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Fmoc Solid-Phase Synthesis of Peptide Thioesters Using an Intramolecular *N,S*-Acyl Shift

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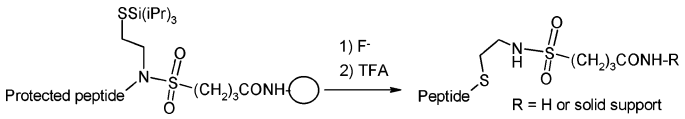
ABSTRACT



We describe the Fmoc solid-phase synthesis of peptide thioesters based on the alkylation of the safety-catch sulfonamide linker with a protected 2-mercaptoethanol derivative. The thioester is generated on the solid phase after the peptide chain assembly as a consequence of an intramolecular *N,S*-acyl shift. Depending on the stability of the spacer separating the sulfonamide linker from the resin toward TFA, treatment of the peptidyl resin with TFA led to a soluble or supported deprotected thioester.

Chemoselective ligation methods such as native chemical ligation[1] or Staudinger ligation[2] allow the assembly of proteins of moderate size (150 amino acids) and provide ready access to natural as well as modified proteins. Native chemical ligation is based on the reaction of a peptide bearing a C-terminal thioester group with an N-terminal cysteinyl peptide. Transthioesterification is followed by an intramolecular *S,N*-acyl shift that leads to the formation of a peptide bond at an X−Cys junction. Application of this chemistry to N-terminal selenocysteine peptides was also described.[3] Methods based on the use of N-linked, thiol-containing, cleavable auxiliaries[4] were used to extend the principle of native chemical ligation to sites other than Cys residues. Ligation of peptide thioesters with N-terminal homocysteine[5] or homoselenocysteine[6] peptides followed by methylation permitted the formation of X−Met or X−Seleno-Met bonds.[7]

The key starting materials for native chemical ligation are unprotected C-terminal peptide thioesters. Peptide thioesters are often prepared using Boc/benzyl solid-phase peptide chemistry.[1c] However, the widespread use of the Fmoc/*tert*-butyl chemistry[8] for peptide synthesis over the Boc/benzyl method has stimulated the development of methods allowing the preparation of peptide thioesters that are compatible with the basic treatments used to remove the Fmoc α-amino protecting group.

The thioester group is unstable in the presence of piperidine, which is usually employed for the removal of the Fmoc

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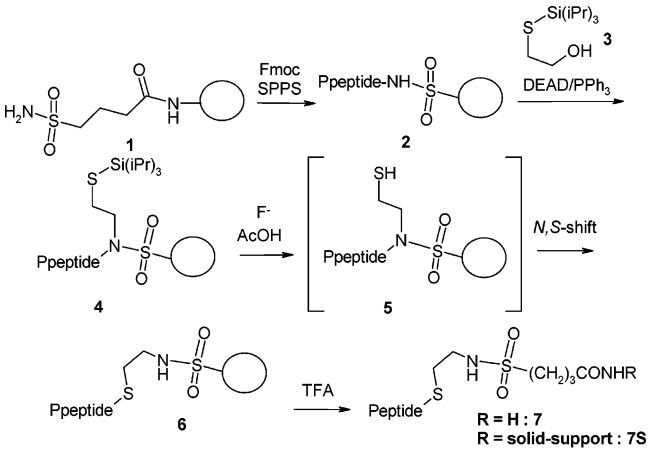
protection after the coupling of each amino acid. Li et al.[9] and Clippingdale et al.[10] have prepared peptide thioesters by replacing piperidine with 1-methylpyrrolidine/hexamethyleneimine/HOBt and DBU/HOBt mixtures, respectively. Brask et al. have prepared peptide thioesters using the backbone amide linker (BAL) and a thioester moiety masked as a trithioortho ester throughout the Fmoc/*tert*-butyl peptide synthesis.[11] Other methods involve the formation of the thioester group after chain assembly to avoid its exposure to basic conditions. Barany et al. have used BAL or esters resins and orthogonal allyl protection for the C-terminal α-carboxylic group for generating thioesters after chain assembly.[12] Hilvert et al. have prepared peptide thioesters using Lewis acid (Me_2AlCl)-catalyzed thiolysis of ester resins.[13,14] Finally, two groups have developed an attractive method exploiting the Kenner safety-catch sulfonamide linker,[15−17] which allowed the assembly of the peptide using standard Fmoc/*tert*-butyl chemistry. Subsequent alkylation of the acylsulfonamide linkage with diazomethane or iodoacetonitrile followed by displacement with an excess of a thiol produced the protected thioester in solution that was further deprotected with concentrated trifluoroacetic acid.[18] The use of an excess of LiBr permitted improvement of the thiolysis step.[19]

We report here a novel method for peptide thioester synthesis that is based also on the use of the sulfonamide safety-catch linker, but where the thioester function is generated on the solid-phase as the consequence of an intramolecular *N*,*S*-acyl shift as depicted in Scheme 1.

