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Fmoc-Based Synthesis of Peptide Thioesters for Native Chemical Ligation Employing a *tert*-Butyl Thiol Linker

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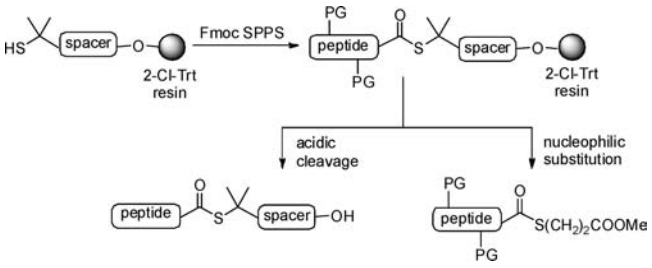
ABSTRACT



***tert*-Butyl thioesters display an astonishing stability toward secondary amines in basic milieu, in contrast to other alkyl and aryl thioesters. Exploiting this enhanced stability, peptide thioesters were synthesized in a direct manner, applying a *tert*-butyl thiol linker for Fmoc-based solid-phase peptide synthesis.**

Peptide thioesters are key molecules in the synthesis of peptides and proteins, for example, as starting materials for native chemical ligation (NCL).[1] NCL is the trans-thioesterification of a C-terminal thioester peptide with an N-terminal cystein peptide, followed by an S → N acyl shift[2] to furnish a native amide linkage. While peptide thioesters can be prepared by Boc chemistry,[3] their synthesis with standard Fmoc-based methods is hampered by the reactivity of thioesters against piperidine resulting in rapid thioester cleavage during Fmoc deprotection. Direct syntheses of peptide thioesters using Fmoc amino acids have been attempted with limited success, e.g., by applying milder deblocking techniques which suffer from low yields and/or incomplete Fmoc deprotection.[4] Instead "safety-catch linkers" were used for the Fmoc-based synthesis of peptide thioesters. These linkers are activated, e.g., by alkylation of acyl sulfonamides (Kenner's linker),[5] oxidation of acyl hydrazides,[6] or intramolecular diacylation.[7,8]

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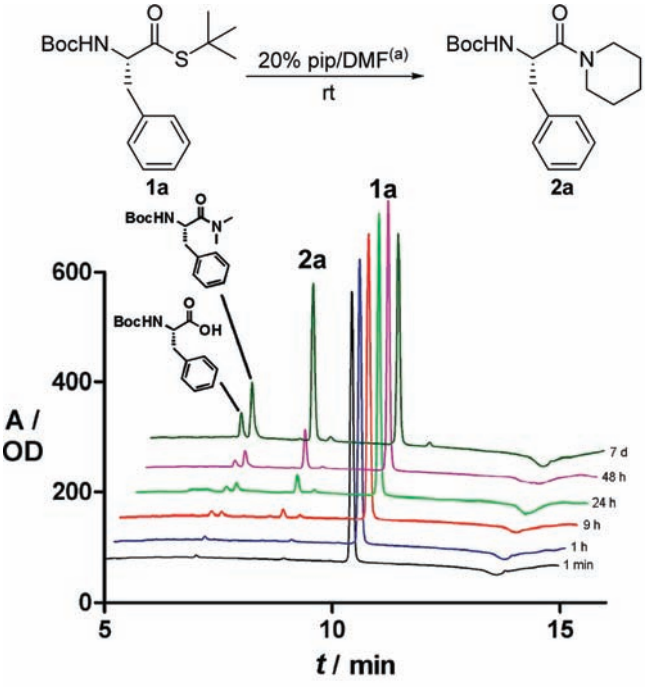
Herein, we describe the first Fmoc-based synthesis of peptide thioesters using a *tert*-butyl thioester as a linker. *tert*-Butyl thioesters are of exceptional stability against nucleophilic bases and strong acids,[9] which distinguishes them from other alkyl thioesters.[10] At the same time, *tert*-butyl thioesters remain smoothly cleavable by thiolates.[11]



