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Fmoc Solid-Phase Synthesis of Peptide Thioesters by Masking as Trithioortho Esters

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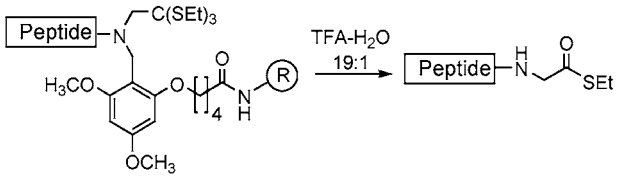
ABSTRACT



Total chemical synthesis of proteins by chemoselective ligation relies on C-terminal peptide thioesters as building blocks. Their preparation by standard Fmoc solid-phase peptide synthesis is made difficult by the lability of thioesters to aminolysis by the secondary amines used for removal of the Fmoc group. Here we present a novel backbone amide linker (BAL) strategy for their synthesis in which the thioester functionality is masked as a trithioortho ester throughout the synthesis.

The development of efficient methods for covalent coupling of *unprotected* peptides (referred to as chemoselective ligation) has dramatically extended the reach of total chemical synthesis of proteins.[1] In "native chemical ligation", originally developed by Kent and co-workers,[2] a peptide thioester[3] is reacted with a peptide carrying an N-terminal Cys moiety to provide, after transthioesterification and rearrangement, a native amide bond connecting the two peptide segments. Peptide thioesters can be accessed by Boc/Bn-based solid-phase synthesis. In contrast, the often favored Fmoc/^{t}Bu strategy includes repeated treatment with piperidine, which will aminolyze thioesters. However, several strategies for overcoming this obstacle to Fmoc-based synthesis of peptide thioesters have recently been reported. A specialized linker (handle), Ellman's modified version of Kenner's sulfonamide[4] linker, has been used.[5,6] While it is stable to aminolysis, treatment with powerful alkylating agents makes it susceptible to nucleophilic displacement by a thiol. Other approaches to utilization of Fmoc/^{t}Bu strategies for synthesis of peptide thioesters have relied on nonnucleophilic reagents for Fmoc removal[7] and conversion of partially protected peptides into their thioesters.[6,8] However, all these methods have inherent limitations (e.g., use of harsh alkylating agents, poor nucleophiles to remove the Fmoc group).

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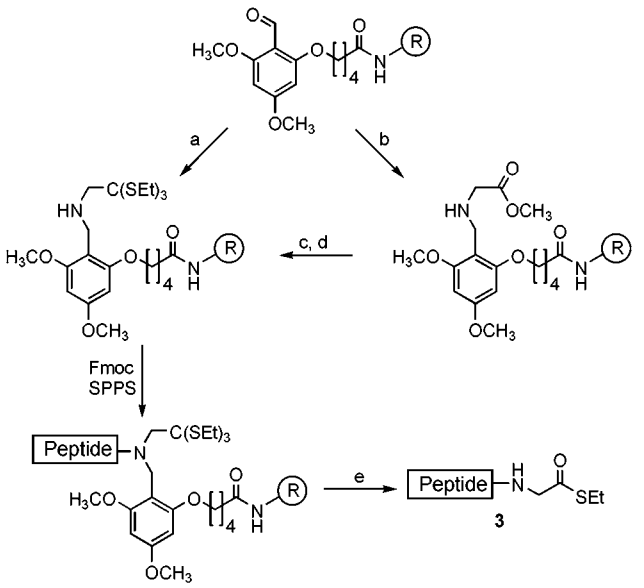
Backbone amide linker (BAL)[9,10] strategies provide general approaches to the synthesis of C-terminal-modified peptides; a BAL strategy for the synthesis of C-terminal peptide thioesters has been reported.[11] Here, the first amino acid was anchored as the allyl ester by reductive amination. Following peptide chain assembly, the orthogonal allyl protection was removed and the liberated carboxylic acid moiety was used to C-terminally extend the peptide with an amino acid thioester. Although this approach was successful in many cases, its scope was somewhat limited by the need for special precautions when coupling the second amino acid to prevent diketopiperazine (DKP) formation and the possible racemization when coupling the amino acid thioester.

