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

Axel Sewing and Donald Hilvert

General Methods. All Fmoc-amino acids and resins were from Novabiochem, reagents for peptide synthesis from Applied Biosystems, native BPTI (aprotinin), trypsin and chymotrypsin from Calbiochem and all other chemicals from Fluka or Aldrich. Dichloromethane was dried over CaH₂. All other reagents were used without purification.

Peptides were synthesized on an Applied-Biosystems ABI 433A peptide synthesizer using standard methods.¹ Amino acid side chains were protected with the following groups: Arg(Pbf), Asn(Trt), Asp(O-tBu), Cys(Trt), Lys(Boc), Ser(tBu), Thr(tBu), and Tyr(tBu). Analytical HPLC was performed on a Nucleosil C-18 column (300 Å, 5 µ, 250 mm x 4.6 mm) with a flow rate of 1 mL min⁻¹; a C-18 column (300 Å, 7µ, 250 mm x 21 mm) was used for preparative HPLC with a flow rate of 10 mL min⁻¹. Linear gradients from 100% 0.1% TFA in water (A) to 60% acetonitrile (B) over 45 min were used. BPTI and its fragments were purified and analyzed on
C-8 columns using a gradient from 15% A to 50% B over 60 min.

**General procedure for synthesizing C-terminal peptide thioesters.** After completion of standard Fmoc-SPPS,\(^1\) dried resin (50-100 mg) was swollen in 1 mL dry CH\(_2\)Cl\(_2\) for 15 min under an inert atmosphere. A cold solution containing 222 µL EtSH and 0.5 mL AlMe\(_3\)\(^{2,3}\) (2 M in hexane) in 3.5 mL CH\(_2\)Cl\(_2\) was added. The resulting suspension was shaken for 5 h at room temperature under argon, then evaporated to dryness. A cleavage cocktail was added slowly while cooling, and after shaking for 2-3 h, the mixture was filtered. Depending on the peptide sequence, different amounts of TFA, H\(_2\)O, EtSH, thioanisole, triisopropylsilane and phenol were used for the cleavage cocktail. The crude peptide thioester was precipitated with cold, dry diethyl ether and purified by preparative HPLC. Resin loading was determined spectrophotometrically as previously described.\(^1\)

**Epimerization.** The extent of epimerization at the C-terminal amino acid during thioester synthesis was quantified in the case of Ac-Gly-Ala-Phe-SEt by \(^1\)H-NMR spectroscopy by integrating the doublets at 1.12 and 1.29 ppm corresponding to the diastereotopic alanine methyl
$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ (ppm) = 7.35-7.17 (m, 5 H), 4.77-4.69 (m, 1 H), 4.40-4.33 (m, 1 H), 3.82-3.80 (d, 2 H), 3.27-3.19 (m, 1 H), 2.96-2.82 (m, 3 H), 1.99 (s, 3 H), 1.29 (d, $^3$J (H,H)=7 Hz, 0.09 H), 1.25-1.19 (m, 3 H), 1.12 (d, $^3$J (H,H)=7 Hz, 0.91 H).

**BPTI Synthesis.** BPTI$^{1-37}$-SEt thioester was obtained from BPTI$^{1-37}$-PAM (50 mg, 10 µmol) as described above. The crude product was purified twice by reverse phase HPLC on a C-8 column to afford the desired compound as a TFA salt (4.1 mg, 0.8 µmol, 8%). The thioester was ligated with 1.1 eq BPTI$^{38-58}$ for 15 h as described by Kent.[4] The product was purified by membrane filtration (Microsep 3K), gel filtration (Superdex® Peptide HR 10/30) and HPLC to give ligated, reduced BPTI$^{1-58}$ (4.2 mg, 63%). The reduced protein was renatured aerobically and purified by FPLC and HPLC.[4,5]

**BPTI Characterization.** Concentrations of BPTI and enzyme solutions were determined spectrophotometrically.[6] CD spectra were recorded at pH 8.2 in 50 mM Tris buffer. Trypsin inhibition was determined by the procedure of Kassel.[7] Inhibition of chymotrypsin-catalyzed hydrolysis of Suc-Gly-Gly-Phe-para-nitroanilide was performed as described.[8]
References:


**Figure 1.** HPLC analysis of typical reaction mixtures obtained by cleaving IFKDG-PAM with AlMe$_2$Cl/EtSH (top) and AlMe$_3$/EtSH (bottom). 1, IFKD(OH); 2, IFKD(SEt); 3, IFKD(imid)G(SEt); 4, IFKD(SEt)G(SEt).