Stereoelectronic Effects on Collagen Stability: The Dichotomy of 4-Fluoroproline Diastereomers

Jonathan A. Hodges and Ronald T. Raines
Department of Chemistry and Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

General Experimental. Commercial chemicals were of reagent grade or better, and were used without further purification. *N*-(*tert*-Butoxycarbonyl)-(2*S*,4*S*)-4-hydroxyproline benzyl ester (BocHypOBn, **1**) was synthesized as described previously.[S1] Anhydrous THF, DMF, and CH_2Cl_2 were obtained from a CYCLE-TAINER® solvent delivery system (Baker). Other anhydrous solvents were obtained in septum-sealed bottles. Flash chromatography was performed with silica gel 60, 230–400 mesh (Silicycle). Preparative HPLC was performed with a Varian Dynamax C-18 reversed-phase column. Analytical HPLC was performed with a Vydac C-18 reversed-phase column. Linear gradients of solvent A (H_2O with 0.1% v/v TFA) and solvent B (CH_3CN with 0.1% v/v TFA) were used. Both ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) were obtained with a Bruker AC+ 300 spectrometer at the University of Wisconsin–Madison Chemistry Instrument Center. All NMR samples were in $CDCl_3$ unless indicated otherwise.

Synthetic routes to *N*-[*N*-[*N*-(fluorenylmethoxycarbonyl)-(2*S*,4*R*)-4-fluoroprolyl]-2*S*-prolyl]-glycine (**10**) and *N*-[*N*-(*N*-*tert*-fluorenylmethoxycarbonyl-(2*R*,4*S*)-4-fluoroprolyl)-2*S*-prolyl]-glycine (**16**) are summarized in Scheme S1 and Scheme S2, respectively.

***N*-(*tert*-Butoxycarbonyl)-(2*R*,4*S*)-4-*p*-nitrophenoxyproline benzyl ester (2).** BocHypOBn (**1**; 4.95 g, 15.4 mmol) was dissolved in dry THF under Ar(g). After cooling the solution in a 0 ºC bath, PPh_3 (4.84 g, 18.5 mmol) and *p*-nitrobenzoic acid (3.07 g, 18.4 mmol) were added. This addition was followed by the slow (*ca*. 15-min) dropwise addition of a solution of DIAD (3.7 g, 18 mmol) in dry THF (10 mL). The resulting solution was stirred for 3 h and then concentrated to a yellow oil by rotary evaporation under reduced pressure. Adding chloroform to the oil resulted in the formation of colorless crystals that were isolated by vacuum filtration. TLC indicated that the crystals were not the desired product **2**, but most likely triphenylphosphine oxide. The remaining material was purified by flash chromatography (1.5% v/v CH_3OH in $CHCl_3$). Chromatography was repeated twice more to complete the separation of **2** from triphenylphosphine oxide and diisopropylhydrazine, and thus afford **2** (6.71 g, 14.3 mmol, 93%) as a colorless oil. ^{1}H NMR δ: 1.39 and 1.49 (s, 9H), 2.43–2.69 (m, 2H), 3.69–3.87 (m, 2H), 4.53 (dd, J = 1.5, 9.6, 0.6H), 4.68 (dd, J = 1.5, 9.2, 0.4H), 5.00–5.30 (m, 2H), 5.53–5.58 (m, 1H), 7.17–7.29 (m, 5H), 7.97–8.07 (m, 2H), 8.18 (dd, J = 1.8, 8.8, 2H).

***N*-(*tert*-Butoxycarbonyl)-(2*R*,4*S*)-4-hydroxyproline benzyl ester (3).** To a solution of **2** (6.5 g, 14 mmol) in dioxane (45 mL) was added a solution (110 mL) of $KHCO_3$ (0.5% w/v in 10% v/v aqueous methanol). After stirring for 14 h, the mixture was concentrated by rotary evaporation under reduced pressure, and the resulting oily solid was partitioned between $CHCl_3$ (100 mL) and water (50 mL). The aqueous layer was extracted further with $CHCl_3$ (2 × 20 mL), and the combined organic extracts were then washed with water (50 mL), dried over $MgSO_4$(s), and concentrated by rotary evaporation under reduced pressure. Flash chromatography (3% v/v CH_3OH in $CHCl_3$) provided 4.33 g of a yellow oil. Analysis by ^{1}H NMR revealed that the oil consisted of a 3.6:1 mixture of **3** and *N*-(*tert*-butoxycarbonyl)-(2*S*,4*S*)-4-hydroxyproline methyl

ester. Anticipating easier separation following fluorination, the mixture containing 3.6 g of **3** (11 mmol, 79%) and 0.75 g (3.0 mmol) of the methyl ester was used in the next step.

