Fmoc-Compatible Solid-Phase Peptide Synthesis of Long C-Terminal Peptide Thioesters**

Axel Sewing and Donald Hilvert*

C-Terminal peptide thioesters are valuable intermediates for the chemical synthesis of proteins by peptide fragment condensation,[1] and for the production of cyclic peptides[2] and peptide dendrimers.[3] They are typically prepared either by solid-phase peptide synthesis (SPPS)[4] or in the case of longer segments, biosynthetically, by intein technology.[5]

Until recently, SPPS of peptide C-terminal thioesters was largely restricted to *tert*-butoxycarbonyl (Boc) methodology[4] because of the sensitivity of peptide thioesters to the basic conditions required to remove the widely used 9-fluorenylmethoxycarbonyl (Fmoc) protecting group.[6] However, several Fmoc-compatible routes to peptide thioesters are now available, including the use of new cleavage cocktails,[7] backbone amide linkers,[8] and activatible sulfonamide linkers.[9, 10] Each approach has advantages and disadvantages, but the need for special linkers, resins and/or complicated procedures remains a general limitation.

We recently reported an alternative Fmoc-compatible strategy for production of peptide thioesters involving direct cleavage of peptides from standard resins using an excess of $AlMe_2Cl$ and EtSH in dichloromethane.[11] This one-pot method yields the corresponding thioesters directly in good yield and purity. However, some problems arising from epimerization of C-terminal residues other than glycine and undesired side reactions, especially formation of side chain thioesters and aspartimide rearrangement, were observed. Here we report substantial improvements in the original procedure that allow its application to long peptides of complex composition.

The synthesis of the pentapeptide thioethyl ester LYRAG-SEt was used as a model system for optimizing the reaction conditions. The influence of the resin (4-hydroxymethylphenylacetamidomethyl-polystyrene (PAM), Wang, and 4-hydroxymethylbenzoic acid (HMBA))[6, 12] and the choice of Lewis acid on yield, side reactions, and epimerization was examined. In our original publication we reported that cleavage of LYRAG from PAM resin with $AlMe_2Cl$/EtSH to give LYRAG-SEt proceeded in 60% yield. With improved handling procedures and more accurate quantification of resin loading[6] this yield could be increased to 87% (Table 1). The HMBA resin afforded comparable yields and purities (84% isolated thioester) but the more reactive Wang resin always gave lower yields of the desired thioester and a higher proportion of the corresponding C-terminal acid. Because preloaded PAM resins are commercially available, they have an advantage over HMBA resins. However, the HMBA resin also allows the protected peptide to be cleaved with triethylamine in methanol to give a methyl ester.[6] The latter can be converted into a thioester in solution with lower excesses of alkylaluminum reagent than used for direct cleavage from the resin, but this two-step procedure provided no advantage with respect to purity or yield of the final product when peptides, rather than single amino acids,[11] were used.

Table 1. Yields of selected C-terminal peptide thioesters.

Peptide	Resin/ Ester	Reagent[a]	Thioester [%]	Acid [%]	Bis-thioester[b] [%]	Aspartimide[c] [%]
LYRAG	PAM	A	87	5	–	–
LYRAG	PAM	B	85	6	–	–
LYRAG	HMBA	A	84	5	–	–
LYRAG	OMe	C	67	17	–	–
IFKDG	PAM	A	22	n.d.[d]	13	25
IFKDG	PAM	D	43	n.d.[d]	15	6
IFKDG	PAM	B	62	n.d.[d]	6	3

[a] Reagent A: 0.2 M $AlMe_2Cl$, 0.6 M EtSH in CH_2Cl_2; reagent B: 0.2 M $AlMe_3$, 0.6 M EtSH in CH_2Cl_2; reagent C: 0.02 M (2 equiv) $AlMe_2Cl$, 0.06 M (6 equiv) EtSH in CH_2Cl_2; reagent D: 0.2 M $AlMe_2Cl$, 0.6 M EtSH, 0.2 M THF in CH_2Cl_2. [b] Bis-thioester IFKD(SEt)G-SEt. [c] Aspartimide-thioester IFKD(imid)G-SEt. [d] n.d.: not determined.

