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# Preparation of peptide thioesters using Fmoc strategy through hydroxyl side chain anchoring

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**Abstract**

In the course of the chemical synthesis of human protein mitogaligin, we present here a simple method to prepare peptide thioesters using Fmoc chemistry. The hydroxyl side chain of serine was reacted with a trichloroacetimidate Wang resin to anchor it on solid phase. After peptide elongation and orthogonal unmasking of the C-terminus, the amino thioester was introduced under optimized conditions to avoid epimerization.
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Since developed by Kent and co-workers in 1994,[1] native chemical ligation technique (NCL) has been used to synthesize various natural and non-natural polypeptides and proteins. The chemoselective ligation between a C-terminal peptide thioester and a N-terminal cysteinyl peptide results in the formation of an amide bond. Up to now, the preparation of the peptide thioester has remained the limiting stage of this methodology. It was originally carried out using the Boc strategy which requires of a strong acid treatment (HF or TFMSA) to release the peptide from the resin. As these conditions are generally not compatible with the obtention of peptide thioesters bearing post-translational modifications, alternative methods have been developed using the milder Fmoc strategy. However, its main pitfall is the thioester lability under the nucleophilic conditions used for the successive Fmoc removals (piperidine). To overcome it, almost all current methods[2–14] involve a post elongation introduction of the thioester which includes the modification of the C-terminus, a known challenging task in peptide chemistry.[15] The coupling of a thiol (thioesterification) or an amino acid thioester (amidation) at the C-terminus of a protected peptide are among the most user-friendly methods for peptide thioester preparation using Fmoc strategy, therefore the most promising ones.[8–14] The key step of these approaches, that is, the thioester introduction, can be performed either in solution[8] or on solid phase.[9–14] The former is limited to small peptides due to solubility problems of large protected peptides. This difficulty can be ruled out by grafting the peptide to a resin through its backbone[9] or through the side chain of a trifunctional amino acid,[10–14] thus allowing the orthogonal unmasking of the C-terminus. The side-chain anchoring onto a Wang resin,[10,11] a *p*-nitrophenylcarbonate Wang resin,[11,12] a 2-chlorotrityl resin,[11] or amide resins[11,13] has been reported to anchor Asp, Glu, Lys, Asn, and Gln amino acids. Serine and threonine derivatives have been reported to be anchored through a silyl ether linker, but this strategy requires a time consuming multi-step

*Abbreviations:* Bpoc, 2-(4-biphenylyl)isopropoxycarbonyl; HATU, 2-(1-*H*-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, hydroxy-7-azabenzotriazole; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; OSu, succinimido ester; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; TFA, trifluoroacetic acid

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procedure to synthesize the protected amino acid-linker conjugate.[14] To extend the applicability of the hydroxyl side chain anchoring, we explored the synthesis of peptide thioesters from a readily commercially available trichloroacetimidate Wang resin and an appropriate serine derivative (Scheme 1).

Our approach was inspired by the methodology initially developed by Hanessian and Xie,[16] and applied by Mayer and co-workers to the on-resin head-to-tail cyclization of peptides.[17] It involves (a) the β-hydroxyl reaction of Ser or Thr to the trichloroacetimidate derivative of Wang resin, (b) a stepwise elongation using Fmoc chemistry, (c) the selective allyl ester deprotection, (d) the solid phase carboxyl activation to couple an amino thioester, (e) a final TFA treatment to release the deprotected peptide from the resin. To demonstrate the feasibility of this technique, we carried out the synthesis of peptide thioester **1** (Scheme 1). A special care was taken to minimize epimerization which can occur during the installation of the thioester moiety.[8f,9a] Peptide **1** is a short fragment from mitogaligin, a cytotoxic human 97 amino acid protein involved in a new cell death program,[18] that we have planned to produce using native chemical ligation. The sequence corresponds to the 50–53 fragment that will be ligated in this model study to the cysteine-containing peptide **5**, the 54–58 fragment (H–CTWSL–OH), affording ligated peptide **6**.

