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Synthesis of peptide thioesters via an *N–S* acyl shift reaction under mild acidic conditions on an *N*-4,5-dimethoxy-2-mercaptobenzyl auxiliary group

Ken'ichiroh Nakamura, Tomoki Kanao, Tomoya Uesugi, Toshiaki Hara, Takeshi Sato, Toru Kawakami, and Saburo Aimoto*

An efficient method of peptide thioester synthesis is described. The reaction is based on an *N*-4,5-dimethoxy-2-mercaptobenzyl (Dmmb) auxiliary-assisted *N-S* acyl shift reaction after assembling a peptide chain by Fmoc-solid phase peptide synthesis. The Dmmb-assisted *N-S* acyl shift reaction proceeded efficiently under mildly acidic conditions, and the peptide thioester was obtained by treating the resulting *S*-peptide with sodium 2-mercaptoethanesulfonate. No detectable epimerization of the amino acid residue adjacent to the thioester moiety in the case of Leu was found. The reactions were also amenable to the on-resin preparation of peptide thioesters. The utility was demonstrated by the synthesis of a 41-mer peptide thioester, a phosphorylated peptide thioester and a 33-mer peptide thioester containing a trimethylated lysine residue.

Supporting information may be found in the online version of this article

Keywords: 4,5-dimethoxy-2-mercaptobenzyl (Dmmb) group; peptide thioester; *N–S* acyl shift; Fmoc SPPS; thioester-producing resin; on-resin preparation

Introduction

Peptide thioesters are important building blocks that are currently used in ligation methodology, including the thioester method [1–5], native chemical ligation [6–8] and extended chemical ligation [9–13]. In addition to their use in polypeptide synthesis, these ligation strategies are also used in dendrimer synthesis [14], the preparation of protein microarrays [15] and for the *N*- or *C*-terminal modification of proteins [16,17].

Peptide thioesters can be directly prepared by *tert*-butyloxycarbonyl (Boc) SPPS [1,18]. The use of Fmoc SPPS, however, is much more suitable for preparing peptides that contain a phosphoryl group and/or an acid-sensitive moiety such as a glycosyl group. The thioester linkage is readily decomposed by treatment with piperidine, which is used for the removal of N^{α}-Fmoc groups. When a weak nucleophilic base is used instead of piperidine, a peptide thioester can be obtained [19], but the amino acid residue adjacent to the thioester moiety gradually undergoes racemization due to the increased acidity of its α-proton [20].

To avoid these problems, several groups have explored indirect methods of peptide thioester synthesis, in which a thioester bond is formed after the peptide chain is assembled [21–39]. Safety catch strategy is now widely used in Fmoc-based peptide thioester synthesis, but some problems have been reported, including methionine ε-cyanilation (approx. 50% yield) [40] and elimination of the peptide chain from the solid support during SPPS [41]. An attractive method in which an intramolecular *O-S* acyl shift reaction was recently utilized to prepare peptide thioesters [30]. However, hydrolysis (11% yield) occurred. Hence, a need continues to exist for a general synthetic procedure for preparing peptide thioesters by Fmoc SPPS.

We recently reported on a new strategy for peptide thioester synthesis that is compatible with Fmoc chemistry. The strategy involves an *N*-4,5-dimethoxy-2-mercaptobenzyl (Dmmb) auxiliary-assisted *N-S* acyl shift reaction, which proceeds under acidic conditions [31–33]. The attractive feature of this method is that racemization is effectively suppressed at the thioester positions (less than 1% for Ala, Phe, Leu or Ser). These results were obtained by the synthesis of some model short-chain peptide thioesters including three to six amino acid residues. When the conditions were applied to the preparation of BPTI(1–29)-$SCH_2CH_2SO_3H$ (BPTI, bovine pancreatic trypsin inhibitor), some side reactions occurred. To optimize the yield and suppress the side reactions, we searched series of different acid treatment conditions for the synthesis of the BPTI thioester. The established conditions were then applied to on-resin peptide thioester preparations. In this article, we report on these mild and effective conditions that can be used to prepare peptide thioesters, both in solution and on a resin.

* *Correspondence to: Saburo Aimoto, Institute for Protein Research, Osaka University, Yamadaoka 3-2, Suita, Osaka 565-0871, Japan.*
E-mail: aimoto@protein.osaka-u.ac.jp

Institute for Protein Research, Osaka University, Yamadaoka 3-2, Suita, Osaka 565-0871, Japan

Materials and Methods

General

Fmoc-amino acid derivatives were purchased from the Peptide Institute Inc (Osaka, Japan). Amino acid derivatives used were of L-configuration except for glycine unless otherwise noted. 4-[*N*-(9-Fluorenylmethoxycarbonyl)phenylalanyloxymethyl]phenoxymethylated polyethylene glycol resin (Fmoc-Phe-Alko-PEG resin) was purchased from Watanabe Chemical Industries Ltd. (Hiroshima, Japan). Aminomethyl-ChemMatrix hydrochloride resin was purchased from Matrix Innovation (Montreal, Canada). NovaPEG amino resin hydrochloride was purchased from Novabiochem (Darmstadt, Germany). 1-Methyl-2-pyrrolidinone (NMP) was peptide synthesis grade (Nacalai Tesque, Kyoto, Japan). Fmoc-Leu-D,L-[Dmmb(Trt)]Ala-OH was synthesized according to our previous study [33]. All the other chemicals were commercially available and were used without further purification.

