MS. The desired TEBA peptide **4** was obtained in 27% yield after purification by RP-HPLC. The low yield was attributed to S-*tert*-butylation of **4** in the final deprotection step and the low yield in the first loading step. Both steps were not optimized. During the peptide cleavage in TFA and HPLC purification in a 0.1% TFA-containing aqueous solution, the TEBA-peptide **4** (N-form) was gradually rearranged to its thioester isomer TEBA-peptide **5** (S-form). This mixture consisting of **4** and **5** (6:4, mol/mol) was used for further reactions. We also found that the ratio of the N- and S-forms is variable, depending on the duration in the treatment of peptides under acidic conditions.

For comparison with the TEBA-group with the MeCys-group of thioester surrogates, we also synthesized the model peptide with the MeCys residue as a thioester surrogate, TIGGIR-MeCys-Gly-NH$_2$ **6**, which commenced with the Rink Amide resin (Scheme 1B). Unlike the synthesis of the TEBA-linker, multiple coupling reactions were necessary in the coupling reaction of Fmoc-Arg(Pbf) with secondary amine **7** in the MeCys-thioester surrogate. This result indicated that the secondary amino group of the TEBA-peptide **2** was more reactive than that of MeCys **7** due to the lack of the carbonyl group. After

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peptide elongation with Fmoc SPPS and cleavage of **6** by TFA/triisopropylsilane (TIS)/H_2O for 1.5 h from the resin, TIGGIR-MeCys-Gly-NH_2 **6** was obtained in 56% yield after purification by RP-HPLC. The N−S acyl shift reaction of the TEBA-thioester surrogate was also faster than that of the MeCys thioester surrogate, because we found only a trace amount (<2%) of its thioester isomer (S-form) **6**.

Scheme 1. (A) Preparation of Model Peptide **4** (N-form), Its Thioester Isomer **5** (S-form), and MES Thioester **9**; (B) Preparation of Model Peptide **6** and MES Thioester **9**
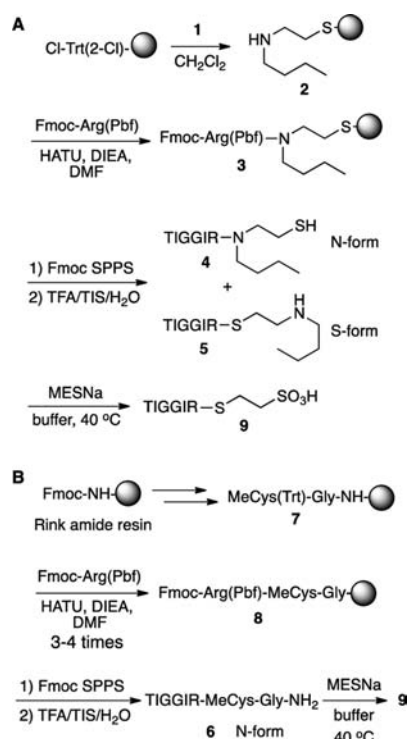
With the model peptides **4** and **6** in hand, we investigated the optimal conditions for their N−S acyl shift reaction followed by a thiol-thioester exchange with an external thiol to afford a stable thioester suitable for ligation reactions. Sodium 2-mercaptoethane sulfonate (MESNa) was selected as an external thiol (Scheme 1). This series of thioesterification involves two independent reactions, an N−S acyl shift and a thiol-thioester exchange. It was found that each reaction with **4** or **5** was pH dependent (Figure 2A). In pH < 2, the N−S acyl shift preferentially occurred whereas the thiol-thioester exchange reaction preferred neutral to basic conditions (pH > 6). The same trend was observed in the MeCys-thioester surrogate. The best yield of MES-thioester **9** from the TEBA-peptides **4/5** was obtained in a pH 3 buffer at 40 °C (Figure 2B), whereas the best result for TIGGIR-MeCys-Gly-NH_2 **6** was observed in a pH 2 buffer at 40 °C (Figure 2C). The thioesterification reaction using the MeCys-thioester surrogate required more acidic conditions than the TEBA-thioester surrogate. This result is consistent with the MeCys residue being less basic than the corresponding tertiary amide found in the

TEBA moiety, attributed to the presence of the electron-withdrawing carbonyl group on the MeCys moiety. Compared to the reactivity of TIGGIR-MeCys-Gly-NH_2 **6**, the conversion of the TEBA-peptides **4/5** to the MES-thioester **9** was faster (Figure 2D), probably due to the higher reaction rate of the N−S acyl shift of **4** to **5**. In practice, the MES-thioester **9** was afforded in 68% yield when the TEBA-peptides **4/5** were treated with MESNa in buffer at pH 3 at 40 °C for 18.5 h. The MES-thioester **9** was successfully subjected to the native chemical ligation (Cys-thioester ligation) with CALVIN **10** in the presence of tris(2-carboxyethyl)phosphine (TCEP) and mercapto-phenyl acetic acid[20] (MPAA) to give **11** (Scheme 2).[1a,b]
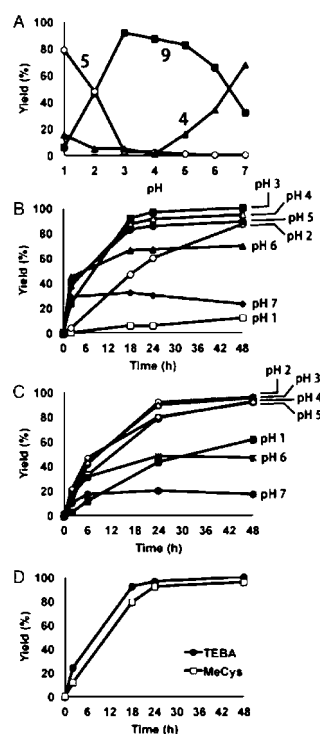

