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Fmoc-Based Synthesis of Peptide Thioacids for Azide Ligations *via* 2-Cyanoethyl Thioesters

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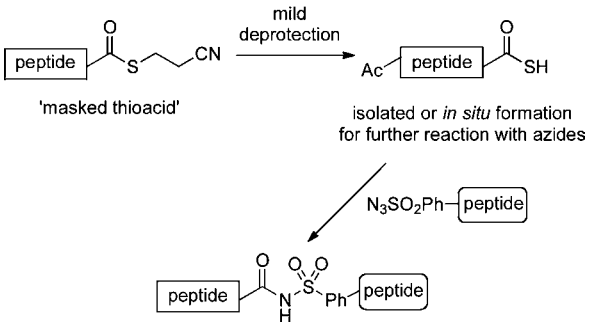
ABSTRACT



Rapid and efficient preparation of peptide thioacids from 2-cyanoethyl peptide thioesters has been accomplished. *S*-2-Cyanoethyl peptide thioesters were obtained cleanly without the need for purification from resin-bound *tert*-butyl peptide thioesters using 3-mercaptopropionitrile as a nucleophile. Elimination of the 2-cyanoethyl group proceeded rapidly ($t_{1/2}$ < 8 min) under mild conditions and furnished peptide thioacids up to the size of a 16-mer. Peptide thioacids could be isolated or formed *in situ* and reacted smoothly with electron-deficient azides yielding an amide as the ligation product.

Peptide thioacids have recently received considerable attention as reactive intermediates and building blocks. For example, they have been applied successfully in the amidation ligation reaction between thioacids and azides.[1] Due to the speed and selectivity of the reaction,[2] the ligation reaction of thioacids and electron-deficient azides has been exploited for protein modification.[3] Furthermore, the thiocarboxylic acid group is orthogonal to many other chemical functionalities and this has been exploited for ligations in more complicated peptide systems.[4] The synthesis of peptide thioacids has previously been described using solid phase Boc-methodology,[5] requiring HF for cleavage from the solid support. Neutral or slightly basic conditions provide a much milder approach to be applied in order to release thioacids from their precursors. Crich et al. introduced a base-labile, β-eliminable 9-fluorenylmethyl (Fm) linker, which enabled the synthesis of peptide thioacids by Boc chemistry without the need for HF cleavage.[6] In contrast, Liu et al. have prepared peptide thioacids by hydrothiolysis of peptide thioesters,[7] which

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(1) (a) Shangguan, N.; Katukojvala, S.; Greenberg, R.; Williams, L. J. *J. Am. Chem. Soc.* **2003**, *125*, 7754–7755. (b) Rosen, T.; Lico, I. M.; Chu, D. T. W. *J. Org. Chem.* **1988**, *53*, 1580–1582. (c) Hosein Hakimelahi, G.; Just, G. *Tetrahedron Lett.* **1980**, *21*, 2119–2122.

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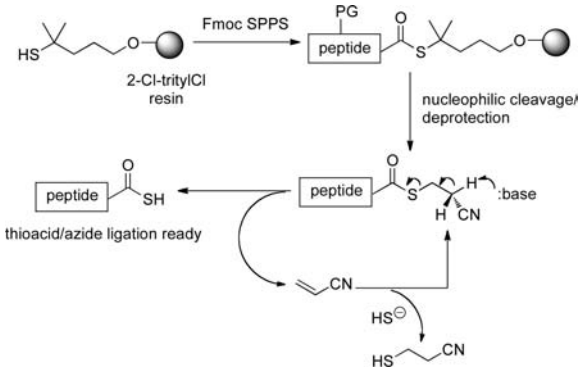
were prepared using an enzymatic approach.[8] They found that efficient release of peptide thioacids was possible at an elevated temperature (42 °C, 2–4 h), whereas at rt reactions remained incomplete after 7 h.

Recently, we have reported the application of a *tert*-butyl thiol linker, 4-mercapto 4-methylpentanol (MMP), for the Fmoc based synthesis of peptide thioesters.[9] The linker was used to construct peptide thioesters which were simultaneously stable against standard Fmoc cleavage conditions (piperidine in DMF (20% v/v)) while being cleavable under mild conditions using thiolates.