***N*-(*tert*-Butoxycarbonyl)-(2*S*,4*R*)-4-fluoroproline benzyl ester (4).** A mixture of **3** (3.6 g, 11 mmol) and *N*-(*tert*-butoxycarbonyl)-(2*S*,4*S*)-4-hydroxyproline methyl ester (0.75 g, 3.0 mmol) was dissolved in 30 mL dry CH_2Cl_2 under Ar(g). The solution was cooled to –78 ºC, and diethylaminosulfur trifluoride (DAST; 5.6 mL, 42 mmol) was than added by syringe. The mixture was stirred for an additional 45 min at –78 ºC and then allowed to warm to room temperature. After 20 h, the mixture was transferred to a separatory funnel and carefully washed with 80 mL of saturated $NaHCO_3$(aq). A considerable amount of gas was released during wash. The organic layer was washed with an additional 2 × 30 mL of saturated $NaHCO_3$(aq) and 30 mL of water, dried over $MgSO_4$(s), and concentrated to a dark oil by rotary evaporation at reduced pressure. Flash chromatography (hexanes/EtOAc 4:1) afforded **4** (1.98 g, 6.12 mmol, 55%) as an oily yellow solid. ^{1}H NMR δ: 1.36 and 1.47 (s, 9H), 1.95–2.22 (m, 1H), 2.49–2.69 (m, 1H), 3.51–4.00 (m, 2H), 4.41–4.58 (m, 1H), 5.07–5.32 (m, 3H), 7.26–7.44 (m, 5H).

***N*-(*tert*-Butoxycarbonyl)-(2*S*,4*R*)-4-fluoroproline (5).** To a solution of **4** (1.98 g, 6.12 mmol) in CH_3OH under Ar(g) was added 1.2 g of palladium on carbon (10% w/w, 1.1 mmol Pd). A balloon filled with H_2(g) was affixed to the reaction flask, and the black suspension was stirred under H_2(g) for 19 h. The mixture was filtered through a pad of Celite, and the colorless filtrate was concentrated to a colorless foam by rotary evaporation at reduced pressure. Compound **5** (1.37 g, 5.86 mmol, 96%) was used without further purification. ^{1}H NMR δ: 1.44 and 1.49 (s, 9H), 1.85–2.75 (m, 2H), 3.43–4.00 (m, 2H), 4.32–4.58 (m, 1H), 5.10–5.35 (m, 1H), 9.50 (br s, 1H).

***N*-(*tert*-Butoxycarbonyl)-(2*S*,4*R*)-4-fluoroproline succinimide ester (6).** *N*-Hydroxysuccinimide (0.74 g, 6.5 mmol) was added to a solution of **5** (1.37 g, 5.86 mmol) in dry dioxane (16 mL) under Ar(g). The mixture was cooled in a 0 ºC bath causing it to become much more viscous. A solution of DCC (1.21 g, 5.87 mmol) in dry dioxane (34 mL) was added. During addition, the mixture became too viscous to stir magnetically, so it was removed from the 0 ºC bath and allowed to warm as the remainder of the DCC solution was added. The reaction mixture was allowed to stir overnight at room temperature, and then placed in a freezer (–20 ºC) for 24 h to facilitate complete precipitation of DCU. After thawing the mixture, the viscous white suspension was filtered under reduced pressure to remove DCU. The filtrate was concentrated to a beige solid by rotary evaporation under reduced pressure. This solid was recrystallized in isopropanol to afford **6** (1.47 g, 4.44 mmol, 76%) as colorless needles. ^{1}H NMR δ: 1.49 (s, 9H), 2.32–2.57 (m, 1H), 2.77–2.90 (m, 5H), 3.52–3.72 (m, 1H), 3.94–4.08 (m, 1H), 4.69–4.84 (m, 1H), 5.15–5.38 (m, 1H).

