Peptide Synthesis

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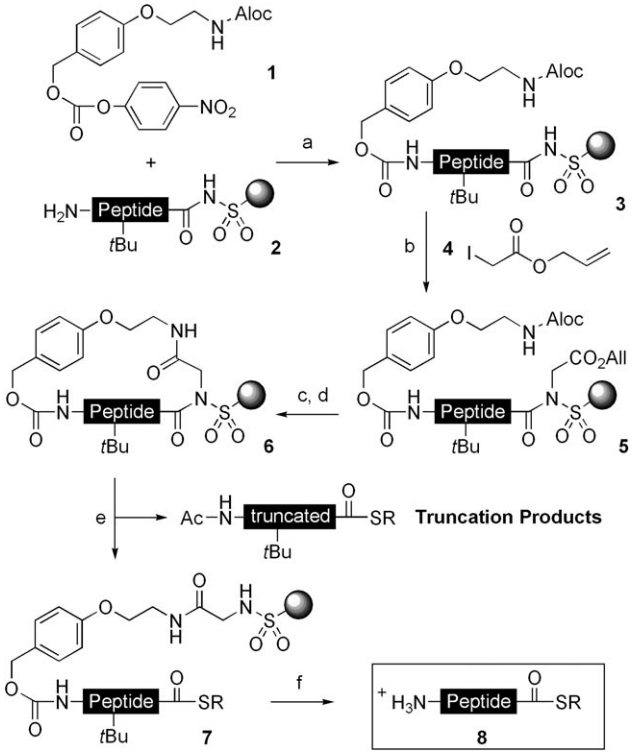
Solid-Phase Synthesis of Peptide Thioesters with Self-Purification**

*Franziska Mende and Oliver Seitz**

Chemical protein synthesis has facilitated studies of functional proteins by allowing the site-selective incorporation of labels, post-translational modifications, and non-proteinogenic amino acids.[1] The most reliable access to proteins and protein domains is provided by fragment ligation techniques with native chemical ligation probably being the most frequently used method.[2] A key requirement of the powerful native chemical ligation chemistry is the accessibility of unprotected peptide thioesters. However, the methods available for the solid-phase thioester synthesis are not as efficient in terms of yield and purity as current techniques for the synthesis of peptide acids and peptide amides.[3] Furthermore, the need for additional steps in solution is a major limitation in automated synthesis of peptide thioesters.

In solid-phase-based methods N-protected amino acid building blocks are coupled from the C-terminal to the N-terminal end. Thus, the truncation products, which accumulate due to failed coupling reactions, also feature a thioester structure. This can complicate purification since each active ester can be subject to side reactions. To obviate the often cumbersome HPLC purification and to enable the direct use of released peptide thioesters in native chemical ligation reactions, we sought a method that would allow the selective detachment of only the full-length peptide thioester. We anticipate that such a method should facilitate applications of native chemical ligation in divergent protein synthesis, for example in chip-based formats. Inspired by cyclization–cleavage approaches used for the inversion of peptide orientation on solid supports,[4,5] we assumed that a combination of on-resin macrocyclization at the N terminus with a thiolysis-induced ring-opening reaction should provide for the desired self-purification effect. A generic protocol involves the coupling of a cyclization linker such as **1** to peptide **2** linked to safety-catch sulfonamide resin (Scheme 1).[6,7] The initially allyloxycarbonyl(Aloc)-protected amino group in **3** tags the full-length peptide for a subsequent macrolactamization reaction. The required carboxy group is introduced upon alkylation of the N-acylsulfonamide in **5** with allyl iodoacetate **4**. Deallylation is followed by macrolactamization to form macrocycle **6**. In the next step, treatment with a mercaptan confers the nucleophilic cleavage of the acyl sulfonamide activated by the prior alkylation. This thiolysis step results in the opening of macrocycle **6**. N-Acetylated truncation products are excluded from the introduction of the cyclization linker and macrolactamization and are thus released into solution at this stage of synthesis. The desired peptide thioester remains on the solid support (**7**) and is liberated upon treatment with trifluoroacetic acid (TFA). It is worth mentioning that macrolactamization is not a strict requirement. The self-purification effect would pertain even if pseudo-intermolecular cross-coupling reactions occurred.



