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A New Approach to the Chemical Synthesis of Keto-Proteins

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GENERAL

Peptide Synthesis. Peptides were synthesized by SPPS using *tert*-Butyloxycarbonyl (Boc) or 9-Fluorenylmethoxycarbonyl (Fmoc) protocols.[1] Peptides were synthesized on either –O-CH_2-Pam (phenylacetamidomethyl) PS-DVB based resin or a thioester-generating resin[2] using *in situ* neutralization protocol for Boc chemistry stepwise solid-phase peptide synthesis and by using established side-chain protection strategies. For Fmoc-based SPPS, a Wang linker was used to generate a peptide acid after cleavage in each case. The reaction scale was either 0.2 or 0.3 mmol. Dithiolane compounds **3-5** were incorporated by a single manual coupling step on an ABI 433 peptide synthesizer. The protected amino acid derivatives (**3-5**) (2.5 equiv), HBTU (2.5 equiv) and DIEA (5 equiv) were preactivated in NMP (1 mL) for 5 min, and then added to the resin along with more NMP (3.5 mL) for resin solvation and vortexed for 1 h. Completion of the coupling was assessed by ninhydrin test. Upon completion of the syntheses, the resins were washed in DCM and dried overnight *in vacuo*.

Peptide Deprotection and Cleavage. Boc-synthesized peptides were cleaved with anhydrous HF containing *p*-cresol (5% *v/v*, 20 mL per gram of resin) for 1 h at 0°C. Fmoc-synthesized peptides were cleaved with Reagent B (TFA/phenol/water/TIPS in ratio 88:5:5:2) for 2 h at room temperature. The crude peptides were isolated by precipitation with ether using standard protocols, dissolved in aqueous acetonitrile solutions and lyophilized to give a white powder.

Reversed-Phase HPLC. Reversed-phase high-performance liquid chromatography was performed on an analytical (C_4, 5 μm, 0.46 cm × 25 cm) or a preparative (C_4, 10 μm, 2.2 cm × 25 cm) column. Chromatographic separations were achieved using linear gradients of buffer B in A (A = 0.1% aqueous TFA; B = 90% ACN, 10% H_2O, 0.09% TFA), typically 5-95% over 35 min at a flow rate of 1 mL/min for analytical analysis and 5-65% over 60 min at 13 mL/min for preparative separations. The purity of the crude peptides was assessed by HPLC with absorbance detection at 214nm. Analytical and preparative HPLC fractions were characterized by ESMS and HPLC, combined and lyophilized to give white powders.

Mass Spectrometry. Mass spectra were acquired on a triple quadrupole mass spectrometer equipped with an Ionspray atmospheric pressure ionization source. Samples (10 μL) were injected into a moving solvent (10 μL/min; 50:50 ACN/0.05% TFA) coupled directly to the ionization source via a fused silica capillary interface (50 μm i.d. × 50 cm length). Sample droplets were ionized at a positive potential of 5 kV and entered the analyzer through an interface plate and subsequently through an orifice (100-

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[1] M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int. J. Pept. Protein Res.* **1992**, *40, 180.*

[2] Hojo, H.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1991**, *64*, 111-117; Hojo, H.; Kwon, Y.; Kakuta, Y.; Tsuda, S.; Tanaka, I.; Hikichi, K.; Aimoto, S. *Bull. Chem. Soc. Jpn.* **1993**, *66*, 2700-06.

120 μm diameter) at a potential of 80 V. Full scan mass spectra were acquired over the mass range 500-2200 Da with a scan step size of 0.1 Da. Molecular masses were derived from the observed *m/z* values. High-resolution mass analyses were acquired on a Bruker Daltonics 9.4T Fourier Transform Mass Spectrometer (FTMS) using external calibration; a 512k data set and zero fill to 1M words. The centroid of the peak was used to measure the mass.

Nuclear Magnetic Resonance. ^{1}H and ^{13}C NMR spectra were acquired on a JEOL Eclipse+ 400 spectrometer in $CDCl_3$, d_6-DMSO, or CD_3OD.