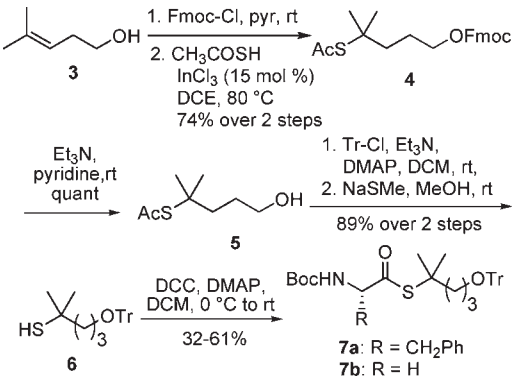
entry	thioester	half life
1	-*tert*-butyl (**1a**)	6.5 days
2	-*iso*-propyl (**1b**)	8.1 h
3	trityl (**1c**)	6.5 h
4	ethyl (**1d**)	5.5 h
5	benzyl (**1e**)	55 min
6	MMP-trt (**7a**)	9.4 days
7	MMP-trt (**7b**)	7.5 h

Figure 1. Stability of various thioesters of Phe during treatment with 20% piperidine in DMF treatment. *tert*-Butyl thioester **1a** yielded the piperidine amide **2a** and the DMF-derived *N*-dimethyl amide. Half-lives were determined by LC−MS at 220 nm.

In order to validate the strategy, various thioesters were investigated under Fmoc-cleavage conditions (20% piperidine in DMF) by HPLC−MS, and the $t_{1/2}$ of *tert*-butyl was > isopropyl > trityl > ethyl > benzyl (Figure 1). The results indicate that thioesters are stabilized by steric hindrance as well by electron donation. For example, the sterically most demanding triphenyl methyl (trityl) thioester was cleaved faster than the smaller but more electron-rich isopropyl and the more bulky benzyl thioester faster then ethyl. The *tert*-butyl thioester of Boc-phenylalanine displayed a half-life of 6.5 days, strongly supporting the feasibility of the envisaged linker system for the direct synthesis of peptide thioesters.

In order to obtain a thioester linkage of sufficient stability, a spacer was introduced and acetyl-protected 4-*m*ercapto 4-*m*ethyl*p*entanol **5** was used as the MMP-linker precursor. Commercially available 4-methylpent-3-en-1-ol **3** was *O*-Fmoc-protected, and indium trichloride[12] catalyzed the regioselective introduction of thioacetic acid yielding the tertiary thioacetate **4** (Scheme 1).

Scheme 1



Deprotection of the Fmoc group afforded the linker precursor **5**. *O*-Tritylation of **5** followed by deacetylation yielded mercaptan **6** which was converted to the model thioester **7a** by S-acylation employing Boc-phenylalanine, *N*,*N*′-dicyclohexylcarbodiimide (DCC), and *N*-dimethylaminopyridine (DMAP).

Thioester **7a** was used as a model compound to investigate the stability of the linker on trityl resin and possessed a $t_{1/2}$ of more than 9 days in the presence of 20% piperidine in DMF. For comparison, the corresponding thioester of Boc-glycine **7b** was prepared and tested. The stability of **7b** toward piperidine was significantly reduced ($t_{1/2}$ = 7.5 h), reflecting the high reactivity of glycine thioesters in NCL.[13] All other amino acid thioesters tested (His, Trp, Gln, Val) showed stability similar to that of the Phe-derived thioester. In conclusion, *tert*-butyl thioesters should be useful as linkers for all C-terminal amino acids with the highest reactivity for glycine.

The MMP linker **5** was attached to 2-chlorotrityl chloride resin in pyridine providing resin **8** in a high yield as determined by elemental analysis of sulfur from a resin sample (Scheme 2). The acetyl protecting group was removed with hydrazine acetate[14] furnishing resin **9**, and the first amino acid was coupled yielding thioester product **10**. Acylation of the thiolinker resin with phenylalanine was investigated with different condensation reagents and