The lability of thioesters to nucleophilic displacement is just one of many instances where it is desirable to protect or mask the sp^2 carbon in a carbonyl moiety as an sp^3 carbon to prevent side-reactions. Corey reported in the 1970s that lactones could be masked as dithioortho esters to prevent nucleophilic attack on the carbonyl and that alkyl esters could be converted to thioesters or trithioortho esters.[12] The oxophilic aluminum reagents affecting this were prepared from $AlMe_3$ and mono- or divalent thiols. Recently, Hilvert and co-workers reported an ingenious application of Corey's chemistry to the synthesis of peptide thioesters.[13] They developed a protocol for synthesis of peptide thioesters in which peptides C-terminally anchored as esters to linkers were first treated with excess $AlMe_3$ or Me_2AlCl together with RSH and then with TFA-containing cocktails to give the peptide thioester. They observed that treatment with a large excess of oxophilic reagent favored formation of trithioortho esters and ketene dithioacetals, which on acidic workup yielded peptide thioesters, albeit with the risk of racemization. However, the need to expose the whole assembled peptide to harsh reagents caused side-reactions to varying degrees, including conversion of side-chain ester groups to their thioesters and formation of aspartimide.

Here we present a novel and safe strategy, which after standard Fmoc/^{t}Bu solid-phase synthesis and TFA deprotection/release directly provides the peptide thioester (Scheme 1). The key element is anchoring of an amino trithioortho ester derived from Gly through an *o*-BAL[14] handle to a solid support in the first step of the synthesis. Two tactics were developed: (i) synthesis of the Gly trithioortho ester in solution followed by anchoring through *o*-BAL or (ii) first anchoring of a Gly ester to *o*-BAL followed by formation of the trithioortho ester on-resin. An additional advantage is that the trithioortho ester is not susceptible to nucleophilic attack and thus that formation of DKP at the dipeptide stage is avoided.

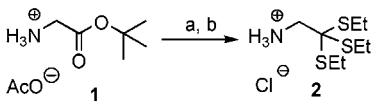
Scheme 1. Synthesis of a Peptide Thioester on the *o*-BAL Handle[a]



[a] Reaction conditions: (a) $H_2NCH_2C(SEt)_3$·HCl (**2**), $NaBH_3CN$, DMF; (b) H-Gly-OMe·HCl, $NaBH_3CN$, DMF; (c) $AlMe_3$ (10 equiv), EtSH (30 equiv), in CH_2Cl_2, 0 °C (to preform the reagent), then (d) 18 h in CH_2Cl_2, rt; (e) TFA−H_2O (19:1).

The first step in the former approach was preparation of the amino trithioortho ester as a shelf-stable compound. Treatment of H-Gly-O^{t}Bu·AcOH (**1**)[15] with the oxophilic reagent in CH_2Cl_2, preformed from $AlMe_3$ and EtSH, afforded after a simple extraction with 1% aqueous HCl followed by lyophilization the trithioortho ester $H_2NCH_2C(SEt)_3$·HCl (**2**) as a NMR pure solid with correct elemental analysis in 43% yield (Scheme 2). Raising the amount of aluminum reagent from 10 to 20 equiv raised the yield to 61%; lowering the amount to less than 10 equiv gave significantly reduced yields. Solid **2** could be stored at −18 °C for 10 months with no signs of degradation.

Scheme 2. Synthesis of Gly Trithioortho Ester[a]



[a] Reaction conditions: (a) $AlMe_3$ (10 equiv), EtSH (30 equiv), in CH_2Cl_2, 0 °C (to preform the reagent), then (b) 18 h in CH_2Cl_2, rt.

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(10) Abbreviations: *o*-BAL, *ortho*-backbone amide linker; DKP, diketopiperazine; MTBE, methyl *tert*-butyl ether; *o*-PALdehyde, 5-(2-formyl-3,5-dimethoxy-phenoxy)pentanoic acid; SPPS, solid-phase peptide synthesis; TG, tentagel; TFA, trifluoroacetic acid. Amino acid symbols denote the L-configuration unless stated otherwise.