Scheme 1. General Principle for Peptide Thioacid Synthesis

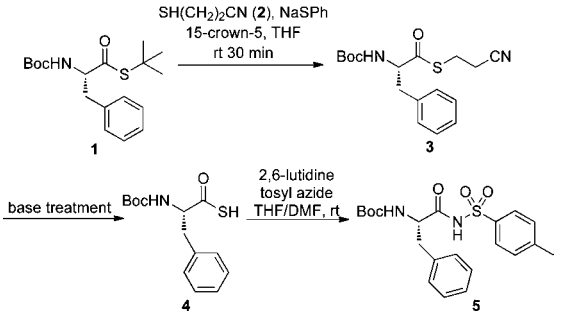


We envisioned that application of this linker could be extended to the synthesis of peptide thioacids by displacing the peptide as a masked thioacid using an appropriate thiolate for nucleophilic cleavage. Although base- and acid-labile protecting groups for thioacids have been previously described,[10] we reasoned that a thiolate specifically activated for the release of the thiocarboxylate functionality by β-elimination should be especially favorable (Scheme 1). Therefore, 2-cyanoethyl was selected for thioacid protection and investigated for the release of the desired thioacid product.

The 2-cyanoethyl protecting group has been previously used for the protection of carboxylic acids[11] but, to our knowledge, has not been used for the protection of thioacids. To test this idea, N-Boc-Phe-*S*-*tert*-butyl thioester **1** was prepared and used as a model compound (Scheme 2). 3-Mercaptopropionitrile **2** was generated by reduction of its commercially available disulfide and was used to treat thioester **1** in the presence of sodium thiophenolate under conditions previously described.[8] Thioester **3** was furnished quantitatively via thiolytic cleavage of **1**.

Initially, deprotection of 2-cyanoethyl thioester in **3** was attempted with various amine bases. When treated with

Scheme 2. Model Reaction with *S*-*tert*-Butyl Thioester **1**



piperidine, the thioester was rapidly converted to the piperidine amide and not the thioacid. In the presence of DIPEA or 2,6-lutidine, the 2-cyanoethyl group was not removed after 24 h and extended reaction times led to hydrolysis of the thioester to the carboxylic acid.

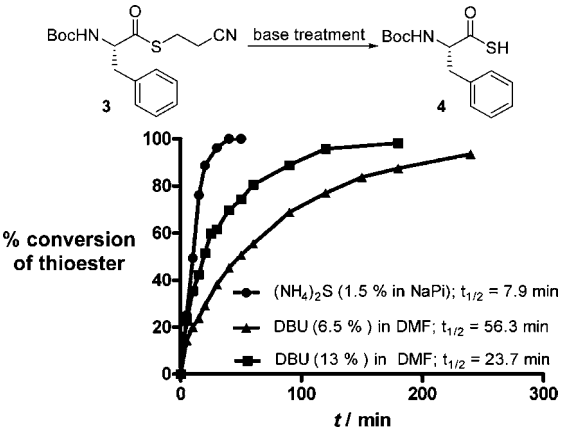


Figure 1. Deprotection of the cyano ethyl protecting group performed at room temperature was most efficient with $(NH_4)_2S$ (●) giving 96% conversion after 30 min. Conversion to the thioacid was also effected with differing concentrations of DBU, 6.5% (▲) and 13% (■) in DMF (v/v).

With the stronger, non-nucleophilic base DBU (6.5%, v/v) deprotection of the cyanoethyl group deprotection occurred in DMF with a reaction half-time of ~1 h (Figure 1). Increasing the DBU concentration to 13% in DMF (v/v) reduced the reaction half-time to 24 min. Conversion of the model thioester **3** to the thioacid **4** occurred immediately with potassium *tert*-butoxide with the reaction finishing in <1 h (see Supporting Information (SI) Table S1, entry 4). As even shorter reaction times were desirable, we investigated alternative conditions to accelerate the rate of the reaction. Considering that the elimination of the 2-cyanoethyl group might be slowed down by the reversibility of the reaction (Scheme 1), it should be possible to accelerate the reaction by the addition of a sulfhydryl nucleophile which can scavenge the released acrylonitrile. Thus, deprotection of 2-cyanoethyl protecting group was

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investigated employing ammonium sulfide as the base (Figure 1). Under these conditions, the half-life of the deprotection was <8 min although the base concentration was significantly reduced (1.5%) and the reaction proceeded in water at pH 9. After 30 min 96% of the thioester was already deprotected to the thioacid **4**, which was stable under HPLC conditions (see SI). Treating the protected amino thioacid **4** with tosyl azide and 2,6-lutidine[1a] afforded the sulfonamide product **5**, which was verified by LC-MS analysis. Thus, S-2-cyanoethyl was proven to be suitable as a protecting group for thioacids.