MALDI-TOF MS spectra were recorded on an Autoflex (Bruker Daltonics, Billerica, US). ESI-MS spectra were recorded on a Thermo Finnegan LCQ DECA XP spectrometer. Fmoc contents were calculated based on the absorbance of *N*-(9-fluorenylmethyl)piperidine at 301 nm, as measured using a Hitachi U-2001 spectrophotometer after treatment of the peptide resin with 50% piperidine in *N*,*N*-dimethylformamide (DMF). Peptide yields were determined by amino acid analysis, which was performed on a Hitachi L-2000 amino acid analyzer after hydrolysis with constant boiling HCl (Nacalai Tesque) at 110 °C for 24 h in an evacuated sealed tube. The extent of the deamination reactions was estimated by the amino acid analysis as described above or by a comparison of peak area of the deaminated peptides with those for the desired peptides. HPLC analyses were carried out on a reversed-phase (RP) column (YMC-Pack ProC18 RS −4.6 × 150 mm or 10 × 250 mm; YMC Co., Ltd., Kyoto, Japan) using a linear increasing gradient of acetonitrile in water/0.1% trifluoroacetic acid (TFA), and peptides were detected by measuring the absorbance at 220 nm. Disposable octadecylsilyl (ODS) cartridges, TOYOPACK ODS-M, were purchased from Tosoh (Tokyo, Japan).

Preparation of Dmmb-Bound Resin 1 and 1′

Fmoc-Leu-D,L-[Dmmb(Trt)]Ala-Phe-Alko-PEG resin (1)

An Fmoc-Phe-Alko-PEG resin (0.27 mmol/g, 370 mg, 0.10 mmol) was washed with NMP (1 min × 3), treated with 20% piperidine in NMP (v/v; 5 min × 2, 20 min) and washed with NMP (1 min × 6). The resin was mixed for 19 h with a solution, prepared by mixing Fmoc-Leu-D,L-[Dmmb(Trt)]Ala-OH (100 mg, 0.12 mmol), *N*,*N*′-diisopropylcarbodiimide (DIPCI); 19 µl, 0.12 mmol) and 1-hydroxybenzotriazole monohydrate (HOBt•H_2O; 18 mg, 0.12 mmol) in DMF (1.0 ml). The resulting resin was washed with NMP (1 min × 3), treated with 10% Ac_2O, 5% *N*,*N*-diisopropylethylamine (DIEA) in NMP (v/v; 10 min), washed with NMP (1 min × 6), MeOH (1 min × 6) and dried under reduced pressure to yield resin **1** (403 mg, Fmoc content: 0.185 mmol/g).

Fmoc-Leu-D,L-[Dmmb(Trt)]Ala-Phe-NHCH$_2$-ChemMatrix resin (1′)

HCl•NH_2CH_2-ChemMatrix resin (0.73 mmol/g, 558 mg, 0.41 mmol) was washed with NMP (1 min × 3), 5% DIEA in NMP (2 min × 3) and NMP (1 min × 3). A mixture of Fmoc-Phe-OH (58.1 mg and 150 µmol) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU; 57 mg, 0.15 mmol), HOBt•H_2O (23 mg, 0.15 mmol) and DIEA (52 µl, 0.30 mmol) in DMF (2.0 ml) was added to the resin and suspension was stirred for 23 h. The resulting resin was washed with NMP (1 min × 3), treated with 10% Ac_2O, 5% DIEA in NMP (v/v; 10 min × 3), washed with NMP (1 min × 6), MeOH (1 min × 6) and dried under reduced pressure to yield the Fmoc-Phe-NHCH$_2$-ChemMatrix resin (493 mg, Fmoc content: 0.248 mmol/g).

The Fmoc-Phe-NHCH$_2$-ChemMatrix resin (0.248 mmol/g, 482 mg, 0.120 mmol) was washed with NMP (1 min × 3), treated with 20% piperidine (5 min × 2, 20 min) and washed with NMP (1 min × 6). The resin was added to the mixture of Fmoc-Leu-D,L-[Dmmb(Trt)]Ala-OH (110 mg, 0.130 mmol), DIPCI (20.9 µl, 0.135 mmol) and HOBt•H_2O (20.7 mg, 0.135 mmol) in NMP (3 ml), and the suspension was stirred for 24 h. The resulting resin was washed with NMP (1 min × 3), treated with 10% Ac_2O, 5% DIEA in NMP (v/v; 10 min × 3), washed with NMP (1 min × 6), MeOH (1 min × 6) and dried *in vacuo* to yield the Fmoc-Leu-D, L-[Dmmb(Trt)]Ala-Phe-NHCH$_2$-ChemMatrix resin (**1**′) (522 mg, Fmoc content: 0.195 mmol/g).