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Next, reductive amination of *o*-PALdehyde-Tentagel (TG, 0.29 mmol/g) resin with amino trithioortho ester **2** proceeded with only 2 equiv in the presence of $NaBH_3CN$. Completeness of the reductive amination was indicated by a recently published color test for resin-bound aldehydes.[16] The Fmoc-protected second residue, Ala, was coupled as the symmetrical anhydride, whereas the remaining residues were coupled using standard protocols, to provide target sequence H-Phe-Val-Lys(Boc)-Glu(^{t}Bu)-Tyr(^{t}Bu)-Ala-N[$CH_2(SEt)_3$]-BAL-Ile-TG. Treatment with TFA−H_2O (19:1) for 2 h, followed by precipitation with methyl *tert*-butyl ether (MTBE) and lyophilization, released the deprotected peptide thioester H-Phe-Val-Lys-Glu-Tyr-Ala-Gly-SEt (**3**) into solution. It was rewarding to see that no trace of ortho ester was observed and that the thioester (correct ESMS) was obtained in a crude HPLC purity of >90% (Figure 1: HPLC, 265 nm). After preparative HPLC purification, pure peptide thioester **3** was obtained in 42% yield.

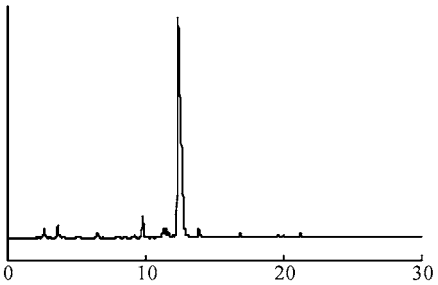

Figure 1. Analytical HPLC chromatogram of crude peptide thioester H-Phe-Val-Lys-Glu-Tyr-Ala-Gly-SEt (**3**) synthesized using preformed **2**.

In the alternative approach, entirely on solid phase, the synthesis commenced with reductive amination of resin-bound *o*-PALdehyde (0.29 mmol/g) with H-Gly-OMe·HCl in DMF in the presence of $NaBH_3CN$. Next, the resin was treated with the aluminum reagent, preformed from $AlMe_3$ and EtSH, in CH_2Cl_2 under Ar for 18 h to give the BAL-anchored trithioortho ester (Scheme 1). Evidence for this transformation was implicit from the eventual synthesis of the peptide thioesters (vide infra). Some solid debris from the reaction, most likely Al_2O_3, remained mixed with the resin. From here, peptide chain assembly followed standard protocols and the heptapeptide sequence was assembled as above. Acidic hydrolysis and amino acid analysis (AAA) of a sample of resin-bound peptide gave amino acid ratios of about unity, including for the internal reference amino acid[17] Ile. This proved both that the *o*-BAL handle was stable to treatment with $AlMe_3$ and that the DKP was not formed to any significant extent. Finally, peptide thioester **3** was obtained after acidolytic release from the support with either TFA−H_2O (19:1) or TFA−phenol−*i*Pr_3SiH−H_2O (88:5:5:2, reagent B)[18] for 2 h with a similarly high HPLC purity as above. Again, ESMS confirmed that the trithioortho ester moiety had been converted to the expected thioester. Purification by preparative HPLC yielded 23% of the peptide thioester **3**, based on the loading determined by AAA. It is noteworthy that this approach was compatible with a variety of peptide side-chain functionalities, including ester moieties, as the on-resin reaction with the aluminum reagent was at the beginning of the synthesis and not on the fully protected peptide after chain-assembly.

In conclusion, both strategies outlined above were successful in producing peptide thioesters. It was rewarding to see that the most widely used cleavage conditions in Fmoc/^{t}Bu SPPS, TFA−H_2O, and a silane-containing cocktail both were effective in converting the trithioortho ester into the thioester and gave the peptide thioester in high purity. The peptide thioester yield was significantly higher for the approach using a preformed trithioortho ester, making this a simple and efficient strategy for the synthesis of peptide thioesters. However, the yield from the alternative strategy with formation of the trithioortho ester on solid phase could possibly be improved. A number of opportunities exist for optimizing the methodology, including different choice of thiol and substitution of $AlMe_2Cl$ for $AlMe_3$. Initially, this strategy was explored for the formation of C-terminal glycine thioesters. Further studies should address the question of whether this strategy can be extended to chiral amino acids in the C-terminal position without racemization. Key to this will be to avoid intermediate formation of the ketene dithioacetal. As the trithioortho ester masking was carried through a whole series of synthetic cycles, these findings may also prove to be of relevance for general protecting group chemistry.

Supporting Information Available: Experimental procedures and analytical data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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