Next, the efficacy of 2-cyanoethyl as a protecting group for the synthesis and isolation of peptide thioacids was investigated. The peptide sequence Ac-SYRGF was prepared applying the MMP linker method furnishing the resin-bound peptide thioester (Figure 2), which was displaced from the resin with 3-mercaptopropionitrile as 2-cyano ethyl thioester **6a**. Cleavage was accomplished again in the presence of sodium thiophenolate and 15-crown-5 and yielded the pure thioester, which could be employed for thioacid release without further purification. Direct cleavage attempts of the peptide from the resin with sulfhydryl equivalents such as Na_2S or NaHS in a THF/H_2O mixture in hopes of obtaining peptide thioacids led only to hydrolysis of the thioester.

The application of either potassium *tert*-butoxide or DBU as a base to the pentapeptide **6a** gave impure products, and no peptide thioacids could be isolated. Since it is well described that both potassium *tert*-butoxide and DBU are effective bases for β-eliminations,[12] these failures were unexpected. Possibly the deprotection reactions were too slow under these conditions and the hydrolysis of thioesters with residual water becomes dominant.

Therefore, again ammonium sulfide was investigated as the base for the release of peptide thioacid **7a** from the 2-CE thioester **6a**. The sulfhydryl equivalent was applied in a phosphate buffer (pH 9) and furnished the clean thioacid with barely any hydrolysis of the thioester (Figure 2). Cleavage of the cyanoethyl group proceeded very rapidly with a reaction half-time of <8 min, finishing in <30 min at rt without the need for elevated temperatures (see SI). For comparison, under the very same reaction conditions Liu et al. had found that peptide thioesters of 3-mercaptopropanoic acid were only partially cleaved after 7 h and required elevated temperatures for completion (42 °C, 2 h).[8] The observed drastic difference in the reactivities of 2-cyanoethyl and 2-carboxyethyl thioesters should be addressed. ^{13}C NMR spectroscopy of the two thioesters indicated virtually identical deshielding of the thioester carbonyl carbon (200.96 ppm for the 2-carboxyethyl thioester (**10**) vs 200.7 ppm for the 2-cyanoethyl thioester **3**; see the SI) and suggested comparable electrophilicity. Thus, the reactivity difference of the two substrates might be explained by different reaction mechanisms. While the 2-carboxyethyl group can be cleaved exclusively via an S_N mechanism, conversion of the 2-cyanoethyl group can occur via β-elimination of the protecting group. The acrylonitrile released by the E mechanism can be expected to react rapidly with a hydrosulfide anion to refurnish 1 equiv of 3-mercaptopropionitrile.

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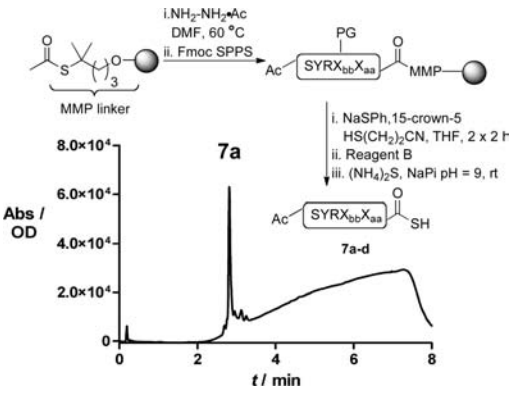

Figure 2. Synthesis of peptide thioacids using the MMP linker.

Next, the concept was extended toward the synthesis of a selection of peptide thioesters (**6a**–**6d**) of the generic structure Ac-SYR$X_{aa}X_{bb}$ containing different amino acids at the C-terminus (Table 1). All these peptide thioesters could be deprotected and isolated by preparative HPLC as peptide thioacids (**7a**–**7d**) in good yields. Only under prolonged treatment (>2 h) with diluted acid did slow decomposition to peptide acids set in. NMR analysis indicated racemization of the carboxy-terminal amino acid, which is due to the nature of the thioester-linker we previously reported.[9] In order to test the efficiency of the synthesis protocol for an elongated peptide thioacid, the 16-mer penetratin-1, a cell-penetrating peptide from the third helix of the homeodomain of the antennapedia protein,[13] was prepared as MMP-linked thioester **8**. Using the same methodology the penetratin 2-cyanoethyl thioester **9a** was afforded (Figure 3). After cleavage of the protecting group with ammonium sulfide, the 16-mer thioacid Ac-RQIKIWFNRRMKWKKF-COSH **9b** was obtained in a yield of 51% based on initial coupling of the amino acid and was purified by preparative RP HPLC.

