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Facile, Fmoc-Compatible Solid-Phase Synthesis of Peptide C-Terminal Thioesters

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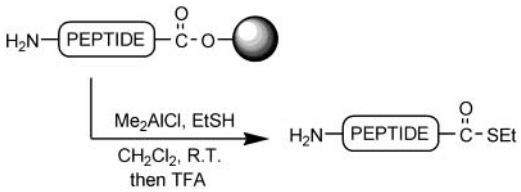
ABSTRACT



A short route to peptide C-terminal thioesters was developed that does not require the use of special linkers or resins and is compatible with standard Fmoc chemistry. Following conventional solid-phase peptide synthesis, an excess of Me_2AlCl and EtSH in dichloromethane cleaves peptides from Wang or Pam resins to give the corresponding thioesters directly in good yield and purity.

C-Terminal peptide thioesters are key intermediates in enzymatic and nonenzymatic peptide bond forming reactions. Their utility in peptide fragment condensations, in particular, has made small and medium-sized proteins readily accessible to total chemical synthesis.[1] These activated species are also valuable for the construction of proteins containing nonnatural backbones[2] and for the synthesis of cyclic peptides[3] and peptide dendrimers.[4]

Peptide C-terminal thioesters can be prepared by standard solid-phase peptide synthesis (SPPS) using *tert*-butoxycarbonyl (Boc) methodology[5] or, for larger peptide fragments and protein domains, molecular biologically with intein technology.[6] Synthetic methods compatible with widely used 9-fluorenylmethoxycarbonyl (Fmoc)-based chemistry[7] would complement these approaches, especially for peptides containing functionalities incompatible with Boc chemistry. However, resin-bound thioesters are unstable to repeated exposure to piperidine, which is used to remove Fmoc groups. The susceptibility of thiol esters to epimerization under the basic conditions of Fmoc-SPPS is an additional concern.

Several strategies for solving these problems have been reported. In one approach,[8] a thioester-compatible Fmoc-cleavage cocktail [i.e., 1-methylpyrrolidine (25%), hexa-

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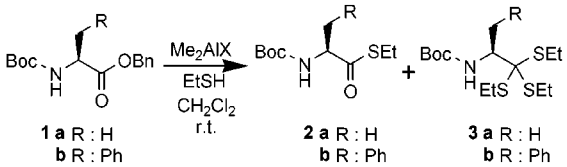
methyleneimine (2%), and 1-hydroxybenzotriazole (HOBt, 2%) in NMP−DMSO (1:1)] has been successfully applied to the synthesis of an unprotected 25-residue peptide thioester in 24% yield. Alternatively, the labile thioester can be introduced at the end of the synthesis, for example, using the backbone amide linker (BAL) strategy.[9] The C-terminal residue of a peptide, anchored to a solid support through its backbone nitrogen, is activated and coupled to an amino acid thioester immediately prior to final cleavage and deprotection. A 7-aa peptide was prepared in this way in excellent yield and acceptable levels of epimerization.[9] A potentially more general method relies on a recent modification of Kenner's sulfonamide "safety-catch" linker.[10] Normal Fmoc-SPPS is followed by activation of the sulfonamide linker by alkylation and cleavage of the peptide from the resin with a thiol nucleophile. This strategy has been used to prepare thioesters of several peptides,[11] including a 24-residue glycopeptide in 21% yield.[12]

As an alternative to these published methods, which may require extensive optimization or exploit expensive or commercially unavailable linkers, we report preliminary results on a short and simple route to unprotected peptide C-terminal thioesters using Fmoc-SPPS on the Wang[13] and Pam[14] resins. Corey has shown that alkylaluminum thiolate, prepared from trimethylaluminum and the corresponding mercaptan, reacts with simple esters in CH_2Cl_2 to produce thioesters in high yield.[15] We reasoned that a peptide assembled by Fmoc procedures on conventional hydroxymethyl resins would similarly yield peptide thioesters upon treatment with an alkylaluminum thiolate. To our knowledge, this has never been tried on peptide esters, in solution or on solid support.[16]