Preparation of the Protected Dmmb-Bound BPTI(1–29) Resin 2 and 2′

Peptide chain elongation was carried out with an Applied Biosystems peptide synthesizer (model 433A) using the *FastMoc 0.25 MonPrevPk* protocol with end capping by acetic anhydride/HOBt/DIEA after the introduction of each amino acid.

Protected BPTI(1–29)-D,L -[Dmmb(Trt)]Ala-Phe-Alko-PEG resin (2)

Starting from the Dmmb-bound resin **1** (0.185 mmol/g, 399 mg, 73.8 µmol), 690 mg of a protected peptide resin corresponding to the sequence of BPTI(1–29), H-Arg(Pbf)-Pro-Asp(^{t}Bu)-Phe-Cys(Trt)-Leu-Glu(^{t}Bu)-Pro-Pro-Tyr(^{t}Bu)-Thr(^{t}Bu)-Gly-Pro-Cys(Trt)-Lys(Boc)-Ala-Arg(Pbf)-Ile-Ile-Arg(Pbf)-Tyr(^{t}Bu)-Phe-Tyr(^{t}Bu)-Asn(Trt)-Ala-Lys(Boc)-Ala-Gly-Leu-D,L-[Dmmb(Trt)]Ala-Phe-Alko-PEG resin, was obtained (Pbf: 2,2,4,6,7,-pentamethyldihydrobenzofuran-5-sulfonyl, Trt: trityl).

Protected BPTI(1–29)-D,L-[Dmmb(Trt)]Ala-Phe-NHCH$_2$-ChemMatrix resin (2′)

Starting from the Dmmb-bound resin **1**′ (0.195 mmol/g, 494 mg, 96.3 µmol), 1.55 g of protected peptide resin corresponding to the sequence of BPTI(1–29), H-Arg(Pbf)-Pro-Asp(^{t}Bu)-Phe-Cys(Trt)-Leu-Glu(^{t}Bu)-Pro-Pro-Tyr(^{t}Bu)-Thr(^{t}Bu)-Gly-Pro-Cys(Trt)-Lys(Boc)-Ala-Arg(Pbf)-Ile-Ile-Arg(Pbf)-Tyr(^{t}Bu)-Phe-Tyr(^{t}Bu)-Asn(Trt)-Ala-Lys(Boc)-Ala-Gly-Leu-D,L-[Dmmb(Trt)]Ala-Phe-NHCH$_2$-ChemMatrix resin (**2**′), was obtained.

Preparation of BPTI(1–29)-D,L-(Dmmb)Ala-Phe-OH (3)

Resin **2** (50.5 mg) was treated with 1.0 ml of Reagent K (TFA, phenol, water, thioanisole and 1,2-ethanedithiol [82.5 : 5 : 5 : 5 : 2.5, v/v/v/v/v]) [42] at room temperature for 2 h. Cold ether was added to the reaction mixture and the suspension was stirred for 15 min and then allowed to stand for an additional 15 min. The resulting precipitate was washed with ether three times and then dissolved in a mixture of water and acetonitrile. The solution was passed through a disposable ODS cartridge and the elute was lyophilized to give a crude white powder (14.9 mg). This crude product was purified by RP-HPLC (column: YMC-Pack ProC18 RS [10 × 250 mm]) to give the Dmmb-bound peptide **3** (18% based on the Fmoc group of the initial resin **1**); MALDI-TOF MS, *m/z* 3763.61,

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calculated for $[M+H]^+$: 3762.43 (average); amino acid analysis: $Asp_{2.0}Thr_{0.90}Glu_{1.1}Pro_{3.9}Gly_{2.0}Ala_{3.3}Ile_{1.2}Leu_{2.0}Tyr_{2.9}Phe_{3.4}Lys_2Arg_{2.9}$.

Peptide Thioester Synthesis in Solution via the *N-S* Acyl Shift Reaction

BPTI(1–29)-$SCH_2CH_2SO_3H$ (4)

The Dmmb-bound peptide **3** (2.41 nmol) was treated with 0.25 M HCl in 50% aqueous (aq) acetonitrile (20 µl) containing 0.5% tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (w/v), prepared by mixing equal volumes of 0.50 M HCl and acetonitrile, at 37 °C for 3 h and 20 µl of 50% aq acetonitrile was then added. Sodium 2-mercaptoethanesulfonate (6.6 mg) was added to this solution, followed by AcONa (1.8 mg), to increase the pH of the solution to 6–7. The reaction mixture was stirred at room temperature for 1 h and purified by RP-HPLC (column: YMC-Pack ProC18 RS [4.6 × 150 mm]) to give the peptide thioester **4** (1.98 nmol) in a yield of 82% based on peptide **3**; MALDI-TOF MS, *m/z* 3486.84, calculated for $[M+H]^+$: 3486.12 (average); amino acid analysis: $Asp_{1.9}Thr_{0.90}Glu_{1.0}Pro_{3.9}Gly_{1.9}Ala_{2.9}Ile_{1.3}Leu_{2.0}Tyr_{2.9}Phe_{2.1}Lys_2Arg_{2.8}$.