***N*-[*N*-(*tert*-Butoxycarbonyl)-(2*S*,4*R*)-4-fluoroprolyl]-*2S*-proline (7).** Proline (0.55 g, 4.8 mmol) was added to 2.2 mL of a methanolic solution of benzyltrimethylammonium hydroxide (2.2 M), and the mixture was concentrated by rotary evaporation under reduced pressure. The flask containing the residual oil was flushed with Ar(g) before the addition of anhydrous DMF (50 mL) and **6** (1.45 g, 4.39 mmol). This solution was stirred under Ar(g) for 14 h, and then concentrated to an oil with an orange tint by rotary evaporation under reduced pressure. The oil was taken up in 5% w/v $KHCO_3$(aq) and washed with ethyl acetate. The aqueous layer was acidified to pH ~ 2 by addition of 2N HCl. The product was extracted with ethyl acetate, and the organic layer was dried over $MgSO_4$(s) and concentrated to a colorless foam by rotary evaporation under reduced pressure, affording **7** (1.18 g, 3.57 mmol, 81%).

^{1}H NMR δ: 1.40 and 1.45 (s, 9H), 2.01–2.34 (m, 5H), 2.43–2.61 (m, 1H), 3.54–3.98 (m, 4H), 4.57–4.72 (m, 2H), 5.14–5.38 (m, 1H), 8.35 (br s, 1H).

***N*-[*N*-[*N*-(*tert*-Butoxycarbonyl)-(2*S*,4*R*)-4-fluoroprolyl]-2*S*-prolyl]-glycine benzyl ester (8).** Glycine benzyl ester hydrochloride (0.76 g, 3.8 mmol) and HOBt (0.52 g, 3.8 mmol) were added to a solution of **7** (1.15 g, 3.48 mmol) in dry DMF (70 mL) under Ar(g). The colorless solution was cooled in a 0 ºC bath before adding DIPEA (0.67 mL, 3.8 mmol), which caused the solution to turn cloudy, and EDCI (0.74 g, 3.8 mmol). The mixture was allowed to stir at room temperature overnight and then concentrated by rotary evaporation under reduced pressure. The resulting oil was dissolved in ethyl acetate (100 mL) and washed with 50 mL each of 5% w/v $KHCO_3$(aq), 5% w/v $KHSO_4$(aq), and water. The organic layer was dried over $MgSO_4$(s) and concentrated to a colorless oil by rotary evaporation under reduced pressure. Flash chromatography (35% v/v CH_3CN in $CHCl_3$) afforded **8** (1.05 g, 2.20 mmol, 63%) as a colorless solid. ^{1}H NMR δ: 1.39, 1.41, and 1.46 (s, 9H), 1.67–2.61 (m, 6H), 3.47–4.35 (m, 6H), 4.49–4.75 (m, 2H), 5.05–5.35 (m, 3H), 7.25–7.59 (m, 5H).

***N*-[*N*-[*N*-(*tert*-Butoxycarbonyl)-(2*S*,4*R*)-4-fluoroprolyl]-2*S*-prolyl]-glycine (9).** Palladium on carbon (10% w/w, 0.41 g Pd/C, 0.39 mmol Pd) was added carefully to a solution of **8** (1.05 g, 2.19 mmol) in methanol (37 mL) under Ar(g). A balloon filled with H_2(g) was affixed to the reaction vessel, and the mixture was allowed to stir overnight under H_2(g). The mixture was filtered through a pad of Celite, and the filtrate was concentrated to a colorless solid by rotary evaporation under reduced pressure. The product **9** (0.83 g, 2.1 mmol, 98%) was used without further purification. ^{1}H NMR (CD_3OD) δ: 1.42, 1.44, and 1.46 (s, 9H), 1.92–2.73 (m, 7H), 3.50–4.08 (m, 6H), 4.49–4.73 (m, 2H), 5.14–5.38 (m, 1H).