Determination of optical purity of amino acid derivatives. The method involves the hydrolysis in 6N HCl/H_2O, suitable derivatization of the free amino acids, and gas chromatographic separation of the enantiomers on CHIRASIL-VAL or Cyclodextrine derivatives. During hydrolysis however, racemization occurs and the amount of racemate determined represents the sum of racemate originally present in the amino acid derivative plus that generated during acidolysis. To determine the optical purity of the amino acids before hydrolysis, the residue is hydrolyzed in 6N DCl/D_2O whereby racemisation is accompanied by deuterium exchange in the α-C position. After exchange of labile deuterium for hydrogen, the amino acid(s) is derivatised (esterification and perfluoroacylation), and gas chromatographically separated on Chirasil-Val capillary using-EI-SIM-mass spectroscopy for detection. The relative amounts of D- and L-enantiomers originally present in the sample (before hydrolysis) are determined by monitoring the non-deuterated molecular ions or suitable fragment ions of both enantiomers. Optical purity is calculated as $\%D = (Area_D/Area_D+Area_L) \times 100$ and $EE = (Area_L-Area_D/Area_L+Area_D) \times 100$.

2-Methyl-1,3-dithiolane-2-propionic acid (DTL-OH), 1. The synthesis of DTL-OH was adapted previous methods[3,4] (Scheme 1). Ethyl levulinate (5.77g, 40 mmol), 1,2-ethanedithiol (5 mL, 60 mmol) and dichloromethane (150 mL) were added to an oven-dried flask (equipped with a rubber septum and a stirring bar) and stirred under a nitrogen atmosphere. Boron trifluoride etherate complex (2 mL) was added dropwise through a syringe over a few minutes and the resultant solution stirred for 24 h under nitrogen. The reaction mixture was then washed with 5% aqueous NaOH (50 mL), water (50 mL) and the solvent evaporated under reduced pressure to give a milky-white oil. The residue was taken up in 1.5M NaOH (50 mL) and heated, with stirring, at 85°C for 2 h. The initially milky solution became clear and colorless (can also be slightly yellow-tinged) and the progress of the ester hydrolysis was assessed by TLC. After cooling, the mixture was washed once with ether (100 mL) to extract impurities. The resulting aqueous layer was neutralized by the drop-wise addition of 37% HCl (about 6 mL) at 4°C to form a cloudy white suspension. Extraction with ether (3 x 100 mL), drying the organic layer over $MgSO_4$, filtration and evaporation of the solvent under reduced pressure gave the target product as a fine white powder (4.6g, 60%). Calc. for $C_7H_{12}O_2S_2$ 192.3 Da (avg.), Exp.

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[3] Westling, M.; Smith, R.; Livinghouse, T. *J. Org. Chem.* **1986**, *51*, 1159-1165.

[4] Mori, T.; Sawada, Y.; Oku, A. . *J. Org. Chem.* **2000**, *65*, 3620-3625.

mass $[M+H]^+$ = 193. *[1]H* NMR (400 MHz, CD_3OD) 1.74 (3H, s), 2.21-2.17 (2H, m including triplet *J* = 7.8 Hz), 2.53-2.57 (2H, m including triplet *J* = 7.8 Hz), 3.30-3.36 (4H, m); ^{13}C NMR (100 MHz, $CDCl_3$) 31.8, 32.9, 39.3, 65.9, 179.9.

2-Methyl-1,3-dithiolane-2-carboxylic acid (DTP-OH), 2. DTP-OH was synthesized from ethyl pyruvate (40 mmol scale) using a method analogous to that described above for the preparation of DTL-OH to give the target compound as a white powder (3.2 g, 48%). Calc. for $C_5H_8O_2S_2$ 164.3 Da (avg.), Exp. mass $[M+H]^+$ = 165. *[1]H* NMR (400 MHz, d_6-DMSO) 1.82 (3H, s), 3.42-3.46 (4H, m), 12.98 (1H, broad); *[13]C* NMR (100 MHz, d_6-DMSO) 26.9, 40.1, 63.5, 173.7.