To investigate the efficiency of the alkylaluminum thiolate-mediated cleavage, several concerns need to be addressed. First, racemization of the C-terminal thioester residue is conceivable under cleavage conditions. Second, poor solvation of the peptide linked to the resin under the reaction conditions (e.g., in CH_2Cl_2) might limit access of the reagent to the cleavage site.[7] Finally, the alkylaluminum reagent might promote undesired reactions on the backbone and/or the side chains of the peptide.

To study the first issue, we applied Corey's conditions[15] to Boc-alanine benzyl ester (**1a**) as a model. Treatment of **1a** with 2 equiv of Me_3Al and 2 equiv of EtSH in CH_2Cl_2 for 5 h at room temperature gave the desired thioester in good yield but with a poor enantiomeric excess (ee) of 67% (Table 1, entry 1). Note that thio-orthoester **3a** is also formed as a byproduct in the reaction. The latter becomes the major product if longer reaction times and an excess of Me_3Al and EtSH are used (Table 1, entry 2).

Table 1. Solution-Phase Synthesis of Amino Thioesters



entry	R	X (equiv)	EtSH (equiv)	time (h)	% **2**	ee (%)[a]	% **3**[b]
1	H	Me (2)	2	5	78	67	8
2	H	Me (6)	18	6	traces	nd	77
3	H	Me (2)	6	3.5	83	77	5
4	H	Me (2)	30	3	84	86	traces
5	H	Cl (2)	6	1.75	86	99.7[c]	traces
6	Ph	Cl (2)	6	1.75	87	98	traces

[a] Determined by optical rotation unless otherwise specified. [b] ee of **3a**,**b** not determined. [c] Determined by chiral gas chromatography.

To circumvent the problem of racemization, less basic conditions were sought. To "buffer" the thiolate solution, an excess of thiol was added (Table 1, entries 3 and 4). When the EtSH/Me_3Al ratio was raised from 2:2 to 30:2, the ee increased to 86%. The results were further improved by replacing Me_3Al with Me_2AlCl. Under these new conditions (2 equiv of Me_2AlCl, 6 equiv of EtSH, 1.75 h., rt), **1a** and Boc-phenylalanine benzyl ester (**1b**) were easily converted to thioesters **2a** and **2b** in good yields (86−87%) and excellent optical purities (99.7% and 98% ee, respectively) (Table 1, entries 5 and 6).

The optimized conditions were subsequently applied to resin-bound peptides. However, only low yields of peptide thioester were obtained. Larger excesses of Me_2AlCl and EtSH were needed to effect the reaction. For example, peptide−resin **4a′** (Leu-Tyr(O*t*Bu)-Arg(Pbf)-Ala-Gly-O−Wang resin), prepared by standard Fmoc solid-phase protocols, was treated with 20 equiv of Me_2AlCl and 60 equiv of EtSH for 5 h. After evaporation of the solvent under vacuum, cleavage of side chain protecting groups [TFA-PhOH−H_2O (95:2.5:2.5), 2.5 h][17] and purification by reverse-phase HPLC, the desired peptide C-terminal thioester Leu-Tyr-Arg-Ala-Gly-SEt **5a** (34%) and the corresponding acid Leu-Tyr-Arg-Ala-Gly-OH **6a** (20%) were isolated (Table 2, entry 1). Use of the more acid-stable Pam resin instead of the Wang resin decreased the amount of free acid formed (3−5%) and thus led to significantly higher yields of peptide thioesters such as **5a** and **5b** (60−63%, Table 2, entries 2 and 3).