Table 1. Yields of Peptide CE-Thioesters and Thioacids with Different Amino Acids at the C-Terminal Position Including the 16-mer Thioester and Thioacid Derivative of Penetratin

entry	peptide	CE-thioester yield (%)[a]	thioacid yield (%)[b]
1	Ac-SYRGF (**6a**,**7a**)	95	79
2	Ac-SYRPV (**6b**,**7b**)	83	62
3	Ac-SYRGQ (**6c**,**7c**)	75	73
4	Ac-SYRGS (**6d**,**7d**)	61	83
5	Ac-RQIKIWFNRR MKWKKF (**9a**,**9b**)	97	51

[a] Yields 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] Yields were determined after HPLC purification.

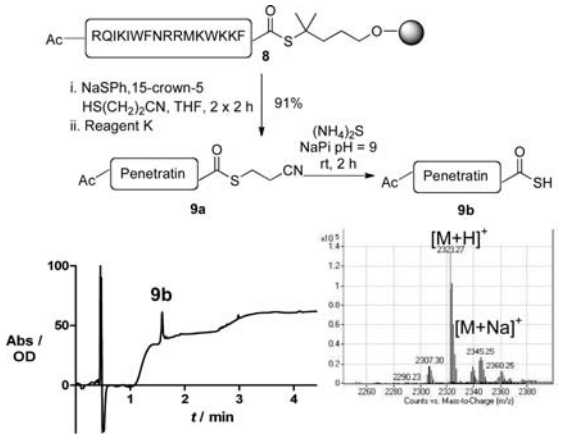

Figure 3. Synthesis of a 16-mer penetratin-1 peptide thioacid from the precursor thioester peptide.

Finally, the viability of the pentapeptide thioacid **7a** synthesized via 2-cyanoethyl thioester was investigated in thioacid–azide ligation reactions (Table 2). For this purpose, two alternative reaction paths were pursued (Scheme 3). First, the isolated peptide thioacid **7a** was ligated with a model sulfonyl azide peptide N_3-$SO_2C_6H_4$-LYRAG-NH_2 **11** in an aqueous buffer system using 2,6-lutidine as a basic catalyst as previously described.[1a] The reaction was complete within 15 min and yielded the corresponding sulfonamide peptide Ac-SYRGF-[$NHSO_2C_6H_4COONH$]-LYRAG-NH_2 **12** in 84% yield after HPLC purification.

Alternatively, the direct conversion of the preceding 2-CE thioester **6a** into the ligation product **13** in a two-step one-pot reaction was explored. For the purpose **6a** was treated with DBU to effect elimination to the thioacid **7a** *in situ*. Addition of tosyl azide to the reaction mixture followed by 2,6-lutidine as the catalyst directly furnished Ac-SYRGF-$NHSO_2C_6H_4CH_3$ **13** as the ligation product. This concept of in situ generation of thioacids for one-pot ligation reactions has been recently applied using the Fm protecting group for the synthesis of sulfonamides with peptidic as well as nonpeptidic structures.[14]

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Scheme 3

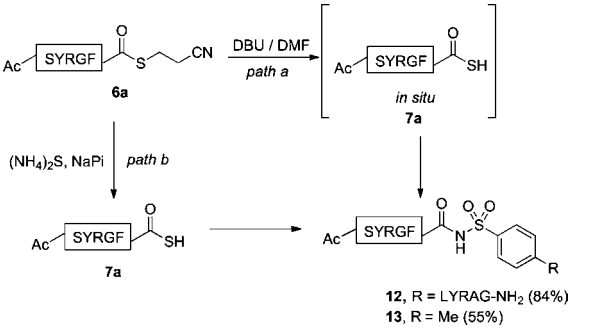

Table 2. Ligation of Ac-SYRGF-SH with Selected Azides

entry	substrate	product	yield (%)[a]
1	N_3-$SO_2C_6H_4$-LYRAG-NH_2	Ac-SYRGF-$NHSO_2C_6H_4$-LYRAG-NH_2 (**12**)	84
2	N_3-$SO_2C_6H_4$-*p*CH_3	Ac-SYRGF-$NHSO_2C_6H_4$-*p*CH_3 (**13**)	55

[a] The product yield was determined by dry weight after HPLC purification of the ligated peptide.

In summary, we have shown that the 2-cyanoethyl group is ideally suited for the protection of thiocarboxylic acids and was applied in the preparation of thioacids and peptide thioacids from their parent thioesters. Furthermore, 2-mercaptopropionitrile serves as an excellent source for the introduction of the thiol functionality, in this case for a masked peptide thioacid by nucleophilic displacement from the MMP linker.

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Supporting Information Available. Experimental procedures, 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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The authors declare no competing financial interest.