Scheme 1. Fmoc-based solid-phase synthesis of peptide thioesters with self-purification. a) **1**, 5% NEt_3/DMF, b) alkylation: **4**, DIPEA, DMF, c) deallylation: Pd^0, d) macrolactamization: PyBOP, e) thiolysis: RSH, f) TFA cleavage. Aloc = allyloxycarbonyl, All = allyl, DIPEA = ethyldiisopropylamine, PyBOP = benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate.

The reaction sequence was optimized by using the minimal sequence of the osteogenic growth peptide (OGP) as target.[8] To facilitate reaction monitoring a double-linker strategy was employed.[9] We chose to attach Ellmann's alkanesulfonamide linker[7] to the trityl resin in **9** (Scheme 2). Mild acidolysis cleaves the trityl ester bond, thereby enabling the analysis and quantification of the released peptidylsulfonamide structures **10**–**12**. The construction of the double-linker resin, loading of the sulfonamide, elongation of the peptide chain, and introduction of the cyclization linker were

[*] Dipl.-Chem. F. Mende, Prof. Dr. O. Seitz
Humboldt-Universität zu Berlin
Institut für Chemie
Brook-Taylor-Strasse 2, 12489 Berlin (Germany)
Fax: (+49) 30-2093-7266
E-mail: oliver.seitz@chemie.hu-berlin.de

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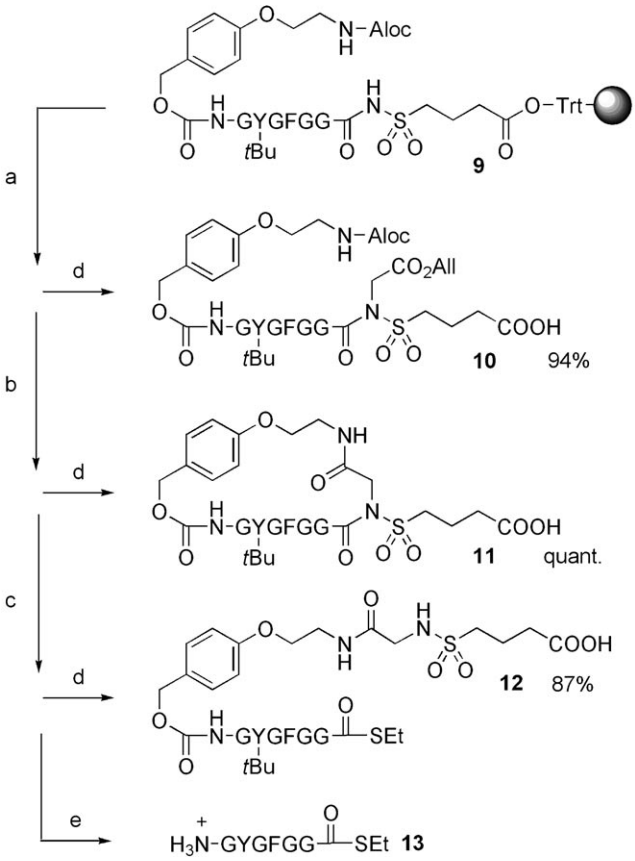

Scheme 2. Double-linker approach: a) 8 M **4**, 2 M DIPEA, DMF; b) 1. 0.02 M $[Pd(PPh_3)_4]$, DMB (0.2 M), CH_2Cl_2; 2. 0.1 M PyBOP, 0.1 M HOBt, 0.3 M DIPEA, CH_2Cl_2; c) 2 M EtSH , 0.12 M NaSPh, DMF; d) 1% TFA/CH_2Cl_2, e) TFA, *m*-cresol, H_2O, EDT (87.5:5:5:2.5). EDT = 1,2-ethanedithiol; HOBt = 1-hydroxy-1*H*-benzotriazole.