By using $AlMe_3$ in place of the more acidic $AlMe_2Cl$, the formation of aspartimides and aspartic acid side chain thioesters can be greatly suppressed (Table 1). For example, attempts to cleave IFKDG-PAM with $AlMe_2Cl$/EtSH produced the thioester IFKDG-SEt in low yield (22%), as well as substantial amounts of the IFKD(SEt)G-SEt bis-thioester (13%) and the aspartimide generated by cyclization of the aspartyl residue (25%). In contrast, treatment of IFKDG-PAM with $AlMe_3$/EtSH gave a 62% yield of the desired thioester, 6% bis-thioester, and only 3% aspartimide thioester. Addition of stoichiometric amounts tetrahydrofuran (THF) to the $AlMe_2Cl$ reagent also reduced aspartimide formation, but did not affect bis-thioester formation. Addition of THF to $AlMe_3$ provided no advantage.

Although $AlMe_3$ is compatible with peptides of complex composition, earlier studies[11] suggested that the extent of epimerization of susceptible C-terminal thioesters would also increase in the presence of this Lewis acid. Indeed, the ratio of L,L/L,D diastereomers of the tripeptide Ac-Gly–Ala–Phe-SEt went from 9:1 to 3.5:1 when $AlMe_2Cl$ was replaced by $AlMe_3$ in the cleavage reaction. This difficulty is circumvented when a cleavage site with a C-terminal glycine can be chosen, as is often the case.[1, 9, 13, 14]

The utility of the optimized $AlMe_3$/EtSH conditions was demonstrated by synthesis of the thioester corresponding to the 37 amino acid long N-terminal fragment of bovine pancreatic trypsin inhibitor (BPTI) (Figure 1). This fragment was previously prepared by Boc methodology and used to synthesize the 58 amino acid long inhibitor by native chemical ligation.[14]

We prepared the $BPTI^{1-37}$ fragment by SPPS on a PAM resin with standard Fmoc chemistry. The optimized synthesis required triple couplings at difficult stretches[15] as well as

[*] Prof. D. Hilvert, A. Sewing
Laboratorium für Organische Chemie
Swiss Federal Institute of Technology (ETH)
Universitätstrasse 16, 8092 Zurich (Switzerland)
Fax: (+41) 1-632-1486
E-mail: hilvert@org.chem.ethz.ch

[**] The authors are grateful to the ETH-Zürich and Novartis Pharma AG for support of this work. Fmoc = 9-fluorenylmethoxycarbonyl.

Supporting information for this article is available on the WWW under http://www.angewandte.com or from the author.

  1433-7851/01/4018-3395 $ 17.50+.50/0

RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGG↓CRAKRNNFKSAEDCMRTCGGA

Figure 1. Sequence of BPTI with ligation site indicated (↓).

increased deprotection and coupling times. After completion of the synthesis, the peptide was cleaved from the resin by treatment with $AlMe_3$ and thiol for 5 h. All side chain protecting groups were subsequently removed with a cocktail containing 89% trifluoroacetic acid (TFA), 4% water, 2% phenol, 2% thioanisole, 2% ethanethiol, and 0.8% triisopropylsilane. Analysis of the crude reaction mixture by HPLC/MS identified BPTI$^{1-37}$-SEt as the major product (Figure 2).