Our synthetic strategy begins with peptide assembly on a 3-carboxypropanesulfonamide linker using standard Fmoc/*tert*-butyl chemistry. Next, alkylation of the acylsulfonamide group is performed using Mitsunobu chemistry with protected mercaptoethanol derivative **3**.[20] Removal of the thiol protecting group gives supported intermediate **5**, which features a thiol nucleophile in close proximity to the activated carboxyl group. Consequently, **5** was found to rearrange spontaneously into **6** as a result of an intramolecular *N*,*S*-acyl shift which

Scheme 1. Synthesis of Thioesters Based on the Use of the Kenner Safety-Catch Linker, Mitsunobu Alkylation with **3**, and Intramolecular *N*,*S*-Acyl Shift



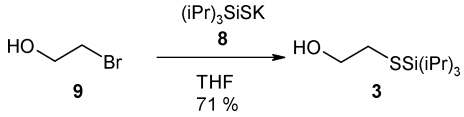
Ppeptide : protected peptide

a : peptide = Fmoc-FA-; **b :** peptide = Ac-ILKEPVHGA-; **c :** peptide = Ac-ILKEPVHGV-

involves a cyclic five-membered intermediate. Thus, a protected peptide thioester is formed that is still attached to the solid-support. Final deprotection of the peptide chain is performed in TFA. The use of a Rink linker between the 3-carboxypropanesulfonamide arm and the solid support leads to the liberation of the peptide thioester **7** in solution. Alternately, the use of a TFA-resistant linker results in the formation of a deprotected peptide thioester **7S** still attached to the resin that can be engaged in a native chemical ligation from the solid phase.[21]

The triisopropyl group was chosen for the protection of the thiol functionality of 2-mercaptoethanol because it is compatible with Mitsunobu chemistry and can be removed selectively in the presence of the standard side-chain protections. Key reagent **3** was prepared as described in Scheme 2

Scheme 2. Synthesis of Alcohol **3**



by reacting the potassium salt of triisopropylsilane thiol **8**[22] with 2-bromoethanol **9** in anhydrous THF.

In a first series of experiments, Fmoc-Phe-Ala dipeptide was assembled on a 3-carboxypropanesulfonamide linker attached to a Rink PEG-PS resin (NovaSyn). Peptidyl resin **2a** (Scheme 1) was then treated with alcohol **3** in the presence of diethylazodicarboxylate (DEAD) and triphenylphosphine (8 equiv of each). Direct treatment of resin **4a** with TFA led to complex mixtures. Alternately, removal of the triisopropylsilyl group of resin-bound peptide **4a** in the presence of

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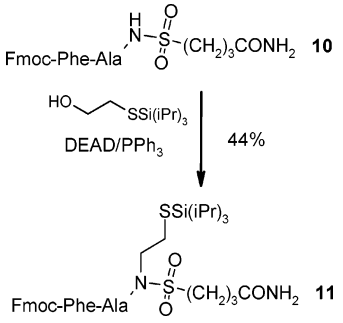
tetrabutylammonium fluoride buffered with acetic acid[23] followed by treatment of the resin with TFA liberated **7a** in solution as the major product, together with peptide Fmoc-Phe-Ala-$NHSO_2(CH_2)_3CONH_2$ **10** due to incomplete Mitsunobu alkylation (**7a**/**10** ratio determined to be 72/28 by RP-HPLC). Peptide **7a** was purified by RP-HPLC (50% yield) and fully characterized.

The ^{1}H NMR COSY spectrum of **7a** showed a SO_2NH proton at 5.27 ppm (triplet) linked to a CH_2CH_2 chain. This signal was not observed in the spectrum of dipeptide **11** (Scheme 3), which was prepared by alkylation of dipeptide

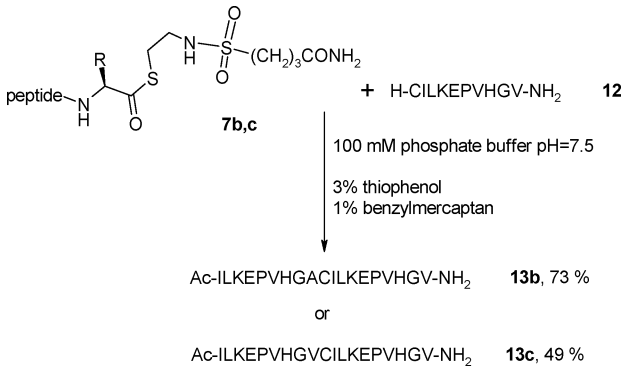
Scheme 3. Synthesis of Peptide **11**[a]



[a] Alkylation was found to be complete after 3 h at room temperature. Partial loss of the acid-sensitive TIPS protecting group during silica gel chromatography accounted for the modest yield obtained in this experiment.