Synthesis of N^{α}-tert-Butyloxycarbonyl-Lys(N^{ε}-DTL)-OH, 3, and N^{α}-tert-Butyloxycarbonyl-Lys(N^{ε}-DTP)-OH, 4. A general description of the syntheses is as follows: L-Lysine was dissolved in water and basic cupric carbonate [$CuCO_3Cu(OH)_2$] added and the solution refluxed. Filtration then washing of the filtrates with hot water gave copper complexed lysine as a light blue powder on evaporation. Either the DTL-OH **1** or DTP-OH **2** acid was dissolved in DMF with 1-hydroxybenzotriazole then dicyclohexylcarbodiimide added and the solution stirred for 30 min at 0°C. The copper complexed lysine was added in acetonitrile, DIEA added and the solution stirred for 3 h at room temperature. After filtration, the solvents were evaporated and the residue taken up in ethyl acetate, washed with sodium bicarbonate, water and dried over $MgSO_4$. The resulting copper-complex was dissolved in boiling water and a stream of hydrogen sulfide gas passed through the solution for 1 h. Additional boiling removed excess H_2S gas dissolved in the solution. HCl was added and the CuS precipitate removed by filtration. The Lys(N^{ε}-DTL) or Lys(DTP) product separated from the solution on cooling and was collected by filtration. The products were N^{α}–amino protected by reaction with Boc anhydride.[5]

***N^{α}-tert-Butyloxycarbonyl-Lys(N^{ε}-DTL)-OH*, 3.** Calc. for $C_{18}H_{32}N_2O_5S_2$ 420.59 Da (avg.), Exp. mass $[M+H]^+$ = 422; High-resolution FTMS: 421.18209 Da (M+H), (calc. monoisotopic for $C_{18}H_{33}N_2O_5S_2$ 421.18254, error 1.1ppm); M.P. 113-115°C; white crystalline solid, $[\alpha]_D$ = +9.35° (c=1.0, $CHCl_3$, 25°C), Rf 0.50 one spot (CH_2Cl_2:MeOH:HOAc, 10:1:0.1), Purity 97.5% (HPLC), ee = 99+% (<0.5% of D-isomer detected); ^{1}H NMR (400 MHz, d_6-DMSO) 1.30 (2H, m), 1.38 (9H, s), 1.50 (2H, m), 1.65 (2H, m), 1.69 (3H, s), 2.08-2.12 (2H, m), 2.26-2.30 (2H, m), 3.00 (2H, dd), 3.28-3.36 (4H, m), 3.84 (1H, m), 7.00 (1H, d), 7.83 (1H, t), 12.40 (1H, s); ^{13}C NMR (100 MHz, C*D*Cl_3) 22.5, 28.4, 28.9, 32.2, 32.7, 34.3, 39.4, 40.2, 40.5, 53.2, 66.3, 80.1, 155.9, 173.3, 175.2.

N^{α}-tert-Butyloxycarbonyl-Lys(N^{ε}-DTP)-OH, 4. Calc. for $C_{16}H_{28}N_2O_5S_2$ 392.54 Da (avg.), Exp. mass $[M+H]^+$ = 394; High-resolution FTMS: 415.13272 Da (M+Na), calc. monoisotopic for $C_{16}H_{28}N_2O_5S_2Na$ 415.13318, error 1.1ppm; M.P. 45-48°C; white powder, $[\alpha]_D$ = -2.93° (c=1.0, $CHCl_3$, 25°C), Rf 0.75 one spot (CH_2Cl_2:MeOH:HOAc, 10:1:0.1), Purity 99% (HPLC), ee = 99+% (<0.5% of D-isomer detected); ^{1}H NMR (400

[5] Tarbell *et al*., *Proc. Natl. Acad. Sci. USA*, **1972**, *69*, 730.

MHz, d_6-DMSO) 1.29 (2H, m), 1.38 (9H, s), 1.50 (2H, m), 1.62 (2H, m), 1.81 (3H, s), 3.05 (2H, dd), 3.34-3.42 (4H, m), 3.80 (1H, m), 7.00 (1H, d), 7.97 (1H, t), 12.38 (1H, s); ^{13}C NMR (100 MHz, C*D*Cl_3) 22.3, 28.4, 28.8, 29.0, 31.6, 39.8, 40.5, 53.3, 67.2, 80.3, 156.0, 171.6, 175.6.

