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Solid-phase synthesis of peptide thioacids through hydrothiolysis of resin-bound peptide thioesters

Xiaohong Zhang, Xiao-Wei Lu, Chuan-Fa Liu *

Division of Chemical Biology and Biotechnology, School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore

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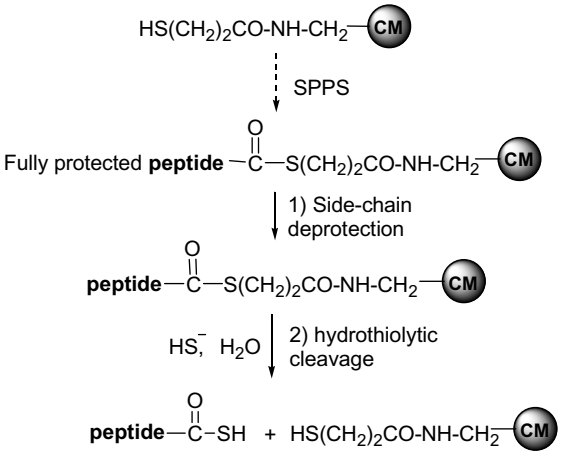
ABSTRACT

We report that solid-phase hydrothiolysis is an efficient method to convert resin-bound peptide thioesters to thioacids in aqueous buffer by using a total PEG-based resin. Also demonstrated is the use of the so-prepared peptide thioacids in chemoselective amide bond formation reactions.

The advent of solid-phase synthesis marked a paradigm shift in the practice of synthetic chemistry.[1] Apt for the preparation of oligomeric compounds such as peptides and oligonucleotides,[2,3] the solid-phase synthesis methodology is also widely used for the synthesis of small molecules,[4] and has been the main platform technology for combinatorial chemistry.[5–7] The versatility of this methodology makes almost any chemical reaction adaptable to solid-phase settings, and the critical task often lies with whether or not one can find a suitable solid support and/or a linker to meet a particular synthetic need. Previously, we reported a simple hydrothiolysis reaction for the synthesis of peptide thioacids[8]—an important class of compounds with increasingly recognized synthetic value.[9–11] When conducted in aqueous buffer at alkaline pH, hydrothiolysis of a peptide thioester by hydrosulfide ions gives rise to the corresponding peptide thioacid in near quantitative yield.[8] We reasoned that, provided with a solid support that has good swelling properties in aqueous media, it should also be possible to conduct solid-phase hydrothiolysis of resin-bound peptide thioesters.

The traditional approach to preparing peptide thioacids is based on the benzhydryl linker chemistry originally developed by Blake et al.[12–14] This approach requires the use of Boc SPPS, and the yield is usually low, because the supernucleophilicity and low pK_a value of the thioacid functionality make it highly susceptible to nucleophilic reactions with cationic electrophiles released from the various side-chain protecting groups during the final HF deprotection and cleavage stage. Such a problem would not exist with a solid-phase hydrothiolysis method, because, through the use of a non acid-labile linker, it is possible to conduct the final deprotection-cleavage in two steps: (1) the acid-mediated deprotection to remove all the side-chain protecting groups and, (2) hydrothiolytic cleavage of the resin-bound peptide thioester (Scheme 1) to give the desired peptide thioacid.



Scheme 1. Synthesis of peptide thioacids by solid-phase hydrothiolysis. CM = ChemMatrix resin.