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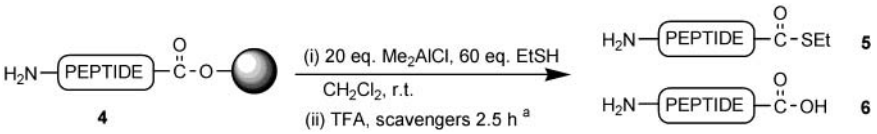
Table 2. Solid-Phase Synthesis of Peptide Thioesters



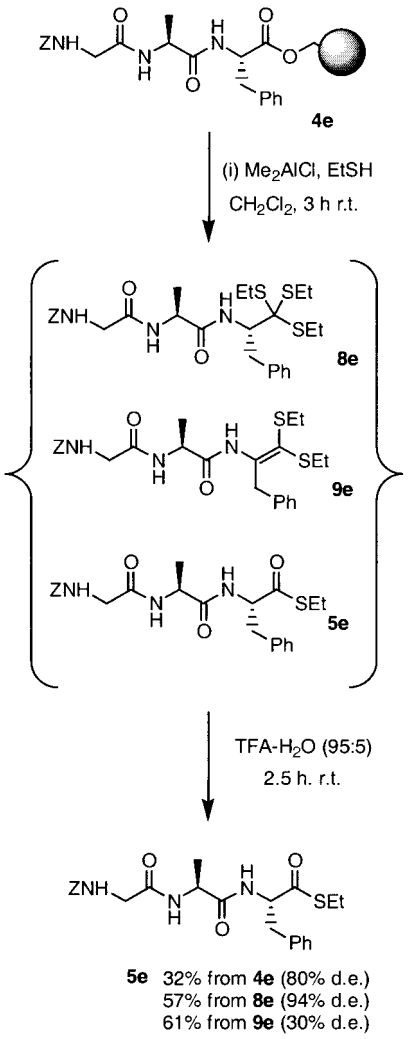
entry	substrate	peptide sequence	resin	time (h) [a]	% **5**[b]	% **6**[b]	% bis-thioester **7**[b,c]
1	**4a′**	LYRAG	Wang	5	34	20	
2	**4a**	LYRAG	Pam	5	60	3	
3	**4b**	HWYQQKSG	Pam	5	63	5	
4	**4c**	IFKDG	Pam	5	26	5	8
5	**4d**	YTKYNDDDTFTVKVG	Pam	3.5	24	nd	*d*

[a] Peptides were cleaved from the resin and the protecting groups subsequently removed. Deprotection conditions: 2.5 h treatment at rt with TFA−PhOH−H_2O (95:2.5:2.5) for **4a**; TFA−thioanisole−EtSH−H_2O (92.5:2.5:2.5:2.5) for **4b** TFA−H_2O (95:5) for **4c** and TFA−EtSH−H_2O (95:2.5:2.5) for **4d**. [b] Isolated yield calculated from the loading capacity of the commercial resin. [c] Ile-Phe-Lys-Asp(SEt)-Gly-SEt. [d] Peptide thioester **5d** was separated from a mixture of other noncharacterized derivatives, probably poly-thioesters, by reverse-phase HPLC.

Aspartic acid-containing **4c** (Ile-Phe-Lys(Boc)-Asp(O*t*Bu)-Gly-O−Pam resin) was used to test the utility of the method with peptides bearing additional ester functionality. The cleavage conditions not only gave the desired thioester **5c** (26%) but also some acid **6c** (5%) and bis-thioester (**7c**, Ile-Phe-Lys-Asp(SEt)-Gly-SEt) (8%) generated by reaction with the *t*-Bu ester of the aspartic acid side chain (Table 2, entry 4), as well as significant amounts of aspartimide rearrangement product.[18,19] Despite these complications, we were able to isolate the 15-residue peptide thioester **5d** containing three aspartic acid residues in 24% yield (Table 2, entry 5). The yield from this procedure is comparable to that reported by Li et al. for the same peptide using a customized linker and special reagents for the removal of Fmoc protecting groups.[8]