N^{α}-(9-Fluorenylmethoxycarbonyl)-Lys(N^{ε}-DTP)-OH, 5. DTP-OH (0.984g, 6 mmol) was dissolved in DMF (3mL) then DCC (0.618g, 3 mmol) added and stirred at room temp for 1 h. The solution was filtered and N^{α}-Fmoc-Lys-OH (1.1g, 3 mmol) added, along with further DMF (3 mL) and stirring allowed to continue for 3 h. The solvent was evaporated under reduced pressure at 40^{o}C to give a yellow oil. This was taken up in chloroform (20 mL) and washed with (2 x 20 mL each) 1N HCl, water and brine then dried over $MgSO_4$. The solution was then evaporated to give a yellow oil and purified using a chromatotron (4-mm silica bed), eluting with a gradient of (0-5%) methanol/chloroform. The purest fractions were combined and evaporated to give an off-white solid. Recrystallization from EtOAc/hexanes gave the title compound as a white powder (0.927g, 63%). Calc. for $C_{26}H_{30}N_2O_5S_2$ 514.66 Da (avg.), Exp. mass, $[M+H]^+$ = 516; High-resolution FTMS: 515.16518 Da (M+H), (calc. monoisotopic for $C_{26}H_{31}N_2O_5S_2$ 515.16689, error 3.3ppm); M.P. 51-53^{o}C, ee = 99+% (0.1% of D-isomer detected); ^{1}H NMR (400 MHz, *d*-$CHCl_3$) 1.42 (2H, m, Hχ), 1.55 (2H, m, Hδ), 1.78 (2H, m, Hβ), 1.95 (3H, s), 3.22-3.27 (4H, m), 3.28-3.34 (2H, m, Hε), 4.19 (1H, t, Hα), 4.37 (3H, m), 5.71 (1H, d), 7.29 (2H, t), 7.38 (2H, t), 7.52-7.60 (3H, m, including d), 7.74 (2H, d); ^{13}C NMR (100 MHz, *d*-$CHCl_3$) 21.35, 22.15, 24.68, 25.12, 25.86, 28.55, 28.85, 31.55, 33.22, 39.83, 40.50, 40.79, 47.16, 53.68, 67.24, 73.18, 119.99, 125.15, 127.13, 127.75, 141.32, 143.72, 143.86, 156.42, 173.17, 175.67.

General procedures for the deprotection of the DTL and DTP groups. CAUTION: Mercury and silver salts and their solutions are highly toxic. All weighing and manipulation of these compounds should be carried out in a fume-hood. Nitrile gloves, lab-coat, safety-glasses and a face-shield are the minimum protective items required. All unused solutions, HPLC waste and solid waste from work-ups which use these salts must be disposed of in accordance with local, state and federal regulations.

Unmasking of DTL and DTP groups with $Hg(OAc)_2$. The following procedure was used for the unmasking of peptides **6**, **7** and **8**. The ketone function was regenerated using 10-15 eq. of $Hg(OAc)_2$ in 10% AcOH (or 50% aqueous ACN). Typically, the peptide was dissolved to concentration around 10 mg/mL. The reaction was rapid and completed within 30-60 min for peptides lacking cysteine, as determined by HPLC and ESMS analyses. These showed the characteristic retention time shift and observed mass for the unmasked product. Generally, the free keto-peptides eluted 1-5 min earlier on a 5-95%B/30 min gradient (peptide dependant) compared to the dithiolane-protected precursor. After completion of the unmasking reaction, β-mercaptoethanol (100 eq, 10-fold over Hg^{2+}) was added to the solution. A white precipitate formed almost immediately and stirring was continued for a further 30 min. The solution was diluted

with 10% aqueous acetonitrile (4.5 mL), filtered then subjected to preparative HPLC to isolate the free keto-peptide.