Peptide Thioester Synthesis via the *N-S* Acyl Shift Reaction on Resin

BPTI(1–29)-SCH_2 CH_2SO_3H (4)

An aliquot of protected Dmmb-bound resin **2′** (15 mg) was treated with Reagent K (400 µl) at room temperature for 2 h and successively washed with dichloromethane (1 min × 3) and acetonitrile (20 s) to give the unprotected BPTI(1–29)-D,L-(Dmmb)Ala-Phe-$NHCH_2$-ChemMatrix resin (**3′**). Resin **3′** was treated with 0.25 M HCl in 50% aq acetonitrile (500 µl) at 37 °C for 3 h after washing with the same hydrochloric acid solution (1 min × 2). The resulting resin was washed with aq acetonitrile (20 s × 3) and treated with sodium 2-mercaptoethanesulfonate (65 mg) in 0.1 M sodium phosphate buffer (pH 7.0) (200 µl) and acetonitrile (200 µl) for 1 h. The resulting resin was washed with 50% aq acetonitrile (400 µl × 3) and the filtrate was subjected on RP-HPLC (YMC-Pack ProC18 RS, 4.6 × 150 mm) to give peptide thioester **4** in a yield of 15% (based on the Fmoc group of initial resin **1′**): MALDI-TOF MS, *m/z* 3485.50, calculated for $[M+H]^+$: 3486.12 (average); amino acid analysis: $Asp_{2.1}Thr_{1.1}Glu_{1.0}Pro_{3.9}Gly_{2.0}Ala_{3.1}Ile_{1.2}Leu_{1.9}Tyr_{2.8}Phe_{1.8}Lys_2Arg_{3.0}$.

TpoR(467–481)-$SCH_2CH_2SO_3H$ (5) (TpoR, thrombopoietin receptor)

Yield, 34% based on the Fmoc-Ala-D,L-[Dmmb(Trt)]Ala-Phe-$NHCH_2$-NovaPEG resin (**1′-a**); ESI-MS, *m/z* 1759.1, calculated for $[M+H]^+$: 1759.7 (monoisotopic); amino acid analysis: $Asp_{1.0}Thr_{0.92}Ser_{2.7}Glu_1Pro_{2.8}Gly_{1.9}Ala_{1.0}Tyr_{1.0}$.

Fmoc-TRF2(1–41)-$SCH_2CH_2SO_3H$ (6) (TRF2, telomere repeat-binding factor 2)

Yield, 12% based on the Fmoc-Gly-D,L-[Dmmb(Trt)]Ala-Phe-$NHCH_2$-NovaPEG resin (**1′-b**); MALDI-TOF MS, *m/z* 4279.49, calculated for $[M+H]^+$: 4279.54 (average); amino acid analysis: $Asp_{0.96}Ser_{5.3}Glu_{2.1}Gly_{13}Ala_{5.6}Leu_1His_{1.0}Arg_{9.0}$.

[$Ser(PO_3H_2)^{10}$]-Histone H3(1–12)-$SCH_2CH_2SO_3H$ (7)

Yield, 31% based on the Fmoc-Gly-D,L-[Dmmb(Trt)]Ala-Phe-OCH_2-Pam-ChemMatrix resin (**1′-c**) (Pam, phenylacetoamide); MALDI-TOF MS, *m/z* 1508.68, calculated for $[M+H]^+$: 1508.67 (monoisotopic); amino acid analysis: $Thr_{2.9}Ser_{0.73}Glu_{1.0}Gly_{1.0}Ala_{2.0}Lys_2Arg_{2.0}$.

[$Lys(Me_3)^9$]-Histone H3(1–33)-$SCH_2CH_2SO_3H$ (8)

Yield, 19% based on the starting resin **1′-b**; MALDI-TOF MS, *m/z* 3562.57, calculated for M^+: 3562.18 (average); amino acid analysis: $Thr_{5.0}Ser_{2.0}Glu_{2.2}Pro_{2.2}Gly_{3.2}Ala_{8.5}Leu_{1.1}Lys_{5.4}Arg_4$.

Results and Discussion

Peptide Thioester Synthesis via the *N-S* Acyl Shift Reaction by 85% Aqueous TFA Treatment