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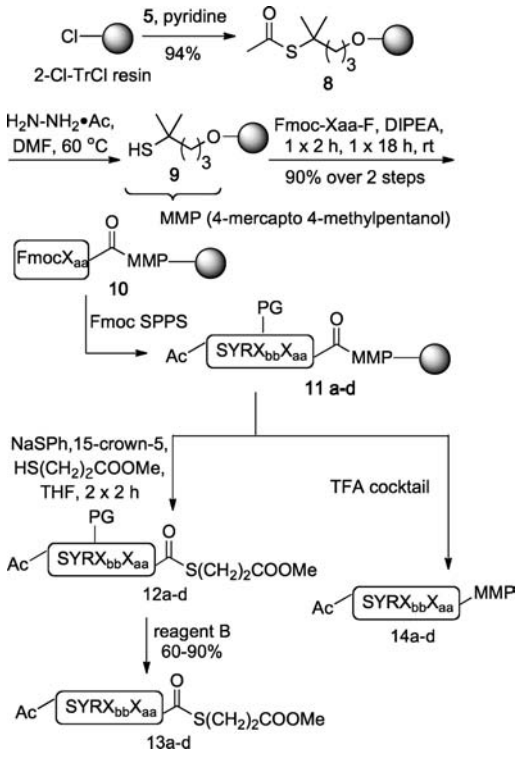
Scheme 2



with acyl fluoride. Reactions were monitored by attenuated total reflection infrared spectroscopy (ATR-IR), and amino acid loadings were quantified by spectrophotometric Fmoc determination following cleavage from a resin sample.

Exclusion of air from the resin was crucial for the success of the first acylation step. Treatment of air-flushed resin with Ellman's reagent[15] indicated a significant decrease of free thiol groups on the resin presumably through oxidative cross-linking.[17] Oxidation of resin **9** was found to be reversible; when treated with sodium bis(2-methoxyethoxy)aluminum hydride (Red-Al) and washed, the Ellman test indicated reduction of the disulfide bridges.

Likewise, the removal of water by azeotropic distillation from the amino acids increased coupling yields of amino acids. Yields between 70 and 80% and final loadings higher than 0.7 mmol/g) were reached for double couplings of dry amino acids[16] to resin **9** with EDC/DMAP under an inert atmosphere. Even better results were obtained from Fmoc-amino acid fluorides[17] delivering yields of up to 90%. Free thiols were capped after the second coupling with Ac_2O and DIPEA. Deprotection of the Fmoc group and coupling of the second amino acid gave quantitative yields. For a detailed validation of the linker concept and the sequence-dependent comparison of reactions, a number of N-acetylated pentapeptides of generic structure Ac-SY-$RX_{bb}X_{aa}$ **11a**−**d** were synthesized and cleaved off the resin using various conditions. Application of sodium ethanethiolate with the cryptand 15-crown-5 in THF followed by treatment with TFA led almost exclusively to the hydrolyzed peptide acid. Applying sodium thiophenolate, 15-crown-5 with an excess of ethanethiol[18] in THF liberated the thioesters from the resin cleanly, and subsequent treatment with TFA delivered thioesters **15a**−**c** without traces of hydrolyzed peptides, however, in only modest yields (14−26%). Nucleophilic cleavage with thiols succeeded best with 3-mercaptopropionic acid methyl ester in the presence of sodium thiophenolate as base and the cryptand 15-crown-5. The protected peptidyl thioesters were treated with TFA and precipitated in methyl *tert*-butyl ether (MTBE) to furnish peptide thioesters **13a**−**d** in high purity (>90%) and excellent yields (65−90%) (Figure 2). Potential formation of diketopiperazine (DKP) products during Fmoc-deprotection of the second amino acid was examined carefully by analyzing the filtrate of the deblocking step. For the C-terminal dipeptides GQ and PV, no DKP formation was detected. Traces of DKP were found only during the washing step after deblocking the second coupled amino acid in the preparation of the pentapeptide Ac-SYRGF **13a** which was still obtained in 69% yield. Coupling of a dipeptide to the singly acylated resin **10** using HATU/HOAt as condensation reagents improved the yield of thioester peptides **13a**,**b**.

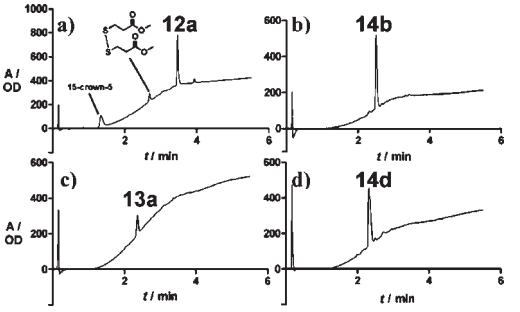


Figure 2. HPLC chromatograms of crude thioesters measured at 220 nm: (a) nucleophilic displacement of **12a** Ac-SYRGF-$S(CH_2)_2COOMe$, (b) total deprotection to **13a**, (c and d) crude MMP thioesters of **14b** and **14d** obtained in good yields and purities.