For the β-hydroxyl anchoring, commercially available trichloroacetimidate Wang resin was reacted with Fmoc–Ser–OAll[19] in anhydrous THF in the presence of $BF_3{\cdot}Et_2O$ as described[16] (Scheme 1, step a). The loading yield (82%) was determined by UV titration of the fluorenylmethylpiperidine adduct after piperidine treatment of resin **2**.[20] After the Fmoc-based peptide elongation (step b), the C-terminal allyl group was removed with $Pd(PPh_3)_4/PhSiH_3$ (step c). The purity of tripeptide **3′** was then checked by analytical HPLC (>95%) and MS[21] after TFA treatment of an aliquot of peptide resin **3**. Next, the amino thioester **4** was installed at the C-terminus. The challenge was to achieve quantitative conversion without affecting the stereochemical integrity of the $C^{\alpha}$ of the C-terminal serine (step d). The epimerization which can possibly occur was evaluated using the commercially available H–Thr(*t*Bu)–$NH_2$ instead of derivative **4**. The two reference peptide amides **7** (Ac–SRST–$NH_2$) and **8** (Ac–SRsT–$NH_2$) were easily synthesized by standard Fmoc chemistry using a Rink resin and characterized by analytical HPLC (Fig. 1a) and MS.[21] Taking advantage of previous studies concerning low epimerization protocols for solid phase carboxyl activation,[9a] HATU/DIEA and HOAt/DCC as coupling reagents were tested under different conditions (Table 1). For each case, tetrapeptide **7** was released from the resin by TFA treatment, and analyzed by HPLC and MS.[21] Purity of crude peptides was estimated by the integration of the HPLC profiles. No significant epimerization (<1%) and good conversion (>99%) were observed (Fig. 1b) except when trying to pre-form the activated ester species by treatment with HATU/DIEA prior to the addition of the threonine derivative (Scheme 1, entry 2) and when a

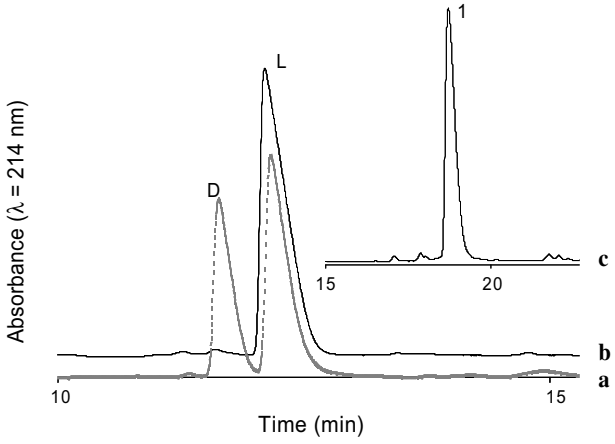
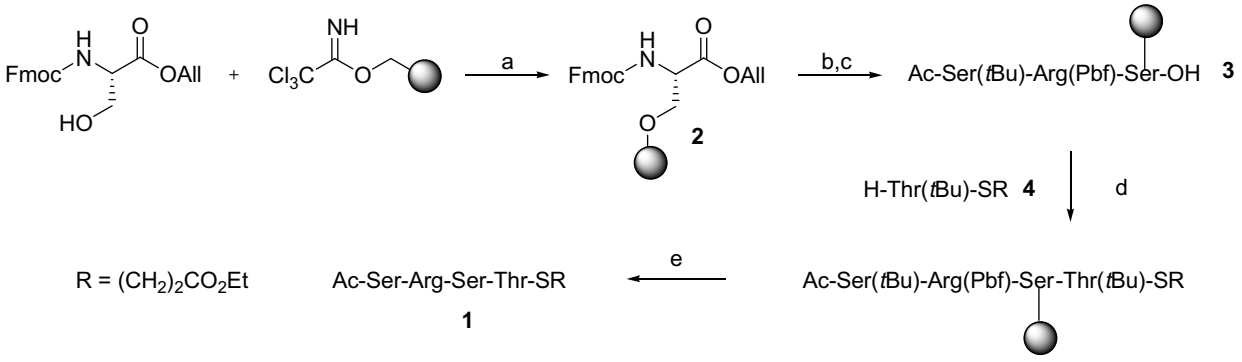



Fig. 1. Analytical RP–HPLC profiles of (a) a mixture of peptide amides **7** (L-form) and **8** (D form) prepared using a Rink amide resin; (b) peptide amide **7** prepared by the hydroxyl side chain anchoring methodology; (c) peptide thioester **1** prepared by the hydroxyl side chain anchoring methodology. Traces a and b: gradient 0–10% B over 30 min; trace c: 0–65% B over 30 min (A: 0.1% TFA in water; B: 0.1% TFA in $CH_3CN$/$H_2O$ 60/40).