performed by using commonly used conditions (see the Supporting Information). The next reaction step comprised the alkylation of the N-acylsulfonamide in **9**, which also served the purpose of introducing the carboxy group needed for macrocyclization. The reagent allyl iodoacetate has a lower reactivity than the commonly used iodoacetonitrile. Nevertheless, alkylation to **10** proceeded in 94% yield when the electrophilic agent was used in excess. The Aloc group and the allyl ester were simultaneously cleaved, initially by using Pd^0-catalyzed allyl transfer to $BH_3{\cdot}Me_2NH$.[10] However, it proved difficult to completely remove the borane–amine adduct from the resin, as evidenced by the formation of dimethylamides during the subsequent macrocyclization reaction (see Figure S1 in the Supporting Information). The use of *N*,*N*′-dimethylbarbiturate (DMB) caused no problems. Macrocyclization reached completion after two consecutive treatments of resin with a solution of PyBOP, HOBt, and DIPEA in CH_2Cl_2. For thiolysis of the activated N-acylsulfonamide bond, macrocycle **11** was exposed to ethanethiol in presence of NaSPh to furnish the resin-bound form of peptide thioester **12** in 87% yield. The final treatment with TFA quantitatively liberated the desired peptide thioester **13**.

After the reaction conditions had been optimized, we returned to the use of the sulfonamide aminomethyl (AM) resin as a single-linker system and investigated the purity of peptide thioesters produced by the cyclization–thiolysis method. In a model synthesis of the OGP thioester **13**, the formation of a truncation product was enforced (see the Supporting Information). In spite of this, HPLC analysis of the crude material showed that thioester **13** was obtained in high 97% purity, providing evidence for the proposed self-purification effect (see Figure S3 in the Supporting Information). To assess the generality of the self-purification method, four more complex peptides were synthesized (Table 1). For comparison, the same peptide benzylthioesters were synthesized according to the conventional method at identical conditions for Fmoc cleavage, coupling and capping, and using iodoacteonitrile for activation of the safety-catch linker rather than allyl iodoacetate.[11] Peptide sequence **14** is derived from the segment 26–37 of ColE1 repressor of primer protein previously used in Kemp's "prior-thiol-capture" ligation chemistry.[12] The crude product **14** synthesized by the linear approach required purification not only because of small amounts of truncation products but also because of contaminations with reagents like NaSPh used in thiolysis of the acyl sulfonamide bond (Figure 1a). After preparative HPLC purification of this material **14** was isolated in 8% yield and 92% purity. The self-purification method furnished crude peptide thioester **14** in 20% overall yield and in 97% purity based on HPLC analysis (Figure 1b).

To further evaluate the usefulness of the self-purification method, a difficult peptide sequence was synthesized. We chose segment 12–37 from bovine pancreatic trypsin inhibitor (BPTI) **15**.[13] The HPLC analysis revealed the expected difficulties of linear solid-phase synthesis (Figure 1c). Major amounts of truncation products were detected. Significant tailing rendered complete removal of by-products by HPLC complicated. Furthermore, the attempts to purify peptide thioester **15** to homogeneity were plagued by the occurrence of cyclic thioesters formed by intramolecular thiol-exchange reactions during the lengthy process of purification (see Figure S2 in the Supporting Information). As a result **15** was isolated in only 6% yield and in 68% purity. The use of the self-purification method solved a significant part of the problems. The isolated crude **15** lacked the $n-19$ truncation product (Figure 1d), which was the dominant by-product formed during linear synthesis. Interestingly, there were still

Table 1: Sequences and yields of selected peptide thioesters.

Substrate	Peptide sequence	M_w [Da]	$[M+H]^+$ (found)	Yield [%][a]	Purity [%][b]
13	GYGFGG^{COSEt}	600.4	601.3	38	97
14	LNELDADEQADL^{COSBzl}	1450.6	1451.6	20	99
15	GPCKARIIRYNAKAGLCQTFVYGG^{COSBzl}	3001.5	3003.9	18	77
16	GATAVSEWTEYKTADGK^{COSBzl}	1918.9	1919.8	3	54
17	AEYVRALFDFNGNDEEDLPFKKG^{COSBzl}	2780.3	2782.2	30	98

[a] Yield was calculated from first amino acid loading. [b] Purity based on HPLC and detection at 210 nm.