Since the hydrothiolysis reaction needs to be conducted in aqueous media, the solid support must have good swelling ability in water. Our first attempt to use a poly(ethylene glycol)-grafted polystyrene (PEG-PS) resin, the TentaGel resin, gave very poor results, with the thiolytic cleavage yield being about 30% at best.[8] This unsatisfactory result is apparently due to insufficient water-swelling of the resin in which the polystyrene core matrix is highly hydrophobic. We therefore turned our attention to a recently developed, totally PEG-based ChemMatrix resin (CM resin).[15] CM resin has a high degree of cross-linking, and exhibits good loading capacity and mechanical stability.[16,17] Most importantly, it is well-solvated in a broad range of nonpolar and polar solvents, including water.[16,17] CM resin has a swelling volume in water of ~11 mL/g.[17] This makes it an excellent candidate resin for solid phase-supported reactions in aqueous media. Herein, we report on the use of CM resin for the synthesis of peptide thioacids through

* Corresponding author. Tel.: +65 6316 2867; fax: +65 6791 3856.
E-mail address: cfliu@ntu.edu.sg (C.-F. Liu).

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hydrothiolysis of resin-bound peptide thioesters. We also demonstrate the synthetic utility of the prepared peptide thioacids in specific amidation with azide compounds and in thioacid capture ligation.

First, thiol-functionalized CM resin was prepared by coupling Trt-SCH_2CH_2COOH onto commercial aminomethyl CM resin followed by detritylation with TFA. Peptides were then assembled on the derived resin using typical SPPS protocols. Although Boc chemistry is the preferred method for synthesizing peptide thioesters, Fmoc chemistry can also be used if one employs Aimoto's Fmoc deprotection method to minimize aminolytic cleavage of the thioester linkage.[18] Thus, peptide **1** (Table 1) was synthesized by Fmoc chemistry using the Fmoc-deprotection mixture of 1-methylpyrrolidine (25%), hexamethyleneimine (2%), HOBt (2%) in NMP/DMSO (1:1). Peptides **2**–**6** were synthesized by using Boc chemistry. For peptides **4**–**6**, the side-chain protecting group for Arg was Mts. Final deprotection was carried out using standard TFA or TFMSA/TFA protocols for Fmoc- or Boc-chemistry synthesized peptides, respectively, to remove all the side-chain protecting groups without cleaving the peptide from the resin. The side-chain deprotected peptide-resin was washed with TFA and acetonitrile, and used immediately for the cleavage with hydrosulfide ions.

Reaction conditions for the solid-phase hydrothiolysis reaction on the resin-bound peptide thioesters were similar to those used in solution reactions reported earlier.[8] Either ammonium sulfide or sodium sulfide was used as the source of hydrosulfide ions. Good hydrothiolytic cleavage yields were achieved for all the CM resin-bound peptide thioesters (Table 1). As seen from Table 1, these peptides contain a diverse set of C-terminal residues, from the smallest Gly to the hindered Ile. For instance, thioacid peptide **2**, which has an Ile residue at the C-terminus, was obtained in excellent yield after a prolonged cleavage reaction time. For most other peptides, the reaction reached its maximum yield after about 3 h, as seen in a simple kinetic study on peptide **4** (Fig. 1).

The cleaved crude products were, in general, of good purity as shown by analytical HPLC analysis. Figure 2 shows representative HPLC profiles of two crude thioacid peptides (Fig. 2A for peptide **4** and Fig. 2B for peptide **3**). It should be noted that peptide **3** was synthesized on recycled mercaptopropionylaminomethyl CM resin as this thiol-derived CM resin is regenerated after thiolytic cleavage and therefore can be reused. Before reuse, the recycled resin was treated with 5% hydrazine in DMF (2.5% mercaptoethanol) for 30 min to ensure complete removal of any uncleaved peptide. The purity of the crude peptide depends solely on the efficiency of SPPS. No S-alkylation side products were observed from HPLC and MS analysis, presumably because all the reactive species that would be derived from the side-chain protecting groups had already been removed and washed away in the previous step. It is worth noting that the solid-phase hydrothiolysis reaction can be conducted under denatured conditions, which are highly desirable for hydrophobic peptides that are prone to aggregation. In this study, thioacid peptides **2**, **3**, and **4** were prepared in the presence of 6 M guanidine–HCl.