To investigate the suitability of our new method for cleaving peptides with an epimerizable C-terminal residue, Z-Gly-Ala-Phe-O−Pam (**4e**) was synthesized. Cleavage of **4e** under the above conditions (Scheme 1), followed by treatment with TFA to quench the reaction, gave Z-Gly-Ala-Phe-SEt (**5e**, 32% yield) together with deprotected Gly-Ala-Phe-SEt (40%). Normal-phase HPLC analysis showed that **5e** was produced as a 9:1 mixture of LL/LD diastereoisomers (80% diastereomeric excess or de). This result contrasts with the low level of epimerization observed for the reaction in solution and reflects the different conditions employed. The large excess of Me_2AlCl and EtSH (20 equiv and 60 equiv, respectively) used to cleave esters from the resin enhances the overall efficiency of cleavage but also favors formation of thio-orthoesters and ketene-thioacetals (see Table 1). Conversion of these adducts to the thioester during acid workup provides an opportunity for epimerization (Scheme 1). Indeed, when the cleavage of Z-Gly-Ala-Phe-O−Pam (**4e**) was quenched with water instead of TFA, we observed three

Scheme 1



(18) Asp-Gly and Asp-Asp are known to be problematic motifs prone to aspartimide formation (Quibell, M.; Owen, D.; Packman, L. C.; Johnson, T. *J. Chem. Soc., Chem. Commun.* **1994**, 2343−2344). In this particular reaction, the aspartimide peptide thioester generated by cyclization of the aspartyl residue during the cleavage reaction is the major product [50% of the total peptide material as judged by integration of HPLC chromatograms and electrospray mass spectrometry (ESMS)].

(19) To suppress the formation of side products (aspartimide and bis-thioester), THF (20 equiv) was added as a weak Lewis base to coordinate the aluminum reagent. This modification reduced the amount of aspartamide side product formed (only 11% of the total peptide material, as determined by HPLC) and increased the overall yield of thioester **5c** up to 37%, but the formation of bis-thioester **7c** was also enhanced somewhat (20%).

products by HPLC and ESMS: thio-orthoester **8e**, thioester **5e**, and ketene thioacetal **9e** in an 8:1:1 ratio (Scheme 1).

After reverse-phase HPLC purification of these compounds, thio-orthoester **8e** and ketene-thioacetal **9e** were separately hydrolyzed by treatment with TFA−H_2O (95:5) for 2.5 h at room temperature. Interestingly, orthoester **8e** was converted to peptide thioester **5e** in 57% yield with little epimerization (LL/LD 97:3 or 94% de) (Scheme 1). This result is remarkable since acid hydrolysis of thio-orthoesters is known to give a stabilized thioacetal cation that readily undergoes β-hydrogen elimination to generate a ketene thioacetal.[20] Analogous treatment of ketene thioacetal **9e** gave rise to the thioester with very low de (30%) (Scheme 1).

In conclusion, Me_2AlCl-mediated thiolate cleavage of peptides from solid-phase supports provides direct access to peptide C-terminal thioesters. This one-pot procedure is compatible with Fmoc-SPPS and does not require special linkers, resins, or complicated protocols. Side reactions with aspartic acid side chains may reduce yields, although the byproducts are easily separated by HPLC. Some epimerization at the site of cleavage also currently limits the method to the preparation of peptides having a C-terminal glycine. Work is in progress to overcome these limitations and further define the scope and the generality of the method. However, given its simplicity, this approach may already prove useful for those ligations involving peptide segments with an activated C-terminal glycine.

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Supporting Information Available: Representative procedures, experimental details, and characterization of the products from the cleavage of Lys(Boc)-Tyr(O*t*Bu)-Arg(Pbf)-Ala-Gly-O−Pam resin (**4a**) and of Z-Gly-Ala-Phe-O−Pam (**4e**) with Me_2AlCl. This material is available free of charge via the Internet at http://www.pubs.acs.org.

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