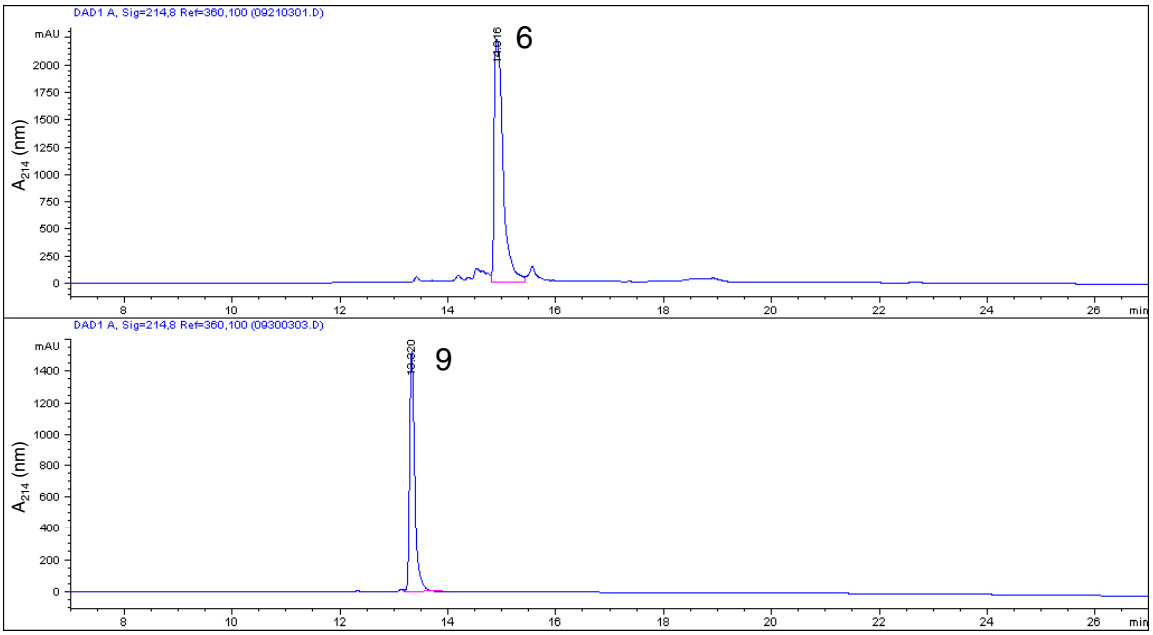


Figure. C_4 reversed phase HPLC analysis of DTL-peptides **6** (top trace) and the levulinic-peptide **9** (lower trace) after purification.