As reported in our previous study, tripeptide thioesters were efficiently prepared via an auxiliary-assisted *N-S* acyl shift reaction by treatment with 85% aq TFA [33]. We employed the same procedure to prepare a model, 29-mer peptide thioester, BPTI(1–29)-$SCH_2CH_2SO_3H$ (**4**) (sequence: RPDF-CLEPPY TGPCKARIIR YFYNAKAGL), as shown in Scheme 1. Dmmb-attached resin **1** was prepared by introducing Fmoc-Leu-D,L-[Dmmb(Trt)]Ala-OH on the H-Phe-Alko-PEG resin. Peptide chain elongation was carried out by standard Fmoc SPPS. The resulting protected peptide resin **2** was treated with Reagent K and purified by RP-HPLC to provide BPTI(1–29)-D,L-(Dmmb)Ala-Phe-OH (**3**) in a yield of 18% based on resin **1**. (Crude product **3** contained about 2–3% of *S*-peptide **9**, whose amount depended on the work-up period.) The Dmmb-attached peptide **3** was treated with 85% aq TFA solution containing 0.5% TCEP at 25 °C for 7 h to produce the corresponding *S*-peptide **9** via the Dmmb auxiliary-assisted *N-S* acyl shift reaction. After removing the solvent by evaporation under reduced pressure, the *S*-peptide **9** was treated with sodium 2-mercaptoethanesulfonate and sodium acetate in aq acetonitrile (pH 6–7) to provide BPTI(1–29)-$SCH_2CH_2SO_3H$ (**4**) and *N*-D,L-(Dmmb)Ala-Phe-OH (**10**) by an intermolecular thiol-thioester exchange reaction. As shown in Figure 1, peptide thioester **4** was produced in 44% yield (isolated yield) based on peptide **3**, although the molecular mass corresponding to BPTI(1–29)-D,L-Ala-Phe-OH (**11**) and

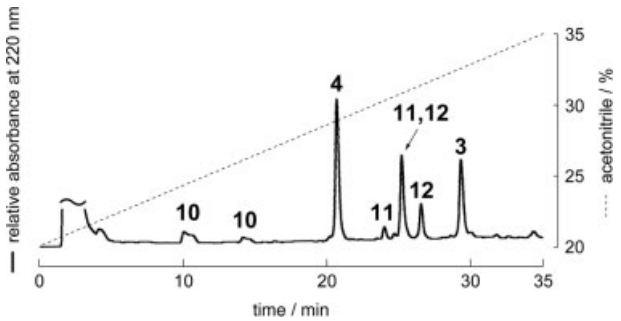


Figure 1. RP-HPLC profile of the reaction mixture of BPTI(1–29)-$SCH_2CH_2SO_3H$ (**4**), in which the *N-S* acyl shift reaction was induced by treatment with 85% aqueous TFA at 25 °C for 7 h. The peak at 2–5 min was derived from nonpeptidic compounds. HPLC conditions; column: YMC-Pack ProC18 RS (4.6 × 150 mm), eluent: 0.1% TFA in aq acetonitrile, flow rate: 1.0 ml/min.

Table 1. Effects of different acid conditions in the *N-S* acyl shift reaction on production of BPTI(1–29)-$SCH_2CH_2SO_3H$ (**4**) and a deamination compound, (N24D)BPTI(1–29)-$SCH_2CH_2SO_3H$ (**13**)

Entry	Acid	Time (h)	Temperature (°C)	Yield of peptide thioester **4** (%)[a]	Yield of **13** (%)[a]
1	85% (11.4 M) TFA	7	25	44	n.d.[b]
2	1.0 M TFA	7	25	68	0.9
3	0.50 M H_2SO_4	7	25	70	0.8
4	1.0 M HCl	7	25	77	3.2
5	1.0 M HCl	3	37	79	8.1
6	0.25 M HCl	3	37	82	0.9
7	0.10 M HCl	3	37	58	0.1

[a] Yields are based on BPTI(1–29)-D,L-(Dmmb)Ala-Phe-OH (**3**).
[b] n.d., not detected. Other by-products **11** and **12** were observed.

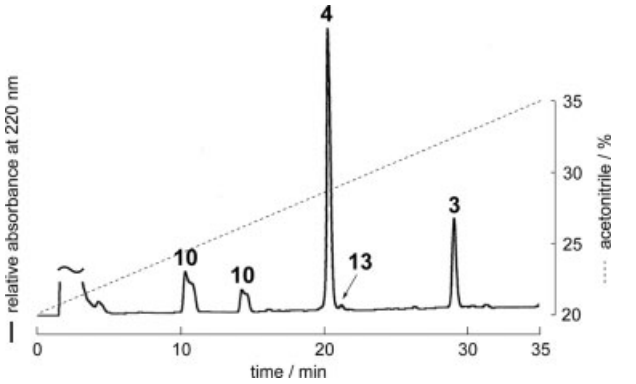


Figure 2. RP-HPLC profile of the reaction mixture of BPTI(1–29)-$SCH_2CH_2SO_3H$ (**4**), in which the *N-S* acyl shift reaction was induced by treatment with a 0.25 M HCl solution at 37 °C for 3 h. The peak at 2–5 min is derived from nonpeptidic compounds. HPLC conditions; column: YMC-Pack ProC18 RS (4.6 × 150 mm), eluent: 0.1% TFA in aqueous acetonitrile, flow rate: 1.0 ml/min.

BPTI(1–29)-D,L-(Dmmb)Ala-OH (**12**) was observed in the RP-HPLC fraction by MALDI-TOF MS. (MALDI-TOF MS, compound **11**: *m/z* 3580.63 (calculated for $[M+H]^+$: 3580.19 [average]); compound **12**: *m/z* 3615.90 (calculated for $[M+H]^+$: 3615.26 [average]).)