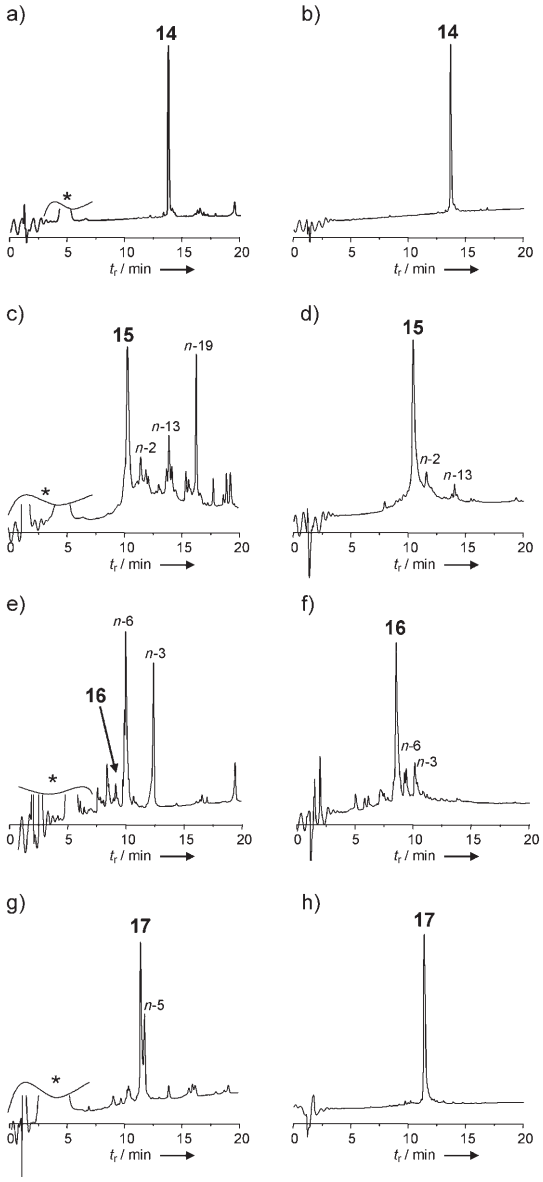

Figure 1. HPLC traces of crude peptide thioesters **14**–**17** in a), c), e), and g) obtained by conventional approach (* NaSPh and DMF) and in b), d), f), and h) obtained by peptide thioester synthesis with self-purification.

minor amounts of the $n-2$ and $n-13$ truncation products. We assume that the truncation products formed insoluble aggregates that hindered extraction by solvents routinely used in solid-phase synthesis. Nevertheless, by omitting making purification unnecessary the cyclization–thiolysis approach furnished peptide thioester **15** in 18% overall yield and in 77% purity, which is superior to both the yield and the purity provided by the conventional synthesis.

We increased the complexity of the synthesis problem by addressing the synthesis of a fragment of the WW domain of the formin-binding protein 28 (FBP28).[14] The 37-mer WW domain of FBP28 is known as an extremely difficult peptide sequence, which is impossible to synthesize by using conventional Fmoc-protected amino acid building blocks.[15] Our attempt to synthesize the thioester of the N-terminal fragment G^1–K^{17} **16** by linear solid-phase assembly also failed. In HPLC–MS analysis trace amounts of the desired peptide thioester **16** were detected as part of a very complex mixture of truncated sequences (Figure 1e). In stark contrast, application of the cyclization–thiolysis approach resulted in a crude material that was pure enough for considering the use in ligation chemistry (Figure 1f).

Recently, Camarero and co-workers reported the synthesis of peptide thioesters on a hydrazine support.[16] This included a peptide thioester similar to **17** that spans the first 23 amino acids of the N-terminal SH3 domain of the c-Crk protein adaptor.[17] We performed the linear synthesis of peptide thioester **17** on the sulfonamide resin and obtained the desired product contaminated by the $n-5$ truncation product (Figure 1g). After HPLC purification thioester **17** was isolated in 13% yield and 95% purity. In contrast, the self-purification procedure provided direct access to peptide thioester **17** in 98% purity (Figure 1h). As HPLC purification was not necessary, peptide **17** was prepared in less time and with higher yield (30%) than by conventional synthesis.