***N*-[*N*-[*N*-(Fluorenylmethoxycarbonyl)-(2*S*,4*R*)-4-fluoroprolyl]-2*S*-prolyl]-glycine (10).** 4N HCl/dioxane (5 mL) was added under Ar(g) to **9** (0.83 g, 2.1 mmol), which dissolved quickly. After stirring for a few minutes, a sticky solid precipitated. After stirring for 1 h, a stream of air was used to evaporate the solvent and HCl. The sticky residue was dissolved in 18 mL of 10% w/v $NaHCO_3$(aq), forming a colorless solution that was cooled in a 0 ºC bath. A solution of FmocOSu (0.70 g, 2.1 mmol) in dioxane (10 mL) was then added. The reaction mixture was stirred for 22 h and then concentrated by rotary evaporation under reduced pressure. The residue was partitioned between 50 mL of 5% w/v $KHCO_3$(aq) and 40 mL of $CHCl_3$, which produced a viscous opaque suspension. The entire mixture was acidified with 2N HCl until the pH of the aqueous layer was lowered to 1.5 which resulted in dissolution of the suspension. The layers were separated, and the aqueous layer was extracted further with $CHCl_3$ (2 × 30 mL). The combined organic extracts were washed with brine (25 mL), dried over $MgSO_4$(s), and concentrated to a colorless foam at reduced pressure. Attempts to purify this foam (1.09 g) by recrystallization were unsuccessful. Preparatory HPLC (linear gradient: 20–50% v/v B over 30 min) was used to isolate **10** (0.19 g, 0.37 mmol, 18%) as a colorless solid. ^{1}H NMR δ: 1.54–2.66 (m, 7H), 3.55–4.80 (m, 11H), 5.22–5.48 (m, 1H), 7.20–7.82 (m, 8H); ESIMS (*m/z*): $[M + H]^+$ 510.

***N*-*tert*-Butoxycarbonyl-(2*R*,4*S*)-4-fluoroproline benzyl ester (11).** Morpholinosulfur trifluoride (morph-DAST; 3.5 mL, 29 mmol) was added by syringe to a solution of **1** (6.5 g, 20 mmol) in dry CH_2Cl_2 (200 mL) under Ar(g) at –78 °C. The solution was allowed to warm to room temperature and stirred overnight. Following a quench with methanol, the reaction mixture was washed with 2 × 100 mL saturated $NaHCO_3$(aq). An emulsion resulted, so the mixture was diluted with H_2O and CH_2Cl_2 and filtered through Celite. The biphasic filtrate was separated, and the organic layer was washed with water, dried over $MgSO_4$(s), and concentrated by rotary

evaporation under reduced pressure. The resulting oil was purified by flash chromatography (1.5% v/v MeOH in CH_2Cl_2), which afforded **11** (3.1 g, 9.6 mmol, 48%) as a yellow oil. An additional 2.32 g of **11** that were contaminated with a minor impurity, as indicated by TLC, were also obtained from the column. ^{1}H NMR δ: 1.35 and 1.46 (s, 9H), 2.20–2.58 (m, 2H), 3.54–3.93 (m, 2H), 4.43–4.65 (m, 1H), 5.05–5.31 (m, 3H), 7.28–7.39 (m, 5H); ^{13}C NMR δ: 28.1, 28.3, 36.5, 36.8, 37.3, 37.6, 52.7, 53.0, 53.1, 53.3, 57.4, 57.7, 66.9, 80.4, 90.0, 90.1, 92.3, 93.3, 128.0, 128.1, 128.3, 128.4, 128.5, 135.6, 153.6, 154.0, 171.2, 171.5; HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{17}H_{22}FNO_4Na$, 346.1431; found, 346.1446.

***N*-*tert*-Butoxycarbonyl-(2*S*,4*S*)-4-fluoroproline (12).** Methanol (95 mL) was carefully added to a mixture of **11** (2.8 g, 8.7 mmol) and palladium on carbon (10% w/w, 1.0 g, 0.94 mmol Pd) under Ar(g). A balloon filled with H_2(g) was affixed to the reaction flask, and the mixture was stirred overnight. After 24 h, unreacted starting material remained, so an additional 0.95 g (0.89 mmol Pd) of Pd/C were added and a fresh balloon filled with H_2(g) was placed on the flask. After stirring overnight, TLC indicated reaction had gone to completion, and the mixture was filtered through Celite. The filtrate was concentrated to a white solid by rotary evaporation under reduced pressure and dried under vacuum, affording **12** (1.8 g, 7.6 mmol, 87%), which was used without further purification. ^{1}H NMR (CD_3OD) δ: 1.44 and 1.48 (s, 9H), 2.34–2.64 (m, 2H), 3.53–3.78 (m, 2H), 4.38–4.48 (m, 1H), 5.11–5.33 (m, 1H); ^{13}C NMR δ: 28.5, 28.7, 37.3, 37.6, 38.0, 38.4, 53.9, 54.2, 54.3, 54.7, 58.6, 59.0, 81.6, 91.6, 92.6, 93.9, 94.9, 155.8, 175.3; HRMS-ESI (*m/z*): $[M - H]^-$ calcd for $C_{10}H_{15}FNO_4$, 232.0985; found, 232.0979.