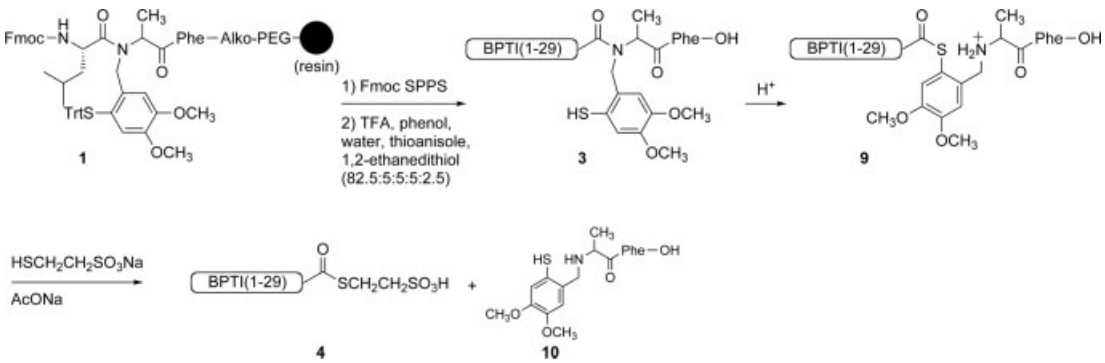
The Dmmb-cleaved peptide **11** was generated by acidolysis of the Dmmb group on the amide during the treatment with the 85% aq TFA solution. The *C*-terminal-missing peptide **12** might be produced via an *N-S* acyl shift reaction at the amide bond between D,L-(Dmmb)Ala and Phe residues, followed by the hydrolysis of the generated thiolactone [32,43–45].

Optimization of Conditions for the Dmmb-Assisted *N-S* Acyl Shift Reaction to Suppress the Side Reactions by Use of a Diluted Acid Reagent

We considered it possible that the strong acidity of 85% (11.4 M) aq TFA (pH < 0 [observed]) might have caused the generation of by-products **11** and **12**, thus reducing the yield of **4** (entry 1 in Table 1). Hojo and coworkers recently reported that an *N-S* acyl shift reaction mediated by an *N*-ethyl cysteine occurs under milder acidic conditions, 5% aq mercaptopropionic acid solution (pH ~1) [35]. Therefore, we reasoned that the use of a more dilute acid reagent might suppress these side reactions. The following conditions were used to prepare peptide thioester **4** (Scheme 1): Peptide **3** was treated with a dilute acid reagent containing 0.5% TCEP (w/v) in aq acetonitrile to give *S*-peptide **9**. Sodium 2-mercaptoethanesulfonate was added to the *S*-peptide **9** reaction mixture, which was then directly neutralized with sodium acetate. The solution was stirred for 1 h to produce the peptide thioester **4**. We found that treatment of peptide **3** with a dilute solution of a strong acid, such as 1.0 M hydrochloric acid, 1.0 M TFA and 0.5 M sulfuric acid (pH 0 ~ 0.2), completely suppressed the generation of by-products **11** and **12**. Among these three acids, a 1.0 M hydrochloric acid solution gave the best yield (77% based on peptide **3**) as shown in entries 2 to 4 in Table 1.

When a 1.0 M hydrochloric acid solution was used, a small amount of deamination compound (N24D)BPTI(1–29)-$SCH_2CH_2SO_3H$ (**13**) was produced, presumably by hydrolysis of the side chain amide at Asn^{24} as shown in entry 4 in Table 1. To suppress the deamination, the reaction period was shortened from 7 h to 3 h and the reaction temperature was increased from 25 to 37 °C, as shown in entry 5 (Table 1). The yield was maintained but the deamination was not suppressed. The generation of by-product **13** was suppressed to less than 1% when the hydrochloric acid concentration was lowered from 1.0 M to 0.25 M, as shown in the RP-HPLC profile (Figure 2) and entry 6 in Table 1. Additional dilution of the acid resulted in a slower *N-S* acyl shift reaction (Table 1, entry 7).

These results suggest that the *N-S* acyl shift reaction proceeds even in a 0.25 M hydrochloric acid solution (pH about 0.6). We conclude that the milder acid reagent increased the selectivity in activation of the carbonyl in the amide containing a Dmmb group by protonation. This is partly because the conjugate acid of a *N,N*-dialkylamide has the highest p*K*a value among primary



Scheme 1. Scheme for the synthesis of BPTI(1–29)-$SCH_2CH_2SO_3H$ (**4**) via an auxiliary-assisted *N-S* acyl shift reaction.

