Facile, Fmoc-Compatible Solid Phase Synthesis of Peptide C-Terminal Thioesters

Dominique Swinnen and Donald Hilvert*

Laboratory of Organic Chemistry, Swiss Federal Institute of Technology (ETH), Universitätstrasse 16, CH-8092 Zürich, Switzerland.

hilvert@org.chem.ethz.ch

SUPPORTING INFORMATION

General Procedures. All Boc-amino acids, Fmoc-amino acids and preloaded Wang resins were purchased from Novabiochem (Switzerland). Preloaded Boc-amino acid Pam resins, 1-methylpyrrolidine (NMP), 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt) and piperidine were purchased from Perkin Elmer (Applied Biosystems). Trimethyl aluminum (2M solution in toluene) was purchased from Fluka, dimethylchloro aluminum (1M solution in hexane) was purchased from Aldrich. Other chemical reagents were obtained from commercial suppliers and used without further purification. CH_2Cl_2 was dried by distillation over CaH_2 prior to use. Protection of amino acids was as follows: Arg(Pbf), Asp(O*t*Bu), Lys(Boc), Tyr(O*t*Bu), His(Boc), Trp(Boc), Asn(Trt), Gln(Trt), Ser(O*t*Bu), Thr(O*t*Bu).

Enantiomeric excess (e.e.) of Boc-Ala-SEt and Boc-Phe-SEt was determined by comparison of their optical rotation with the value measured for an authentic sample made directly from the respective (L)-amino acid, DCC, HOBt and EtSH. For Boc-Ala-SEt with more than 99% e.e. (Table 1, entry 5), the enantiomeric excess was determined by chiral gas chromatography on a Supelco β-DEX 120 column (30 m, 2.5 mm) starting at a temperature of 100°C for 3 min then increasing up to 150°C in 50 min following a linear gradient of 1°C/min.

Peptides were synthesized in stepwise fashion on an ABI 433A peptide synthesizer by established machine assisted solid phase methods using HBTU/HOBt/NMP activation protocols for Fmoc chemistry (FastMoc® protocol, Applied Biosystems) on Fmoc-aminoacyl-O-Wang resins or on aminoacyl-O-Pam resins (obtained by TFA treatment of the Boc-aminoacyl-O-Pam resins followed by neutralization by DIEA). Peptide deprotection was accomplished by 2.5 h treatment at room temperature with TFA-H_2O-PhOH (95:2.5:2.5) for **4a**, with TFA-H_2O-thioanisole-EtSH (92.5:2.5:2.5:2.5) for **4b**, TFA-H_2O (95:5) for **4c** and **4e**, and with TFA-H_2O-EtSH (95:2.5:2.5) for **4d**. Yields of peptides were calculated from resin loading capacities as reported by the commercial venders.

Reverse-phase HPLC was performed on a Waters HPLC system with 220 nm UV detection, using a Waters analytical column (Novapak, 3.9 mm × 150 mm, C_{18} reversed phase) at a flow rate of 1 mL/min or a Vydac preparative column (218TP1022, 22 mm × 250 mm, C_{18} reversed phase) at a flow rate of 10 mL/min. All analytical runs used linear gradients of 60% B in A over 45 min (A = water containing 0.1% TFA, B = acetonitril containing 0.1% TFA) (*condition A*), or of 80% B in A (*condition B*) over 45 min, or of 40% B in A (*condition C*) over 45 min. All preparative runs used linear gradients of 60% B in A over 45 min (*condition D*). Normal-phased HPLC was performed on a Macherey Nagel Si60 analytical column (Nucleosil 100-5, 4 mm × 250 mm, normal phase) at a flow rate of 1 mL/min of 45% ethyl acetate in hexane (*condition E*). All synthetic peptides were greater than 95% pure by analytical reversed-phase HPLC and each had the expected mass compared with the calculated mass (average isotope composition), as determined by electrospray ionization mass spectrometry (ESMS) or high resolution fast atom bombardment mass spectrometry (FAB).

ESMS was performed on a Finnigan TSQ7000 Triple-Quad Mass Spectrometer and FAB on a Micromass VG-ZAB-2SEQ BEqQ-Hybrid Mass spectrometer. ESI-MS values are accurate within ± 1 Da. Calculated masses were based on average isotope composition and were derived using the program SynthAssist Software (Applied Biosystems).