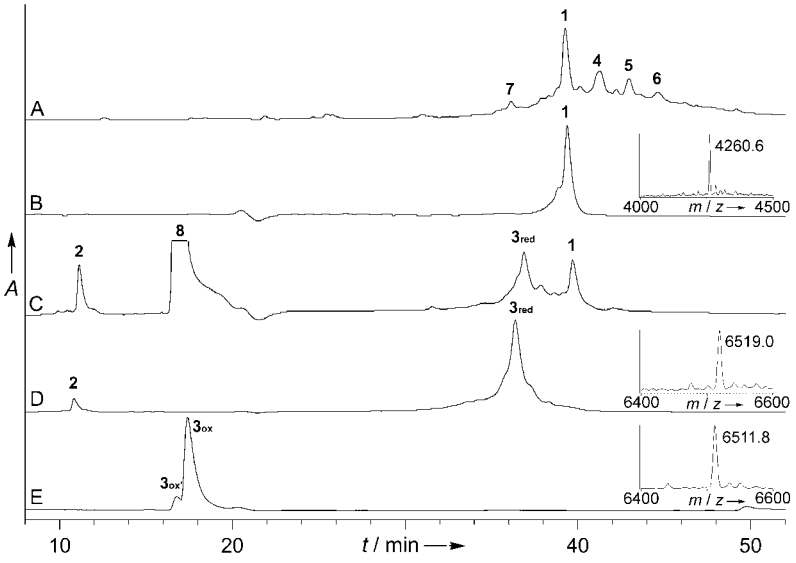

Figure 2. Synthesis of BPTI$^{1-37}$-SEt (**1**) and its native chemical ligation with BPTI$^{38-58}$ (**2**) monitored by analytical HPLC. A) Crude product **1**; B) isolated and purified **1**; C) ligation ($t = 8$ h) of **1** with **2** to form BPTI$^{1-58}$ (**3**$_{red}$); D) ligation mixture ($t = 16$ h) after gel filtration to remove small molecules; E) native, oxidized BPTI$^{1-58}$ (**3**$_{ox}$) after gel filtration and HPLC purification. Masses of key species calculated from the multiple charged ion mass peaks are shown as insets. Side products and reagents: **3**$_{ox}$, oxidized BPTI$^{1-58}$ containing methionine sulfoxide; **4**, BPTI$^{1-37}$-bisthioester; **5**, *t*Bu adduct of BPTI$^{1-37}$-SEt; **6**, acetyl-capped deletion fragment Ac-BPTI$^{15-37}$-SEt; **7**, acetyl-capped deletion fragment Ac-BPTI$^{6-37}$-SEt; **8**, thiophenol.

The Asp^3(SEt) bis-thioester, a *tert*-butyl adduct of the desired thioester, and two capped deletion fragments, Ac-BPTI$^{6-37}$-SEt and Ac-BPTI$^{15-37}$-SEt, were observed as minor side products. The desired thioester is poorly soluble, making purification difficult. After two preparative HPLC runs on a C-8 column, however, it was obtained in 8% overall yield, which can be compared with the 15% yield for the same peptide obtained by the Boc method. The mass of this fragment was determined to be 4260.6 Da by electrospray mass spectrometry (ESI-MS), in good agreement with the calculated mass of 4261.0 Da.

To show that the synthetic BPTI$^{1-37}$-SEt fragment is identical to the previously prepared material, it was condensed with BPTI$^{38-58}$ according to literature protocols to give reduced, denatured BPTI in 63% yield (Figure 2). After oxidation and folding,[14, 15] synthetic BPTI was purified by gel filtration and HPLC. The experimentally determined masses of the oxidized (6511.8 Da) and reduced (6519.0 Da) protein were in good agreement with the calculated values (6512.5 Da and 6518.5 Da, respectively). Synthetic and commercial BPTI co-eluted on a C-8 HPLC column and exhibited identical circular dichroism spectra (data not shown). Moreover, the synthetic protein inhibited trypsin stoichiometrically[16] and chymotrypsin[14, 17] with a K_i of 1.3×10^{-8} M (lit:[14] 1.2×10^{-8} M).

Lewis acid activated cleavage of peptides from commercial resins provides a facile route to C-terminal peptide thioesters. The method is compatible with standard Fmoc procedures, employs commercially available reagents and resins, and does not require special handling procedures. Use of $AlMe_3$ as the Lewis acid minimizes side reactions like aspartimide and bis-thioester formation, although some epimerization at the site of cleavage occurs. Given its simplicity, this method should be useful for preparing thioesters of a wide variety of peptide segments having a C-terminal glycine, which themselves are widely used for protein synthesis.[1, 9, 13, 14]

Received: April 6, 2001 [Z16912]

[1] For a recent review, see: P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923–960.