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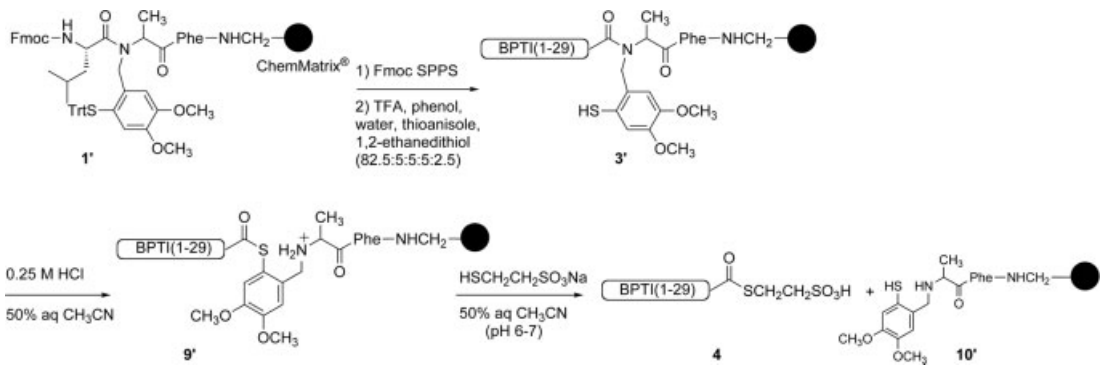

Scheme 2. Synthetic route for the preparation of peptide thioester **4** via an on-resin *N*-*S* acyl shift reaction.

amides, *N*-alkylamides and *N*,*N*-dialkylamides (e.g. the p*K*a values of the protonated forms of acetamide, *N*-methylacetamide and *N*,*N*-dimethylacetamide are −0.6, −0.4 and −0.3, respectively [46]). Hence, the generation of by-products **12** and **13** was suppressed. Acidolysis of the Dmmb group to produce **11** was clearly suppressed under dilute acid conditions.

On-Resin Preparation of Peptide Thioester

For a more convenient preparation of peptide thioester **4** than that in solution, we applied the developed acid conditions (entry 6 in Table 1) to an on-resin *N*-*S* acyl shift reaction, as shown in Scheme 2. Since a peptide chain is directly loaded onto the aminomethylated resin without an acid-sensitive linker in this strategy, the Dmmb-bound peptide **3** and product **10** are not cleaved from the resin, as is the case in resins **3**′ and **10**′, and the peptide thioester **4** is the only compound eluted in the solution phase. ChemMatrix resin (or NovaPEG resin) was used when the reactions were carried out in aqueous media. These resins are all PEG-based resins and are well-solvated in water [47].

The protected BPTI(1–29)-D,L-[Dmmb(Trt)]Ala-Phe-NHCH$_2$-ChemMatrix resin (**2**′) was prepared by a procedure similar to that used for the preparation of the protected BPTI(1–29)-D,L-[Dmmb(Trt)]Ala-Phe-Alko-PEG resin **2** (see Material and Methods section). The protected peptide resin **2**′ was treated with Reagent K for 2 h to give the BPTI(1–29)-D,L-(Dmmb)Ala-Phe-NHCH$_2$-ChemMatrix resin (**3**′), which was washed and then stirred with 0.25 M HCl in 50% aq acetonitrile at 37 °C for 3 h. The resulting *S*-peptide resin **9**′ was quickly washed and then treated with 1.0 M sodium 2-mercaptoethanesulfonate in a mixture of sodium phosphate buffer (pH 7.0) and acetonitrile (1 : 1, v/v) for 1 h to give peptide thioester **1**. (4-Mercaptophenylacetic acid (MPAA) efficiently reacted with *S*-peptide resin **4**′ to provide the corresponding peptide *S*-aryl thioester, which is known to be a useful building block for native chemical ligation [48].) The filtrate was subjected to RP-HPLC and the elution profile is shown in Figure 3. The peptide thioester **4** was obtained in good purity (15% yield based on initial resin **1**′). This yield was nearly equal to that for peptide thioester **4** prepared via the *N*-*S* acyl shift reaction in solution. No evidence was found for the presence of the *C*-terminal-missing peptide **12** in the reaction mixture. The generation of the deamination product **13** was in 0.5% based on the Fmoc content of the initial resin **1**′ (the ratio of **4** to **13** was 97 to 3; Figure 3, peak †). No racemization of Leu^{29} adjacent to the thioester moiety was detected by RP-HPLC (Supporting Information, Figure S1).

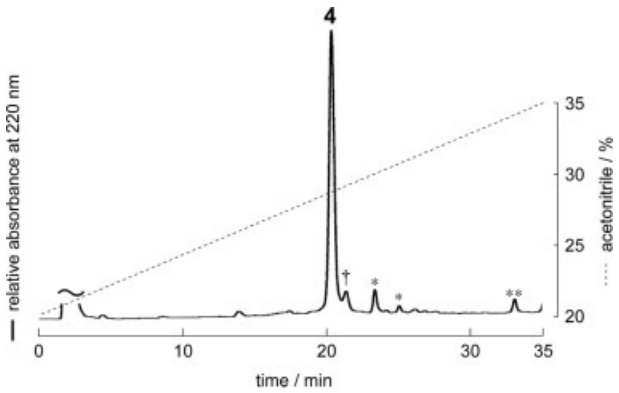

Figure 3. RP-HPLC profiles of the reaction mixture of BPTI(1–29)-SCH$_2$CH$_2$SO$_3$H (**4**) prepared via an on-resin *N*-*S* acyl shift reaction, according to Scheme 2. Peak † contained Ac-BPTI(6–29)-SCH$_2$CH$_2$SO$_3$H and **13**, peaks * contained a ^{t}Bu group adduct with peptide thioester **4** and peak ** contained a trityl (Trt) group adduct with peptide thioester **4**, which were determined by MALDI-TOF MS. The peak for 2–4 min was derived from nonpeptidic compounds. HPLC conditions; column: YMC-Pack ProC18 RS (4.6 × 150 mm); eluent: 0.1% TFA in aqueous acetonitrile; and flow rate: 1.0 ml/min.