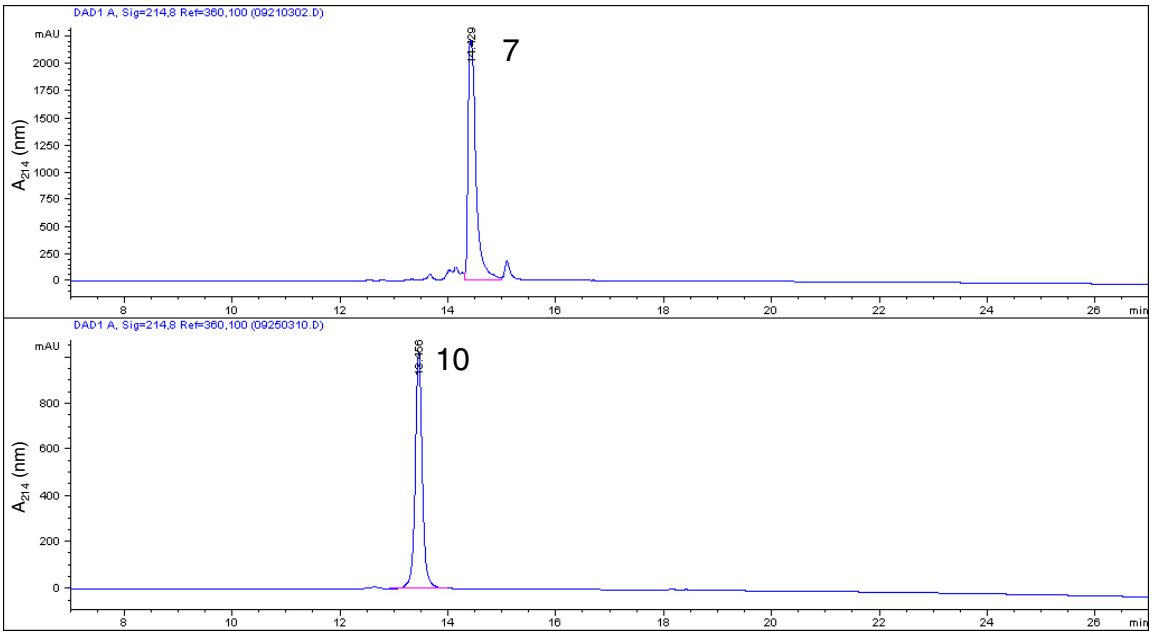


Figure. C_4 reversed phase HPLC analysis of DTP-peptides **7** (top trace) and the pyruvic-peptide **10** (lower trace) after purification.

Table: Summary of synthesized DTL- and DTP-peptides.

Compound	Peptide sequence	HPLC purity*	Calc. mass (Avg) Da	Exp. observed mass (Avg) Da	HPLC R_t (min.)**
6	YGGFMTSEK(DTL)SQTPLVT	>90%	1920	1921	14.9
7	YGGFMTSEK(DTP)SQTPLVT	>90%	1892	1893	14.5
8	AEK(DTP)ITKA	>95%	905	906	12.5
9	YGGFMTSEK(Lev)SQTPLVT	>90%	1844	1845	13.3
10	YGGFMTSEK(Pyr)SQTPLVT	>90%	1768	1769	13.5

* Percentage of crude product after cleavage or dithiolane deprotection; **analytical HPLC as previously described above.

*Unmasking of DTL-peptide **6** with AgOTf to give levulinic-peptide **9**:* DTL-Peptide **6**, YGGFMTSEK(N^{ε}-DTL)SQTPLVT, (19 mg, 10 μmol) was dissolved in 50% acetonitrile/water (0.5 mL) at 40°C and solid silver (I) trifluoromethansulfonate (257 mg, 1.0 mmol, 100 eq) added in small portions with vigorous stirring over 5 min until a clear solution was obtained at 40°C. The solution was stirred overnight in a sealed glass vial at this temperature. The solution was allowed to cool to room temperature then β-mercaptoethanol (0.28 ml, 4 mmol, 4 eq over Ag^+) added. A white precipitate formed almost immediately and stirring was continued for a further 30 min. The solution was diluted with 10% aqueous ACN (4.5 ml), filtered then subjected to preparative HPLC to isolate the free keto-peptide **9**, YGGFMTSEK(N^{ε}-Lev)SQTPLVT, after combination and lyophilization of the pure fractions, as a white solid (13 mg, 72%).

Synthesis of RANTES(1-68)[M67Lys$^{(DTL)}$], **13**. RANTES(1-33) thioester-peptide, SPYSSDTTPCCFAYIARPLPRAHIKEYFYTSGK (**11**), (50 mg, approx. 12μmol) was dissolved in 6M guanidine.HCl containing 0.2M sodium phosphate, pH 7.0 (9 mL). Separately, a solution of RANTES(34-68)[M67Lys$^{(DTL)}$], N-terminal cysteine-peptide CSNPAVVFVTRKNRQVCANPEKKWVREYINSLEK$^{(DTL)}$S, **12** (38 mg, approx. 9 μmol) was also dissolved in 6M guanidine.HCl containing 0.3M sodium phosphate, pH 7.0 (9 mL). Both solutions were mixed, thiophenol (0.1 mL) added and the resulting solution stirred vigorously overnight at room temperature. The progress of the reaction was followed by HPLC inspection of small aliquots of this solution and the end-point determined to be at 20 h. After this time, β-mercaptoethanol (1 mL) and tris(2-carboxyethyl)phosphine (0.2 g) were added and stirring continued for a further 12 h to form a clear solution. The crude reaction mixture was purified by preparative HPLC to give the reduced ligated protein, RANTES(1-68)[M67Lys$^{(DTL)}$] **13**, as a white fluffy powder in 54% yield (39 mg obtained, theoretical 72 mg). Calculated mass (Avg.) 8024 Da; Experimental mass, 8025 Da; Retention time (R_t) 16.0 min.