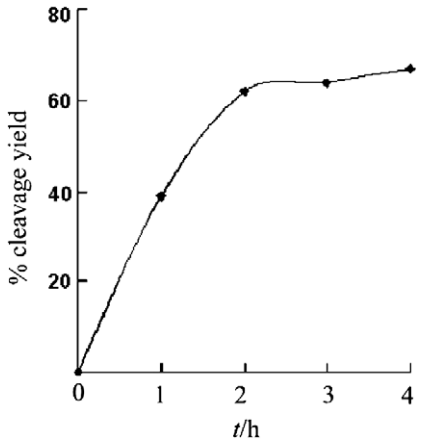

Figure 1. Cleavage yield of thioacid peptide **4** as a function of time. See Table 1 footnote for cleavage conditions.

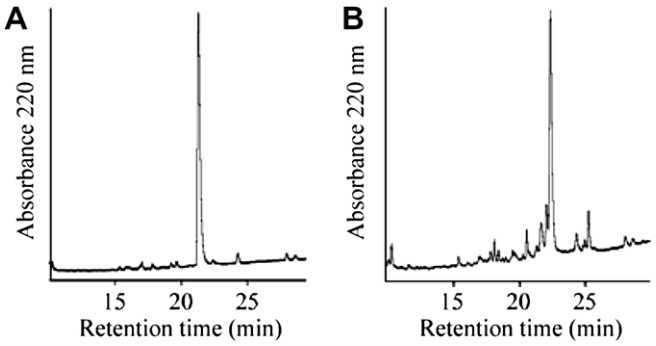

Figure 2. HPLC profiles of crude thioacid peptides **4** and **3** cleaved from CM resin. (A) Thioacid peptide **4** after 5 h cleavage. (B) Thioacid peptide **3** after 3 h cleavage. Peptide **3** was synthesized on recycled CM resin. HPLC linear gradient: 0–60% of buffer B (90% CH_3CN in H_2O containing 0.05% TFA) in buffer A (H_2O containing 0.05% TFA) in 30 min on an analytical C_{18} column (Jupiter, 250 × 4.6 mm).

Table 1
Synthesis of peptide thioacids through hydrothiolysis of CM resin-bound peptide thioesters[19]

Peptide	Sequence	Cleavage yield[a] (%)	MW[b] calcd	m/z[b] found
1	ARTKQTARKSTG[c]	68 (2 h)	1319.7	1320.8
2	GIGDPVTCLKSGAI[d]	85 (8 h)	1345.7	1347.4
3	VGLFEDTNL[d,f]	70 (3 h)	1022.5	1023.4
4	RLLLPGELA[d]	67 (5 h)	996.6	997.8
5	APKRYKANY[e]	80 (3 h)	1125.6	1127
6	DSARAGS[c]	80 (3 h)	678.3	679.6

All resin-bound peptide thioesters are of the general structure: peptide-CO-SCH_2CH_2CO-$NHCH_2$-CM resin.

[a] Cleavage yield was calculated using the quantitative ninhydrin test (absorbance at 570 nm) on peptide-resin before and after cleavage; values in brackets are the cleavage time.

[b] Calculated isotopic molecular weight and found ($[M+H]^+$) m/z value of the peptide thioacid products.

[c] Cleavage conditions: 0.2 M $(NH_4)_2S$, 0.3 M HEPES buffer, pH 8.6, rt.

[d] Cleavage conditions: 0.2 M $(NH_4)_2S$, 0.3 M HEPES buffer, 6 M guanidine–HCl, pH 8.6, rt.

[e] Cleavage conditions: 0.1 M Na_2S, 0.3 M HEPES buffer, pH 7.6.

[f] Synthesized on recycled $HSCH_2CH_2CO$-$NHCH_2$-CM resin.

It is recommended that, once the peptide resins are fully deprotected, they should be cleaved with the thiol reagent as soon as possible, as we have noticed a decrease in the cleavage yield if the peptide resin is stored over time. This is likely due to interchain interactions and aggregation of the resin-bound peptide. In such cases, the deprotected peptide resin must be soaked in TFA for 15–20 min to re-swell the resin thoroughly and break up any interwinding peptide chains and then washed with acetonitrile and DMSO before thiolytic cleavage.