Scheme 1. Synthesis of peptide thioester **1** through hydroxyl side chain anchoring using a trichloroacetimidate Wang resin. Reagents and conditions: (a) $BF_3{\cdot}Et_2O$, THF, 0 °C; (b) (i) Fmoc SPPS; (ii) $Ac_2O$/HOBt/DIEA/DMF; (c) $Pd(PPh_3)_4/PhSiH_3/CH_2Cl_2$; (d) see text; (e) TFA/TIS/$H_2O$.

Table 1
Coupling of H–Thr(*t*Bu)–$NH_2$·HCl onto peptide resin **3** using HATU/DIEA and HOAt/DCC as coupling reagents

| | H–Thr(*t*Bu)–$NH_2$·HCl (equiv) | Coupling reagent | Method[a] | DIEA (equiv) | Conversion[b] (%) | Epimerization[b] (%) |
|---|---|---|---|---|---|---|
| 1 | 10 | HATU (10 equiv) | A | 20 | >99 | <1 |
| 2 | 10 | HATU (10 equiv) | B | 20 | —[c] | —[c] |
| 3 | 5 | HATU (5 equiv) | A | 8 | >99 | <1 |
| 4 | 5 | HOAt/DCC (5 equiv) | A | 8 | >99 | <1 |
| 5 | 5 | HOAt/DCC (5 equiv) | C | 8 | >99 | <1 |
| 6 | 2 | HOAt/DCC (2 equiv ) | A | 4 | 40 | nd |

[a] Method A: H–Thr(*t*Bu)–$NH_2$·HCl/DIEA in $CH_2Cl_2$ and coupling reagents in $CH_2Cl_2$/DMF (8:2) were successively added to the resin bed. Method B: a solid phase pre-activation procedure which consisted in adding first HATU/DIEA in $CH_2Cl_2$/DMF (8:2). After 5 min mixing, H–Thr(*t*Bu)–$NH_2$·HCl was added in solid form. Method C: a solid phase pre-activation procedure which consisted in the adding of HOAt/DCC in $CH_2Cl_2$/DMF (8:2), followed by a 5-min mixing and then the addition of H–Thr(*t*Bu)–$NH_2$·HCl/DIEA in $CH_2Cl_2$.

[b] Estimated by integration of the HPLC peaks at 214 nm.

[c] The preformed activated ester proved to be unstable under basic conditions (DIEA). Only traces of the expected amide were detected by HPLC, and the complex mixture of by-products was not characterized.

low excess of coupling reagents (2 equiv) was used (Scheme 1, entry 6).

Before synthesizing peptide thioester **1**, the preparation of amino thioester **4** was undertaken. If the thioesterification of an aliphatic amino acid is straightforward, the thioesterification of a side chain-protected tri-functional amino acid like threonine has not been reported yet. Our synthesis of **4** involves the commercially available Bpoc–Thr(*t*Bu)–OSu derivative and 3-mercaptopropionic acid ethyl ester as the thiol partner.[22] Peptide thioester **1** was then prepared using amino thioester **4** under the conditions similar to entry 4 (Table 1), leading to a 94% conversion. Repeating the same protocol was necessary to reach completion without any detectable epimerization (Fig. 1c).

Finally, HPLC-purified peptide thioester **1** was engaged in the native chemical ligation with cysteine-containing peptide **5** (Fig. 2). After 22 h, the ligation nearly reached completion affording peptide **6** judging by HPLC. Such low reaction kinetics is well known for peptide thioesters containing a C-terminal β-branched residue like threonine.[23]

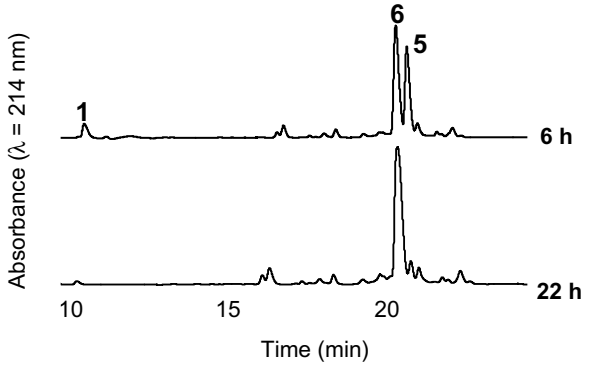



Fig. 2. Analytical RP-HPLC profiles of the ligation reaction between peptide thioester **1** and cysteine-containing peptide **5** yielding peptide **6**. Ligation procedure: Peptides were dissolved in 200 mM $NaH_2PO_4$, pH 7.5, to a final concentration of 2 mM. Benzylmercaptan (1.5%), thiophenol (1.5%), and 20 mM TCEP were added to maintain reducing conditions and to accelerate ligation. Gradient 10–40% B over 30 min (A: 0.1% TFA in water; B: 0.1% TFA in $CH_3CN$).