***N*-(*N*-Fluorenylmethoxycarbonyl-2*S*-prolyl)-glycine benzyl ester (14).** To a solution of *N*-fluorenylmethoxycarbonyl-2*S*-proline (**13**) (3.37 g, 9.99 mmol) in 100 mL of dry CH_2Cl_2 under Ar(g) were added HOBt•H_2O (1.53 g, 9.99 mmol) and DCC (2.06 g, 9.99 mmol). Within minutes, the solution became viscous and opaque. After 20 min, glycine benzyl ester tosylate (3.65 g, 14.8 mmol) and DIPEA (1.7 mL, 9.8 mmol) were added to the mixture. The mixture was stirred for 6 h and then filtered under vacuum to remove DCU. More DCU precipitated from the filtrate, so it was filtered a second time. The filtrate was then washed with 100 mL each of 5% w/v $KHCO_3$(aq), 5% w/v $KHSO_4$(aq), and water. The organic layer was dried over $MgSO_4$(s), and concentrated by rotary evaporation under reduced pressure. The oily residue was purified by flash chromatography (hexanes/EtOAc 1:1.4) and then crystallized from EtOAc/hexanes, which afforded 2.74 g (5.65 mmol, 57%) of **14** as an off-white powder. ^{1}H NMR δ: 1.79–2.42 (m, 4H), 3.35–3.56 m, 2H), 3.76–4.52 (m, 6H), 5.14 (br s, 2H), 6.17–6.27 (br s, 0.4H), 7.03–7.15 (br s, 0.6H), 7.22–7.64 (m, 11H), 7.69–7.81 (m, 2H); HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{29}H_{28}N_2O_5Na$, 507.1876; found, 507.1907.

***N*-[*N*-(*N*-*tert*-Butoxycarbonyl-(2*S*,4*S*)-4-fluoroprolyl)-2*S*-prolyl]-glycine benzyl ester (15).** Diethylamine (6 mL, 60 mmol) was added with stirring to a solution of **14** (0.73 g, 1.5 mmol) in CH_3CN (50 mL). After 90 min, the solution was concentrated to an oil by rotary evaporation under reduced pressure. The reaction vessel was filled with Ar(g), and the oil was then dissolved in dry CH_2Cl_2 (25 mL). Compound **12** (0.35 g, 1.5 mmol), PyBOP (0.78 g, 1.5 mmol), and DIPEA (0.78 mL, 4.5 mmol) were then added. The resulting solution was stirred for 14 h and then concentrated by rotary evaporation under reduced pressure. The resulting oil was dissolved in EtOAc (100 mL) and washed with 5% w/v $KHSO_4$ (aq; 3 × 50 mL). The organic layer was then diluted with 30 mL CH_2Cl_2 to dissolve material that had oiled out and washed with water (50 mL). After drying over $MgSO_4$(s) and concentrating by rotary evaporation under reduced pressure, the resulting oil was purified by flash chromatography (3% v/v CH_3OH in CH_2Cl_2) affording **15** (0.57 g, 1.2 mmol, 80%). Chromatography did not

remove two minor impurities, as indicated by TLC, but crude **15** was used without further purification. ^{1}H NMR δ: 1.40 and 1.47 (s, 9H), 1.71–2.55 (m, 6H), 3.40–4.17 (m, 6H), 4.25–4.84 (m, 2H), 4.97–5.29 (m, 3H), 7.27–7.58 (m, 5.8H), 8.23–8.31 (m, 0.2H); HRMS-ESI (*m/z*): $[M + Na]^+$ calcd for $C_{24}H_{32}FN_3O_6Na$, 500.2173; found, 500.2197.