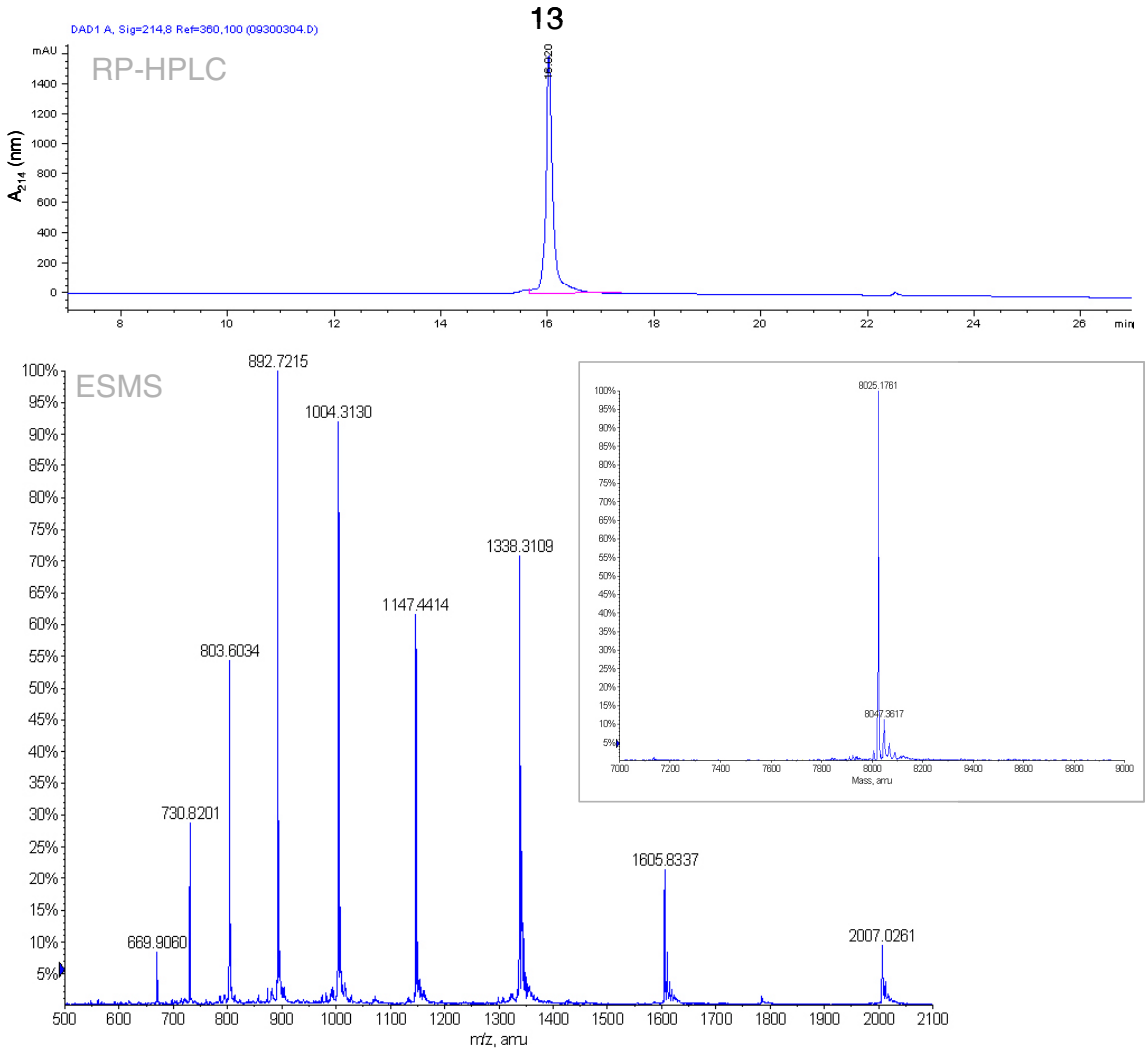
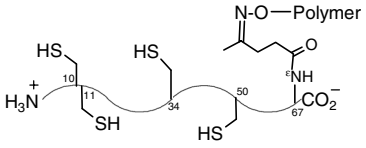


Figure. C-4 reversed phase HPLC (top) and ESMS (lower left and right middle) analysis of *RANTES(1-68)[M67Lys$^{(DTL)}$],* **13** after purification.