In conclusion, our results show that the side chain anchoring through the reaction of a β-hydroxyl amino acid with a trichloroacetimidate Wang resin is a valuable methodology to prepare peptide thioesters by standard Fmoc chemistry. The risk of epimerization has been prevented by using efficient procedures for the on-solid phase α-COOH activation. This work extends the strategy based on side chain anchoring of tri-functional amino acids already described and will be adapted in the future to the synthesis of large peptide thioesters.

*Note*: During the redaction of this manuscript, a related paper by Wong and co-workers has been published but using a bromo Wang resin instead of a trichloroacetimidate for the hydroxyl side chain anchoring.[24]

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21. *Mass spectrometry data*: Tripeptide **3′** (Ac–SRS–OH): [M] cald for $C_{14}H_{26}N_6O_7$ 390.19, found (ESI MS): 390.13; tetrapeptide amides **7** (Ac–SRST–$NH_2$) and **8** (Ac–SRsT–$NH_2$) prepared using a Rink resin: [M] cald for $C_{18}H_{34}N_8O_8$: 490.25, found (ESI MS): 490.27 (**8**, D-form), 490.17 (**7**, L-form), **7** prepared by the hydroxyl side chain anchoring methodology: found (ESI MS): 490.44; Peptide thioester **1**: [M] cald for $C_{23}H_{41}N_7O_{10}S$: 607.26, found (ESI MS): 607.21; Cysteine-containing peptide **5** (H–CTWSL–OH): cald for $C_{27}H_{40}N_6O_8S$: 608.26, found (ESI MS): 608.43; Ligated peptide **6** (Ac–SRSTCTWSL–OH): [M] cald for $C_{45}H_{71}N_{13}O_{16}S$: 1081.49, found (MALDI/TOF MS, matrix: α-cyano-4-hydroxycinnamic acid): 1081.48.
22. H–Thr(*t*Bu)–S–$(CH_2)_2CO_2Et$ **4**: 500 mg (0.98 mmol) Bpoc–Thr(*t*Bu)–OSu (commercially available from Bachem) was dissolved in 10 mL $CH_2Cl_2$ under an argon atmosphere. The mixture was cooled to 0 °C and DIEA (171 μL, 2 equiv), then 3-mercaptopropionic acid ethyl ester (252 μL, 2 equiv) were added. The resulting solution was stirred at rt for 18 h then filtered through a short pad of silica gel. The silica gel was further washed with 30 mL of a 9:1 $CH_2Cl_2$/AcOEt mixture, and the combined effluents were concentrated under reduced pressure to yield a yellow oil. This crude product was then dissolved in 4 mL $CH_2Cl_2$ containing 2% TFA. After stirring for 40 min at rt, solvents were evaporated in vacuo and the resulting gum was extracted with water (3 × 5 mL). The water layer was then subjected to standard semi-preparative RP–HPLC to yield after evaporation then lyophilization pure **4** as a white solid (trifluoroacetate salt, 64 mg, 16%). The yield was not optimized. Selected data for **4**: RP-HPLC: tr: 18.6 min (gradient 10–70% B over 30 min (A: 0.1% TFA in water; B: 0.1% TFA in $CH_3CN$); ESI MS: [M] cald for $C_{13}H_{25}N_4S$ 291.15, found: 291.20. $^1H$ NMR (500 MHz, $D_2O$): $\delta$ 1.20 (s, 9H, $Me_3C$), 1.26 (t, 3H, $CH_3CH_2$, $J_{CH_3-CH_2} = 7.1$ Hz), 1.32 (d, 3H, $CH_3^{\gamma}$, $J_{CH_3^{\gamma}-CH\beta} = 6.6$ Hz), 2.71–2.84 (m, 2H, $CH_2CO$), 3.20–3.36 (m, 2H, $CH_2S$), 4.18 (q, 2H, $CH_2CH_3$), 4.28 (d, 1H, $CH^{\alpha}$, $J_{CH\alpha-CH\beta} = 3.1$ Hz), 4.34–4.41 (m, 1H, $CH^{\beta}$). $^{13}C$ NMR (125.8 MHz, $D_2O$): $\delta$ 12.8, 19.0, 23.7, 27.0, 33.0, 61.7, 63.6, 66.1, 75.6, 173.6, 196.1.
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