Cleavage of Leu-Tyr(O*t*Bu)-Arg(Pbf)-Ala-Gly-O-Pam resin 4a with Me_2AlCl followed by TFA-H_2O-PhOH treatment. 92.13 mg of Leu-Tyr(O*t*Bu)-Arg(Pbf)-Ala-Gly-O-Pam resin (dried 48 h at high vacuum at room temperature, 0.0485 mmol) in 1.0 mL of dry CH_2Cl_2 was stirred for 15 min under argon in a 5 mL flask. In a second flask, 1.0 mL of Me_2AlCl (1M in hexane, 1.0 mmol) diluted in 3.0 mL of dry CH_2Cl_2 was cooled at 0°C under argon. 0.23 mL of EtSH (d: 0.84, 190 mg, 3.0 mmol) was added dropwise and the resulting mixture was allowed to stir for 15 min at 0°C. This solution was added at once to the suspension of peptide-

resin in DCM. After 5 h of stirring at room temperature, the peptide resin was directly poured into 2.0 mL of a solution of TFA-PhOH-H_2O (95:2.5:2.5) and evaporated (rotavap). 3.0 mL of the TFA-PhOH-H_2O (95:2.5:2.5) solution was added to the oily residue and stirred 2.5 h at room temperature. After filtration, the peptide resin was washed twice with TFA and the combined TFA filtrates were evaporated (rotavap). The crude peptide was precipitated by addition of 10 mL of cold ether (0°C), then isolated by centrifugation (5 min at 3000 g). The precipitated peptide was washed with 10 mL of cold ether and centrifuged. The washing-centrifugation procedure was repeated twice. The residue was dried overnight at high vacuum to give 45.6 mg of crude peptide. Purification by preparative reverse-phase HPLC (*condition D*) followed by lyophilization of the collected fractions afforded the thioester Leu-Tyr-Arg-Ala-Gly-SEt **5a** (24.7 mg; 60% yield based on the resin loading capacity) and Leu-Tyr-Arg-Ala-Gly-OH **6a** (1.2 mg; 3%). The purified peptide thioester **5a** was characterized by analytical C_{18} reversed phase HPLC (t_R 15.7 min; >95% purity; *condition A*), by ESI-MS: observed mass 623.4 Da ($[M+H]^+$; calculated average isotope composition for $C_{28}H_{47}N_8O_6S$, 623.8 Da ($[M+H]^+$)) and by high resolution FAB: observed 623.334 Da ($[M+H]^+$; exact mass for $C_{28}H_{47}N_8O_6S$, 622.334 Da ($[M+H]^+$)). The purified peptide acid **6a** was characterized by analytical C-18 reversed-phase HPLC (t_R 10.7 min; >95% purity; *condition A*) and by ESI-MS: observed 579.3 Da ($[M+H]^+$; calculated average isotope composition for $C_{26}H_{43}N_8O_7$, 579.7 Da ($[M+H]^+$)).

Cleavage of Leu-Tyr(O*t*Bu)-Arg(Pbf)-Ala-Gly-O-Pam resin 4a with Me_2AlCl followed by TFA-PhOH treatment. The above cleavage procedure applied to 95.0 mg of Leu-Tyr(O*t*Bu)-Arg(Pbf)-Ala-Gly-O-Pam resin **4a** (0.050 mmol), followed by TFA-PhOH (95:5) instead of TFA-PhOH-H_2O (95:2.5:2.5) for 2.5 h gave 49.1 mg crude peptide. Purification by preparative C-18 reverse-phase HPLC (*condition D*) and lyophilization of the collected fractions afforded the peptide thioester **5a** (7.8 mg; 18%) accompanied by the ketene acetal Leu-Tyr-Arg-Ala-NH-CH=C(SEt)$_2$ **9a** (15.4 mg; 34%) and the peptide acid **6a** (1.4 mg; 4%). The ketene acetal **9a** was characterized by analytical C-18 reversed-phase HPLC (t_R 25.1 min; >95% purity; *condition A*), by ESI-MS: observed 667.5 Da ($[M+H]^+$; calculated average isotope composition for $C_{30}H_{51}N_8O_5S_2$, 667.9 Da ($[M+H]^+$)), and by high resolution FAB: observed 667.343 Da ($[M+H]^+$; exact mass for $C_{30}H_{51}N_8O_5S_2$, 667.342 Da ($[M+H]^+$)).