***N*-[*N*-(*N*-*tert*-Fluorenylmethoxycarbonyl-(2*S*,4*S*)-4-fluoroprolyl)-2*S*-prolyl]-glycine (16).** Crude **15** (0.57 g, 1.2 mmol) was mixed with palladium on carbon (10% w/w, 0.23 g, 0.22 mmol Pd) under Ar(g). Methanol (20 mL) was carefully added to the mixture. A balloon filled with H_2(g) was affixed to the reaction vessel, and the mixture was stirred overnight (17 h). The mixture was filtered through Celite, and the filtrate was concentrated to a solid by rotary evaporation under reduced pressure. The mass of crude BocflpProGly was 0.43 g (1.1 mmol). A solution of 4 N HCl in dioxane (5 mL) was added to the solid, causing it to dissolve. Within a few minutes, a sticky white solid began to form on the sides of the flask. After 2 h, the mixture was concentrated by rotary evaporation under reduced pressure, taken up in dioxane (~10 mL), and concentrated again by rotary evaporation under reduced pressure. The colorless residue was dissolved in water (6 mL), and then dioxane (12 mL) was added to the solution. To the stirred mixture was added FmocOSu (0.37 g, 1.1 mmol) followed by $NaHCO_3$ (0.28 g, 3.3 mmol). After stirring for 13 h, the mixture was concentrated by rotary evaporation under reduced pressure, and the resulting oil was dissolved in 5% w/v $KHSO_4$ (aq; 90 mL). The solution was washed with ethyl acetate (3 × 35 mL) and then acidified by addition of 2N HCl until a pH ~ 2 was obtained. The product was then extracted with CH_2Cl_2 (3 × 35 mL). The organic layer was washed with 5% w/v $KHSO_4$ (aq; 2 × 20 mL) and then concentrated to an oil by rotary evaporation under reduced pressure. Trituration with methanol resulted in a white solid that was recrystallized from methanol providing **16** as colorless needles (0.36 g, 0.71 mmol, 59%). ^{1}H NMR (d_6-DMSO) δ: 1.72–2.37 (m, 5H), 2.45–2.74 (m, 1H), 3.20–3.87 (m, 6H), 3.94–4.66 (m, 5H), 5.10–5.45 (m, 1H), 7.24–7.47 (m, 4H), 7.52–7.72 (m, 2H), 7.84–7.94 (m, 2H), 7.98–8.07 (m, 0.8H), 8.23–8.30 (m, 0.1 H), 8.45–8.52 (m, 0.1H); ^{13}C NMR (d_6-DMSO) δ: 24.3, 28.9, 34.9, 35.1, 35.8, 36.1, 40.6, 46.2, 46.4, 46.6, 46.8, 52.7, 53.0, 53.3, 53.6, 56.6, 57.1, 59.2, 59.4, 66.4, 66.8, 89.5, 90.5, 91.8, 92.8, 120.1, 120.2, 124.9, 125.2, 127.1, 127.2, 127.7, 127.7, 140.7, 140.7, 143.7, 143.8, 143.8, 143.9, 153.7, 169.8, 169.0, 171.2, 171.8, 171.9; HRMS-ESI (*m/z*): $[M - H]^-$ calcd for $C_{27}H_{27}FN_3O_6$, 508.1884; found, 508.1872.

Attachment of FmocflpProGly (16) to 2-chlorotrityl resin. Under Ar(g), 160 mg (0.17 mmol) of 2-chlorotrityl resin (loading: 1.04 mmol/g) were swelled in dry CH_2Cl_2 (2 mL). A solution of **16** (52 mg, 0.10 mmol) and DIPEA (0.070 mL, 0.40 mmol) in dry CH_2Cl_2 (1.6 mL) was added by syringe. An additional 1 mL of dry CH_2Cl_2 was used to ensure complete transfer. After 90 min, 1 mL of anhydrous CH_3OH was added to the mixture to cap any remaining active sites on the resin. The resin-bound peptide was isolated by gravity filtration, washed with several portions of dry CH_2Cl_2 (~10 mL), and dried at reduced pressure over KOH. The mass of the resin-bound peptide was 193 mg. Loading was measured by ultraviolet spectroscopy[S2] to be 0.46 mmol/g.

Attachment of FmocFlpProGly (10) to 2-chlorotrityl resin. Tripeptide **10** was loaded onto 2-chlorotrityl resin in similar fashion to that described for **16**.

Synthesis of $(FlpProGly)_7$ and $(flpProGly)_7$. The two 21-mer peptides were synthesized by segment condensation of the corresponding Fmoc-tripeptides (**10** and **16**) on solid phase using an Applied Biosystems Synergy 432A Peptide Synthesizer at the University of Wisconsin–Madison Biotechnology Center. The first trimer was loaded onto resin as described above. Fmoc-deprotection was accomplished by treatment with 20% (v/v) piperidine in DMF. The

trimers were converted to active esters by treatment with HBTU, DIPEA, and HOBt. All couplings were allowed to occur for 45–60 min at room temperature.