[2] a) Y. Shao, W. Y. Lu, S. B. H. Kent, *Tetrahedron Lett.* **1998**, *39*, 3911–3914; b) J. A. Camarero, T. W. Muir, *Chem. Commun.* **1997**, 1369–1370.

[3] a) J. Shao, J. P. Tam, *J. Am. Chem. Soc.* **1995**, *117*, 3893–3899; b) L. S. Zhang, J. P. Tam, *J. Am. Chem. Soc.* **1999**, *121*, 3311–3320.

[4] S. Aimoto, *Biopolymers* **1999**, *51*, 247–265.

[5] For a recent review, see: C. J. Noren, J. M. Wang, F. B. Perler, *Angew. Chem.* **2000**, *112*, 458–476; *Angew. Chem. Int. Ed.* **2000**, *39*, 450–466.

[6] *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, *Vol. 222* (Eds.: W. C. Chan, P. D. White), Oxford University Press, Oxford, **2000**.

[7] X. Li, T. Kawakami, S. Aimoto, *Tetrahedron Lett.* **1998**, *39*, 8669–8672.

[8] a) J. Alsina, T. S. Yokum, F. Albericio, G. Barany, *J. Org. Chem.* **1999**, *64*, 8761–8769; b) K. J. Jensen, J. Alsina, M. F. Songster, J. Vagner, F. Alberico, G. Barany, *J. Am. Chem. Soc.* **1998**, *120*, 5441–5452; c) J. Alsina, T. S. Yokum, F. Albericio, G. Barany, *Tetrahedron Lett.* **2000**, *41*, 7277–7280.

[9] Y. Shin, K. A. Winans, B. J. Backes, S. B. H. Kent, J. A. Ellman, C. R. Bertozzi, *J. Am. Chem. Soc.* **1999**, *121*, 11684–11689.

[10] R. Ingenito, E. Bianchi, D. Fattori, A. Pessi, *J. Am. Chem. Soc.* **1999**, *121*, 11369–11374.

[11] D. Swinnen, D. Hilvert, *Org. Lett.* **2000**, *2*, 2439–2442.

[12] B. A. Bunin, *The Combinatorial Index*, Academic Press, San Diego, **1998**, pp. 9–76.

[13] a) L. Z. Yan, P. E. Dawson, *J. Am. Chem. Soc.* **2001**, *123*, 526–533; b) A. Brik, E. Keinan, P. E. Dawson, *J. Org. Chem.* **2000**, *65*, 3829–3835; c) W. Y. Lu, M. Randal, A. Kossiakoff, S. B. H. Kent, *Chem. Biol.* **1999**, *6*, 419–427; d) G. J. Cotton, B. Ayers, R. Xu, T. W. Muir, *J. Am. Chem. Soc.* **1999**, *121*, 1100–1101; e) P. E. Dawson, M. J. Churchill, M. R. Ghadiri, S. B. H. Kent, *J. Am. Chem. Soc.* **1997**, *119*, 4325–4329; f) M. Baca, S. B. H. Kent, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11638–11642; g) H. Hojo, S. Aimoto, *Bull. Chem. Soc. Jpn.* **1992**, *65*, 3055–3063.

[14] W. Y. Lu, M. A. Starovasnik, S. B. H. Kent, *FEBS Lett.* **1998**, *429*, 31–35.

[15] M. Ferrer, C. Woodward, G. Barany, *Int. J. Pept. Protein Res.* **1992**, *40*, 194–207.

[16] B. Kassel, *Methods Enzymol.* **1970**, *19*, 844–852.

[17] J. W. Williams, J. F. Morrison, *Methods Enzymol.* **1979**, *63*, 437–467.

  1433-7851/01/4018-3396 $ 17.50+.50/0