10 in the presence of **3**/DEAD/PPh_3. Furthermore, an aliquot of peptide **7a** was reacted with peptide **12** (Scheme 4) in a

Scheme 4. Ligation of Peptides **7b**,**c** with Peptide **12**



pH 7.5 phosphate buffer in the presence of thiophenol/benzylmercaptan. The resulting product Fmoc-FACILKEPVHGV−NH_2 (MALDI-TOF $[M + H]^+$ calcd monoisotopic 1533.8, found 1534.0) was isolated by RP-HPLC, treated with piperidine, and sequenced using Edman chemistry that confirmed the formation of an alanine−cysteine peptide bond. Taken together, these data strongly support the structure proposed for peptide **7a**. Additional results presented below (ligation experiments between peptides **7b**,**c** and peptides **12** and **14**, solid-phase native chemical ligation between peptidyl resin **7Sb** and peptide **12**) confirmed the *N*,*S*-acyl shift following TBAF treatment and the formation of a thioester group at the C-terminus of the peptide.

Next, sequence Ac−ILKEPVHGA or Ac−ILKEPVHGV was assembled on sulfonamide resin **1** (Rink PEG-PS, NovaSyn) to give peptidyl resins **2b**,**c**, which were alkylated using **3** and Mitsunobu activation and treated with TBAF/AcOH and then TFA as described before to give successfully thioesters **7b**,**c**. RP-HPLC profile of the crude thioesters **7b**,**c** showed that the conversions of resins **2b**,**c** into alkylated acylsulfonamide resins **4b**,**c** were similar (ca. 67%) to the conversion of peptidyl resin **2a** into **4a**. For comparison, solution-phase alkylation of dipeptide **10** using similar experimental conditions was complete after 3 h at room temperature (Scheme 3). These results show that the alkylation of the sulfonamide did not depend on the nature of the first amino acid Ala or Val, probably because the site of alkylation is far enough from the C_α. The whole process worked equally well for both sequences (overall isolated yields of purified products, including the SPPS, Mitsunobu alkylation, TIPS cleavage and rearrangement, resin cleavage, and deprotection steps: 23 and 21% for **7b** and **7c**, respectively).

Peptides **7b**,**c** were then engaged in native chemical ligation experiments with Cys-peptide **12** as described in Scheme 4. Control experiments were performed in the same way by treating peptides **7b**,**c** with the peptide H−SILKEPVHGV−NH_2 **14**, where Ser has replaced the Cys residue in peptide **12**. Ligation proceeded well in the presence of Cys−peptide **12**, whereas no reaction was observed with Ser−peptide **14**. Peptides **13b** and **13c** were isolated with 73 and 49% yield, respectively, following RP-HPLC purification. They displayed the expected *m*/*z* values by MALDI-TOF analysis and were found to be identical by RP-HPLC and CZE to reference compounds prepared by standard Fmoc/*tert*-butyl solid-phase peptide synthesis (see Supporting Information).

The formation of supported C-terminal thioester following removal of the TIPS group in the presence of TBAF opens the possibility to perform a native chemical ligation from the solid phase, which should result in the liberation of the ligation product in solution. Camarero et al. have explored this approach using a poly(ethylene glycol)-poly(*N*,*N*-dimethylacrylamide) copolymer support and peptide thioesters assembled using the Boc/benzyl strategy.[21]

Fully deprotected peptidyl resin **7Sb** was prepared as described before starting from a NovaSyn TG amino resin. Deprotection of the peptide chain was performed in concentrated TFA containing dimethyl sulfide, anisole, and water as scavengers. Resin **7Sb** was suspended under argon in pH 7.5 phosphate buffer and reacted with Cys−peptide **12** in the presence of thiophenol and benzylmercaptan as for the

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solution-phase ligation experiments. After 23 h of reaction at room temperature, peptide **13b** was isolated with a 23% yield following RP-HPLC purification (calculated starting from Fmoc-Ala-sulfonamide TG NovaSyn resin and including the peptide elongation step, the Mitsunobu reaction with **3**, TIPS removal, the *N*,*S*-acyl shift, the deprotection in TFA, and the native chemical ligation reaction). Again, this product was found to be identical to the reference peptide synthesized using standard Fmoc/*tert*-butyl stepwise solid-phase chemistry.

In conclusion, we have presented a novel method for synthesizing C-terminal peptide thioesters based on the use of the safety-catch sulfonamide linker and an intramolecular solid-phase *N*,*S*-acyl shift that results in the formation of the thioester bond after the assembly of the peptide chain using standard Fmoc/*tert*-butyl chemistry. The procedure worked equally well for peptides presenting an Ala or Val residue at the C-terminus and thus seems to be insensitive to the bulkiness of the amino acid directly attached to the sulfonamide linker. The thioesters were found to be useful for native chemical ligations. Interestingly, the methodology could be applied to the preparation of a supported and deprotected peptide thioester that permitted a native chemical ligation to be performed from the solid-support.

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Supporting Information Available: Experimental section; ^{1}H or COSY, ^{13}C, and HRMS data for compounds **3**, **7a**, **11**; RP-HPLC for **10**; and for **7b**,**c**, **12**, **13b**,**c** (ligation products and reference compounds), and **14**, RP-HPLC using two different columns and capillary zone electrophoresis (CZE) and a copy of the coelutions between the ligation products and reference compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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