Figure 2. (A) Yield of **4**, **5**, and **9** after incubation for 18 h at a different pH. (B) Rate of conversion of **4** to **9** at a different pH. (C) Rate of conversion of **6** to **9** at a different pH. (D) Comparison of the effect of TEBA and MeCys thioester surrogates on the reaction rate of conversion of **4/5** at pH 3 and **6** at pH 2 into **9**, respectively.

Next, we applied the TEBA-thioester surrogate in the synthesis of the prototypic cyclotide, kalata B1 (kB1, GLPVCGETCVGGTCNTPGCTCSWPVCTRN). Kalata B1, an end-to-end cyclic peptide isolated from the African plant *Oldenlandia affinis*,[21] contains 29 amino acid residues and three disulfide bonds which form a cyclic cystine-knot structure.[22] Cyclotide has frequently been

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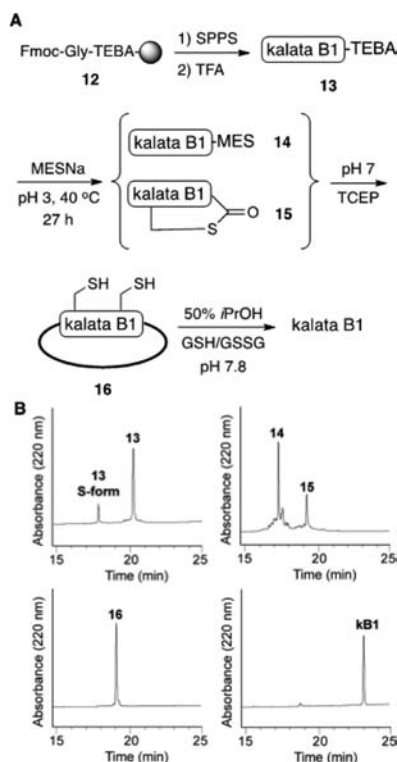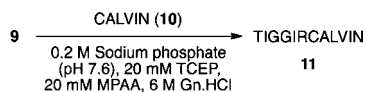
Figure 3. (A) Synthesis of kalata B1 employing the TEBA thioester surrogate. (B) HPLC chromatogram of synthetic intermediates and kalata B1.

used in our laboratory as a model peptide to test novel thioester surrogates and oxidative folding conditions.[23] The Gly18-Cys19 site between loops 3 and 4 of a linear kB1-TEBA peptide **13** as the thioester precursor was selected as a ligation point via the thia-zip cyclization (Figure 3A).[24] After peptide elongation from **12**, the resin-attached protected peptide was globally deprotected

using TFA/TIS/H_2O for 1.5 h to afford the linear kB1-TEBA **13**. The tandem acyl shift reactions of an N−S acyl shift followed by the thiol-thioester exchange of **13** were concurrently performed in the presence of MESNa in phosphate buffer containing 6 M guanidine hydrochloride at pH 3 at 40 °C for 27 h to give the MES-thioester **14** along with several thiolactones **15** formed via the thiol-thioester exchange of **14** with the intramolecular thiols (Figure 3B, 20% on HPLC analysis). Both thioester **14** and thiolactones **15** were directly subjected to the thia-zip cyclization by increasing the reaction solution to pH 7 with 2 N NaOH in the presence of TCEP to obtain the cyclized form **16**. Thia-zip cyclization proceeded through ring expansion of thiolactones, ultimately leading to the macrolactam formation by the N-terminal Cys residue via an S−N acyl shift.[24d] The oxidative folding reaction of the cyclized **16** was carried out by 50% isopropanol in 0.1 M $AcONH_4$ (pH 7.8) under redox conditions using GSH/GSSG.[25] The synthetic kB1 was identical to the native kB1 as determined by coelution in HPLC and ^1H NMR.

In conclusion, we developed a novel thioester surrogate using the TEBA-group for Fmoc chemistry. The TEBA-thioester surrogate eliminates the β-elimination side reaction observed in a MeCys-thioester surrogate in Fmoc deprotection steps during the peptide assembly steps. The efficiency of the coupling reaction of a C-terminal amino acid with the secondary amino group on the TEBA-resin was higher than that of the MeCys(Trt)-Gly-resin, and the TEBA-group could readily be prepared from a commercial source at low cost. Furthermore, a tandem N−S acyl shift followed by a thiol-thioester exchange on the TEBA-thioester surrogate is more efficient than the MeCys-thioester surrogate. By employing the TEBA-thioester surrogate we successfully prepared the cyclic cystine-knot peptide kB1. The proposed TEBA-thioester surrogate provides a simple, practical, and cost-effective method to prepare a peptide thioester suitable for the synthesis of cyclic cystine-rich peptides and post-translationally modified peptides and proteins.

Supporting Information Available. General procedures and additional HPLC, MS data. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.