Cleavage of Z-Gly-Ala-Phe-O-Pam resin 4e with Me_2AlCl followed by TFA-H_2O treatment. The procedure described for the cleavage of **4a** was applied to 106.4 mg of Z-Gly-Ala-Phe-O-Pam resin **4e** (0.0484 mmol), followed by treatment with TFA-H_2O (95:5) for 2.5 h instead of TFA-PhOH-H_2O (95:2.5:2.5), and gave 31.55 mg of crude peptide. Purification by preparative C-18 reverse-phase HPLC (*condition D*) and lyophilization of the collected fractions afforded the peptide thioester Z-Gly-Ala-Phe-SEt **5e** (7.37 mg; 32% yield based on the resin loading capacity) and the deprotected Gly-Ala-Phe-SEt **10e** (8.80 mg; 40%). The purified peptide thioester **5e** was characterized by analytical C-18 reversed-phase HPLC (t_R 44.7 min; >95% purity; *condition C*), by ESI-MS: observed 494.3 Da ($[M+Na]^+$; calculated average isotope composition for $C_{24}H_{29}N_3NaO_5S$, 494.6 Da ($[M+Na]^+$)). The peptide thioester **5e** could be separated by normal-phase HPLC as a 90:10 mixture of LL:LD diastereoisomers (80% diastereomeric excess (d.e.); LL: t_R 31.8 min and LD: t_R 34.3 min; *condition E*). The purified deprotected peptide Gly-Ala-Phe-SEt **10e** was characterized by analytical C-18 reversed-phase HPLC (t_R 21.1 min; >95% purity; *condition A*), by ESI-MS: observed 338.2 Da ($[M+H]^+$; calculated average isotope composition for $C_{16}H_{24}N_3O_3S$, 338.5 Da ($[M+H]^+$)).

Cleavage of Z-Gly-Ala-Phe-O-Pam resin 4e with Me_2AlCl followed by H_2O treatment. The above cleavage procedure applied to 106.4 mg of Z-Gly-Ala-Phe-O-Pam resin **4e** (0.0484 mmol), followed by treatment with H_2O alone for 2.5 h instead of TFA-H_2O (95:5) gave 37.43 mg of crude peptide. Purification by preparative reverse-phase HPLC (*condition D*) and lyophilization of the collected fractions afforded the peptide thioester **5e** (1.12 mg; 5%) accompanied by the orthoester Z-Gly-Ala-Phe-C(SEt)$_3$ **8e** (15.1 mg; 54%) by the ketene acetal Z-Gly-Ala-C(CH_2Ph)=C(SEt)$_2$ **9e** (1.50 mg; 6%) and by the deprotected Gly-Ala-Phe-SEt **10e** (not isolated, t_R 21.1 min; *condition A*). The peptide orthoester **8e** was characterized by analytical C-18 reversed-phase HPLC (t_R 28.3 min; >95% purity; *condition B*), by ESI-MS: observed 600.3 Da ($[M+Na]^+$; calculated average isotope composition for $C_{28}H_{39}N_3NaO_4S_3$, 600.8 Da ($[M+Na]^+$)) and by high resolution FAB: observed 600.200 Da ($[M+Na]^+$; exact mass for $C_{28}H_{39}N_3NaO_4S_3$, 600.200 Da ($[M+Na]^+$)). The ketene acetal Z-Gly-Ala-C(CH_2Ph)=C(SEt)$_2$ **9e** was characterized by analytical C-18 reversed-phase HPLC (t_R 39.8 min; >95% purity; *condition A*), by ESI-MS: observed 539.2 Da ($[M+Na]^+$; calculated average isotope composition for $C_{26}H_{33}N_3NaO_4S_2$, 538.7 Da ($[M+Na]^+$)).

Hydrolysis of the orthoester Z-Gly-Ala-Phe-C(SEt)$_3$ 8e by TFA-H_2O treatment. 10.0 mg of peptide orthoester **8e** (0.019 mmol) were stirred in 5.0 mL of a solution of TFA-H_2O (95:5) for 2.5 h at room temperature then evaporated (rotavap). The crude residue was purified by preparative C-18 reverse-phase HPLC (*condition D*) and the collected fractions were lyophilized to afforded the peptide thioester Z-Gly-Ala-Phe-SEt **5e** (5.1 mg; 57%) and the deprotected Gly-Ala-Phe-SEt **10e** (not isolated, t_R 21.1 min; *condition A*). The peptide thioester **5e** could be separated by normal-phase HPLC as a 97:3 mixture of LL:LD diastereoisomers (94% d.e.; LL: t_R 31.8 min and LD: t_R 34.3 min; *condition E*).