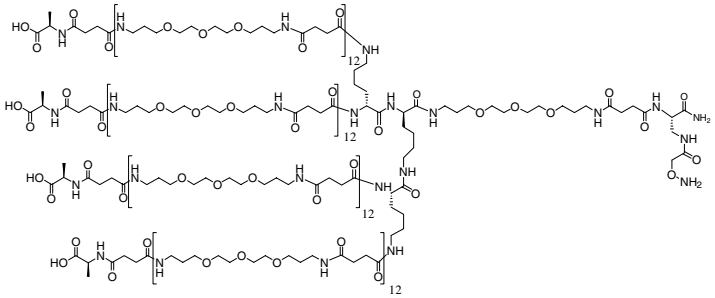
*Deprotection and Polymer-Conjugation of RANTES(1-68)[M67Lys$^{(DTL)}$] (reduced precursor to **15**).* The oximation reaction using the free intermediate keto-protein **14** derived from DTL-protein, RANTES(1-68)[M67Lys$^{(DTL)}$] **13**, with the branched polymer (BP, see structure below) was straightforward. These reactions were conducted in two ways: either by simple batch-wise methods, where the purified *RANTES(1-68)[M67Lys$^{(Levulinic)}$],* **12** (4 mg, approx. 0.5 µmol) and BP (8 mg, approx. 0.5 µmol) were mixed together at the highest possible concentrations in 50% ACN (0.25 ml) and incubated at 40°C for 24 h. The conversion to the polymer-conjugated full-length unfolded RANTES protein was around 40% (5 mg) after HPLC purification. The second method involved *in situ* deprotection of RANTES(1-68)[M67Lys$^{(DTL)}$] **13** (8 mg, 1.0 µmol) and oximation **of** the free intermediate keto-protein **14** with BP (16 mg, 1.0 µmol) in a solution of AgOTf (51 mg, 200 eq., 0.2 mmol) in 50% ACN (0.5 ml), again for 24 h. This *in situ* method generally gave cleaner results and higher yields, where the

unmasking to form the keto-protein and the oximation reaction were concurrent. The work-up for each method involved the addition β-mercaptoethanol (0.14 ml, 2.0 mmol, x10 excess over Ag^+) to the solution leading to the formation of a white precipitate. The solution/suspension was allowed to stand for 4 h at room temperature with occasional vortexing then the solution was diluted with 10% aqueous acetonitrile (4.5 mL), filtered then subjected to preparative HPLC. Using this method, the reduced, unfolded polymer-conjugate RANTES(1-68)[M67Lys$^{(Polymer)}$] (structure below) derivative was isolated in 62% yield (15 mg). Calculated mass (Avg.) 23,990 Da; Experimental mass, 23,991 Da; Retention time (R_t) 23.2 min.

RANTES(1-68)[M67Lys$^{(Polymer)}$]



Branched-Polymer Construct



Disulfide Folding of RANTES(1-68)[M67Lys$^{(Polymer)}$], ***15****.* For folding, a portion of the lyophilized linear conjugate (2.0 mg) was dissolved in buffer (1 mL) containing 2M Guanidine.HCl in 100 mM Tris, pH 8.0, containing cysteine (8 mM) and cystine (1 mM). The folding mixture was stirred gently at room temperature and the progress of the folding monitored by HPLC and ESMS. The folding was judged completed within 5 h, as evidenced by a pronounced shift in HPLC retention time, a sharpening of the peak shape and a change in the mass corresponding to the formation of two disulfide bonds. The mixture was acidified with glacial acetic acid and purified by semi-preparative HPLC. The final pooled material was analyzed by RP-HPLC and ESMS (results shown in Figure 2) and lyophilized to give the final folded product **15** (1.3 mg). Calculated mass (Avg.) 23,986 Da; Experimental mass, 23,987 Da; Retention time (Rt) 22.7 min.