Acidic cleavage and deprotection of the resin-bound thioester peptides **11a**−**d** using TFA and precipitation with MTBE furnished clean products **14a**−**d** in good yields and purities >90% (Table 1). Cleavage of the MMP linker was not observed in any of these products, neither were truncated products or fail sequences detected. High-resolution NMR spectra of **14a** were fully assigned by the use of TOCSY, HMQC, and HMBC spectra (see the Supporting Information) and indicated partial racemization of the C-terminal residue and conservation of the thioester carbonyl after TFA cleavage, thus excluding an

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Table 1. Yields of Selected Crude Peptide Thioesters

entry	peptide thioester	yield[a] (%)
1	Ac-SYRGF-S$(CH_2)_2$COOMe **(13a)**	69/89[b]
2	Ac-SYRGW-S$(CH_2)_2$COOMe **(13b)**	78[b]
3	Ac-SYRGQ-S$(CH_2)_2$COOMe **(13c)**	86
4	Ac-SYRPV-S$(CH_2)_2$COOMe **(13d)**	67
5	Ac-SYRGF-MMP **(14a)**	54/83[b]
6	Ac-SYRGW-MMP **(14b)**	90[b]
7	Ac-SYRGQ-MMP **(14c)**	53
8	Ac-SYRPV-MMP **(14d)**	88
9	Ac-SYRGF-SEt **(15a)**	14[c]
10	Ac-SYRPV-SEt **(15b)**	20
11	Ac-SYRGQ-SEt **(15c)**	26

[a] Yields were calculated relative to the loading of the first amino acid. Purities of products were determined via HPLC at 220 nm with UV/vis spectroscopy to be >90%. [b] This peptide yield was attained with the coupling of a dipeptide to the initial amino acid. [c] After HPLC purification.

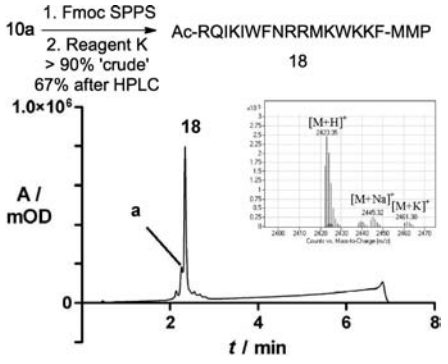

Figure 3. HPLC with accompanying mass spectrum of the crude 16mer penetratin derivative **18**. A small portion of the peptide, **a**, was oxidized at the methionine residue to give the sulfoxide (+16 *m*/*z*).

S → O transesterification of the peptide C-terminus. The deprotected peptide thioester **13a** was reacted with the N-terminal cysteine peptide CGRFLAVR-NH_2 **16** in 0.1 M phosphate buffer (pH 7.4) and delivered the ligation product Ac-SYRGFCGRFLAVR-NH_2 **17** smoothly. Alternatively, the MMP thioester **14a** was activated in situ with sodium thiophenolate and 15-crown-5[19] also furnishing product **17** upon addition of cysteine peptide **16** (see the Supporting Information). Persistence of the MMP linker during synthesis of a longer peptide was finally tested for a derivative of the 16mer penetratin-1, a cell-penetrating peptide from the third helix of the homeodomain of the antennapedia protein.[20] The product Ac-RQIKIWF-NRRMKWKKF-MMP **18** (Figure 3) was obtained in 67% yield after HPLC purification.

In summary, the MMP-linker can be obtained quickly from well-accessible starting materials and can be applied for the preparation of protected and unprotected peptide thioesters displaying a broad range of sequences. The products can be directly used in native chemical ligations and possibly in other reactions of peptidyl thioesters. Currently, the use of the linker for further C-terminal modifications of peptides is under investigation.

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Supporting Information Available. Experimental procedures, stability tests, NMR analyses for key compounds, and LC−MS data for important reactions.This material is available free of charge via the Internet at http://pubs.acs.org.

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