The thioacid functionality is a very soft and powerful nucleophile, which makes it useful in organic synthesis. For instance, the reaction between a thioacid and an azide has received renewed attention recently, because it can be used for facile preparation of complex amides. A thorough mechanistic study has suggested that the reaction proceeds through a prior capture step leading to the formation of a thiatriazoline intermediate, which decomposes to give the amide product.[20] Notably, the reaction was reported to

be especially efficient with electron-deficient azides such as sulfonazides.[10] Although so far thioacid/azide coupling has only been demonstrated with small thioacids such as thioacetic acid and N-protected amino thioacid compounds,[10,11] its unique reaction features imply that it should have the required chemical orthogonality to work in a chemoselective manner with large and complex thioacid compounds containing other functional groups, such as the peptide thioacids listed in Table 1. Therefore, to demonstrate the synthetic utility of the prepared peptide thioacids, peptide **4**, H-ArgLeuLeuLeuProGlyGluLeuAla-COSH, was subjected to amidation with tosyl azide in wet methanol in the presence of 2,6-lutidine.[21] HPLC analysis showed a clean and near quantitative conversion (>95%) of the thioacid peptide to the desired *N*-peptidyl sulfonamide product after 2 h reaction at 23 °C (Fig. 3). Similarly, reaction of peptide **5**, H-AlaProLysArgTyrLysAlaAsnTyr-COSH, with tosyl azide in wet methanol was complete in 2 h to give the expected *N*-acyl-sulfonamide (*m/z* $[M+H]^+$ found: 1263.5, MW calcd: 1262.6) in excellent yield (data not shown). It should be noted that the reaction was conducted in a very dilute solution (ca. 0.2 mM) of the thioacid peptide. Clearly, the high reaction efficiency in such a dilute solution can only be explained by a mechanism of the prior capture type proposed earlier by Williams and co-workers[20] The reaction appeared highly chemoselective despite the presence of a number of side-chain functional groups and a free *N*-terminal amine in the two peptides. These results further validate the utility of the thioacid/azide amidation reaction as a new method to introduce a C-terminal peptide modification with, for example, a biophysical probe.

Peptide thioacids are also the key building blocks for 'mini' thiol capture ligation (or thioacid capture ligation).[9] The key element of this method consists of specific capture of a C-ter thioacid of the first peptide by an activated disulfide from an Npys-modified N-ter Cys side chain of the second peptide to form an acyl disulfide intermediate, which undergoes rapid intramolecular acylation to generate an amide bond.[9] The final product with a native Cys residue at the ligation site is obtained after a simple thiolytic reduction. Figure 4 shows the thioacid capture ligation reaction between thioacid peptide **4** and an Npys-modified cysteinyl peptide (in ~2-fold molar excess), which gave a very good yield of the 35-residue ligation product after 10 min of reaction.[22] Of all the chemoselective ligation methods developed thus far, the thioacid-capture ligation technique is probably the most efficient in terms of reaction rate. The main drawback of this method is associated with the difficulty in obtaining the thioacid building blocks. The development of the solid-phase hydrothiolysis technique for peptide thioacid synthesis will therefore make the thioacid capture ligation a more useful method for protein synthesis.