Demonstration of the Preparation of Four Different Peptide Thioesters

To validate the utility of this method, several peptide thioesters were prepared according to the same procedure on a resin as in Scheme 2, starting from the Fmoc-Xaa-D,L-[Dmmb(Trt)]Ala-Phe-NHCH$_2$-NovaPEG resin (**1**′-**a** [Xaa: Ala], **1**′-**b** [Xaa: Gly]) or the Fmoc-Gly-D,L-[Dmmb(Trt)]Ala-Phe-OCH$_2$-Pam-ChemMatrix resin (**1**′-**c**). The thrombopoietin receptor (TpoR)(467–481)-SCH$_2$CH$_2$SO$_3$H (**5**) containing acid-sensitive moieties, Trp residue [49] and the Asp-Pro sequence [50], was synthesized without any significant side reactions in a yield of 34% based on the starting resin **1**′-**a** (entry 1 in Table 2). A peptide thioester consisting of 41 amino acid residues, a telomere repeat-binding factor 2 (TRF2)(1–41)-SCH$_2$CH$_2$SO$_3$H (**6**), was also prepared in a yield of 12% (entry 2 in Table 2). Site-specific chemically modified peptide thioesters, such as [Ser(PO$_3$H$_2$)^{10}]-histone H3(1–12)-SCH$_2$CH$_2$SO$_3$H (**7**) and [Lys(Me$_3$)^9]-histone H3(1–33)-SCH$_2$CH$_2$SO$_3$H (**8**), were also efficiently prepared in yields of 31% and 19%, respectively (entry 3 and 4, Table 2). The deamination of the Gln residues of peptide thioester **5** and **7** was in 1.4 and 1.1%, respectively (the ratios of the desired peptide thioester **5** or **7** to the corresponding deaminated peptide were 97 to 3 or 96 to 4, respectively; see Supporting Information). In the purification of peptide thioesters

Table 2. Preparation of several peptide thioesters via an on-resin *N-S* acyl shift reaction

Entry	Peptide thioester	Peptide sequence	Yield (%)[a]
1	TpoR(467–481)-SR (**5**)	GPTYQGPWSSWSDPA	34
2	Fmoc-TRF2(1–41)-SR (**6**)	AGGGGSSDGSGRAAGRRASR SSGRARRGRHEPGLGGPAERG	12
3	[Ser(PO_3H_2)^{10}]-H3(1–12)-SR (**7**)	ARTKQTARKS(PO_3H_2)TG	31
4	[Lys(Me_3)^9]-H3(1–33)-SR (**8**)	ARTKQTARK(Me_3)STGGKAPRKQL ATKAARKSAPATG	19

[a] Overall yield [based on starting resin **1′-a** (entry 1), **1′-b** (entry 2 and 4) and **1′-c** (entry 3)].
-SR, -$SCH_2CH_2SO_3H$.

4, **5**, **7** and **8** containing the Asn or Gln residues, the desired peptide thioesters were cleanly separated from the deamination peptides by RP-HPLC. These results indicate that the preparation of a peptide thioester via the Dmmb-assisted *N–S* acyl shift reaction constitutes a useful and viable methodology.

Conclusion

We describe herein a method for the synthesis of peptide thioesters compatible with Fmoc SPPS via a Dmmb auxiliary-mediated *N-S* acyl shift reaction. The Dmmb-bound peptide was converted to the corresponding *S*-peptide by an *N-S* acyl shift reaction under mild acidic conditions, i.e. a 0.25 M hydrochloric acid solution. The *S*-peptide was treated with a thiol compound to give a peptide thioester by an intermolecular thiol-thioester exchange reaction, without significant side reaction products. The results indicate, in this and in previous reports [31,33], that peptide thioesters prepared by this method involve minimal epimerization of the amino acid residue adjacent to the thioester moiety. The *N-S* acyl shift reaction was successfully applied to the on-resin preparation of peptide thioesters. We were able to successfully synthesize a variety of peptide thioesters using this strategy, such as peptide thioesters containing 41 amino acid residues, Cys residues, Trp residues, Asp-Pro sequence, a phosphorylated serine residue or a trimethylated Lys residue. These results indicate that the Dmmb-assisted *N-S* acyl shift reaction provides a useful and promising route to the preparation of peptide thioesters.

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Supporting information

Supporting information may be found in the online version of this article.

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