The direct use of crude peptide thioester was explored in the native chemical ligation of 23-mer thioester **17** with the SH3 domain C-terminal segment **18** (c-Crk, residues 157–191, CILRIRDKPEEQWWNAEDSEGKRGMIPVPYVEKYG) (see the Supporting Information). The N-terminal aspartic acid residue was replaced by a cysteine to enable ligation. For ligation both peptides **17** and **18** were dissolved in a degassed phosphate buffer containing 6 M guanidinium hydrochloride and 100 mM NaH_2PO_4 at pH 7.5 to a final concentration of 1 mM. Benzylmercaptan (1%), thiophenol (3%), and 20 mM TCEP were added to maintain reducing conditions and to accelerate ligation. After 15 h the ligation nearly reached completion (Figure 2). The subsequent HPLC purification furnished the pure synthetic SH3 protein in 53% yield. The molecular mass m/z 6851.5 determined by MALDI-TOF-MS analysis is in agreement with the calculated mass m/z 6850.6 $[M+H^+]$.

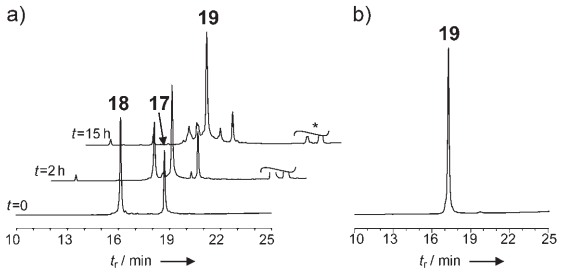

Figure 2. a) HPLC traces of ligation of **17** with **18** at 0 h, 2 h, and 15 h (* thiols). b) HPLC trace of purified **19**.

None of the previously applied methods for the Fmoc-based solid-phase synthesis of peptide thioesters included "self-purification".[18] The more commonly used approaches involve thioester synthesis as one of the last steps after completion of the peptide assembly. For example, thioesterification of protected peptide acids has been performed, but the need for additional reactions in solution, the laborious

purification of protected peptides, and the danger of C-terminal racemization can render this approach cumbersome.[19] More direct access to peptide thioesters is provided by methods that combine resin cleavage with thioester-bond formation such as the reaction induced upon treatment of resin-bound peptide esters with alkylaluminum thiolates.[13] The formation of aspartimides and side-chain thioesters has been reported. The oxidative activation of peptide hydrazides and subsequent aminolysis offers an interesting alternative.[16] This strategy requires preformed amino acid thioesters. The most frequently used method relies on the activation of sulfonamide safety-catch linkers by alkylation and subsequent thiolysis.[11] In both the hydrazide and the conventional sulfonamide safety-catch resins global deprotection is performed in solution. By contrast, there are no time-consuming solution steps necessary in the presented method. The most important hallmark of the cyclization–thiolysis approach is the self-purification effect. The implementation of this tactic involves two additional coupling reactions on the solid phase, which can be readily incorporated into usual protocols of automated synthesis. However, the synthesis of the difficult peptides from BPTI and FBP28 revealed that insoluble truncation products can still cause problems. Repeated washings after the thiolysis step reduced their content (see Figure S4 in the Supporting Information); nevertheless, complete removal proved difficult. It should be possible to improve the purity of such crude, difficult peptides by using optimized solvents or resins.

The results from five different syntheses demonstrate the efficacy of the cyclization–thiolysis approach. The desired products were obtained in better yield and purity than by conventional synthesis on the sulfonamide resin. Purification steps other than ether precipitation are required only for those sequences that form insoluble truncation products. The establishment of the entire SH3 domain (134–191) of the c-Crk protein demonstrated that the released peptide thioesters can be used directly in native chemical ligation. In future studies we will explore the self-purifying method in a parallel format to enable the synthesis of protein arrays by divergent segment ligation.

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