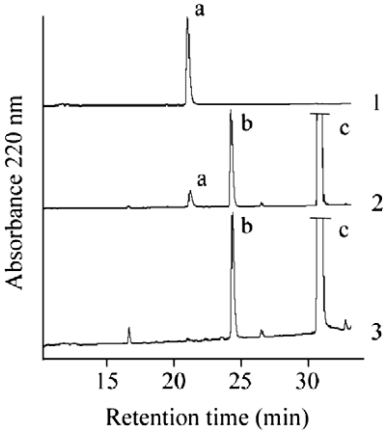

Figure 3. HPLC monitoring of the reaction of peptide thioacid **4** (H-**RLLLPGELA**-COSH) with tosyl azide. Trace 1—the thioacid peptide (peak a; *m/z* $[M+H]^+$ found: 997.7; MW calcd: 996.6). Trace 2—reaction mixture at 1 h. Peak b: reaction product H-**RLLLPGELA**-CONH-SO_2-Ph-CH_3 (*m/z* $[M+H]^+$ found: 1135.8, MW calcd:1133.6). Peak c: *p*-MePh-SO_2-N_3 in excess. Trace 3—reaction mixture at 2 h. HPLC gradient: 0–80% buffer B (90% CH_3CN in H_2O containing 0.05% TFA) in buffer A (H_2O containing 0.05% TFA) for 40 min.

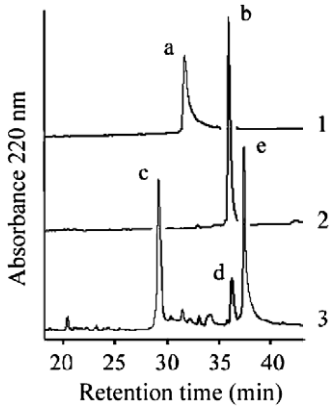

Figure 4. HPLC monitoring of ligation between H-**RLLLPGELA**-COSH and Npys-CA-26. Trace 1, peak a is Npys-CA-26 or H-**C(Npys)AIHAK(ac)RVTIMPKDIQLARRIRGERA**-COOH (*m/z* $[M+3H]^{3+}$ found: 1067.8, MW calcd: 3197.7). Trace 2, peak b is H-**RLLLPGELA**-COSH. Trace 3, TCEP-reduced reaction mixture after 20 min ligation. Peak c is reduced CA-26 (*m/z* $[M+2H]^{2+}$ found: 1523.6, MW calcd: 3043.7). Peak d is the hydrolysis product of the peptide thioacid (*m/z* $[M+H]^+$ found: 981.8, MW calcd: 980.6). Peak e is the ligation product, H-**RLLLPGELACAIHAK(ac)RVTIMPK-DIQL-ARRIRGERA**-COOH (*m/z* $[M+3H]^{3+}$ found: 1337.2, MW calcd: 4006.3). HPLC linear gradient: 0–40% buffer B (90% CH_3CN in H_2O containing 0.05% TFA) in buffer A (H_2O containing 0.05% TFA) for 40 min.

We have demonstrated herein that solid phase-supported hydrothiolysis is an efficient method to convert a CM resin-bound peptide thioester to its corresponding thioacid in aqueous media. The simplicity of this new method should make peptide thioacids easily available for use in organic synthesis by using, for example, thioacid/azide coupling and thioacid capture ligation. It should also further stimulate the development of new synthetic methods based on the use of peptide thioacids.

Acknowledgments

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19. For a typical reaction, 25–50 mg of peptide-resin was suspended in 1 mL of hydrothiolysis buffer in an Eppendorf vial with gentle shaking. The reaction was stopped by acidification with 20% TFA/H_2O with ice cooling. The mixture was subjected to HPLC purification to give the purified thioacid peptide. All operations should be performed in a well-ventilated fume hood.
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21. HPLC purified peptide thioacid (*ca.* 0.25 mM) was mixed with tosyl azide (*ca.* 2 mM) and 2,6-lutidine (*ca.* 2 mM) in wet methanol, and the reaction mixture was monitored by analytical HPLC.
22. Thioacid peptide **4** (*ca.* 0.5 mM) was mixed with Npys-modified CA-26 (*ca.* 1 mM) in CH_3CN/H_2O (0.05% TFA, pH 2). A yellow color developed immediately. The pH of the reaction solution was then adjusted to about 6 with 0.2 M phosphate buffer. TCEP was added after 10 min for thiolytic reduction, and the reaction mixture was subjected to HPLC purification.