Preparative HPLC was used to purify both peptides, $(FlpProGly)_7$ (gradient: 15% B to 45% B over 40 min) and $(flpProGly)_7$ (gradient: 10% B to 50% B over 45 min). Analysis by analytical HPLC (gradient: 10% B to 50% B over 60 min) and MALDI-TOF determined both peptides to be ≥90% pure.

Circular dichroism of $(FlpProGly)_7$ and $(flpProGly)_7$. The peptides were dried under vacuum for at least 48 h before weighing and dissolving in 50 mM acetic acid. Concentrations were confirmed by measuring the absorbance of the solutions at 214 nm ($\varepsilon = 6.4 \times 10^4$ M^{-1} cm^{-1}).[S3] The solutions were allowed to incubate at ≤4 °C for at least 24 h before their CD spectra were acquired using an Aviv 62A DS Circular Dichroism Spectrometer at the University of Wisconsin–Madison Biophysics Instrumentation Facility (http://www.biochem.wisc.edu/bif). Spectra were measured with a 1 nm bandpass. During the denaturation experiments, CD spectra were acquired at intervals of 3 °C. At each temperature, the solutions were allowed to equilibrate for a minimum of 5 min before data was collected.

Sedimentation equilibrium of $(FlpProGly)_7$ and $(flpProGly)_7$. Sedimentation equilibrium experiments were performed with a Beckman XL-A Analytical Ultracentrifuge at the University of Wisconsin–Madison Biophysics Instrumentation Facility (http://www.biochem.wisc.edu/bif). Samples were diluted to approximately 0.1 mM in 50 mM acetic acid, and then allowed to equilibrate at ≤4 °C for at least 24 h. Double-sector charcoal-filled Epon centerpieces with 3-mm path lengths were used. (The short path lengths reduce the total absorbance of the acetic acid buffer.) Equilibrium data were collected at multiple speeds at both 4 and 37 °C. Gradients were monitored at 220 nm. A partial specific volume of 0.684 cm^3/g was calculated by composition with a correction for the fluorine atoms.[S4,S5] Solvent densities of 1.000528 and 0.993546 g/mL at 4 and 37 °C, respectively, were measured by an Anton Paar DMA5000 density meter. Data were analyzed with programs written for IgorPro (Wavemetrics) by Dr. Darrell R. McCaslin (University of Wisconsin–Madison Biophysics Instrumentation Facility).

A semilog plot of the absorbance versus the square of the distance from the center of rotation is shown in Figure S1. In this plot, the slope at any point is proportional to molecular weight. A single ideal species is characterized as a single molecular weight at all speeds and concentrations. At both temperatures, $(FlpProGly)_7$ appears to be a monomer. The apparent molecular weights calculated from these plots are lower than expected (calcd MW 1903, vide infra) but are more consistent with a monomeric species than a complex. At 4 °C, $(flpProGly)_7$ appears to exist as a complex. From these plots, its apparent molecular weight is 2.7-fold greater than that of $(FlpProGly)_7$. Thus, $(flpProGly)_7$ appears to be a trimer at 4 °C, consistent with CD data (Figure 1). The presence of a small amount of monomeric $(flpProGly)_7$ at 4 °C could make the observed mass appear to be 2.7- rather than 3.0-fold greater than that of $(FlpProGly)_7$. Raising the temperature to 37 °C causes a reduction in the molecular weight of $(flpProGly)_7$ to one similar to that of $(FlpProGly)_7$ at either 4 or 37 °C. These data indicate that the $(flpProGly)_7$ trimer unfolds to monomers at ≤37 °C, again consistent with CD data (Figure 1).

All of the apparent molecular weights are somewhat lower than expected. This discrepancy can be attributed to several factors. First, the estimation of the partial specific volume (0.684 cm^3/g) could be incorrect, thus lowering the apparent molecular weights. Second, both peptides display non-ideal sedimentation behavior, which likely results from their shape being rod-like rather than spherical. Regardless, the sedimentation equilibrium data indicate that

$(flpProGly)_7$ but not $(FlpProGly)_7$ forms a triple helix at 4 °C, in gratifying agreement with the CD data.

References

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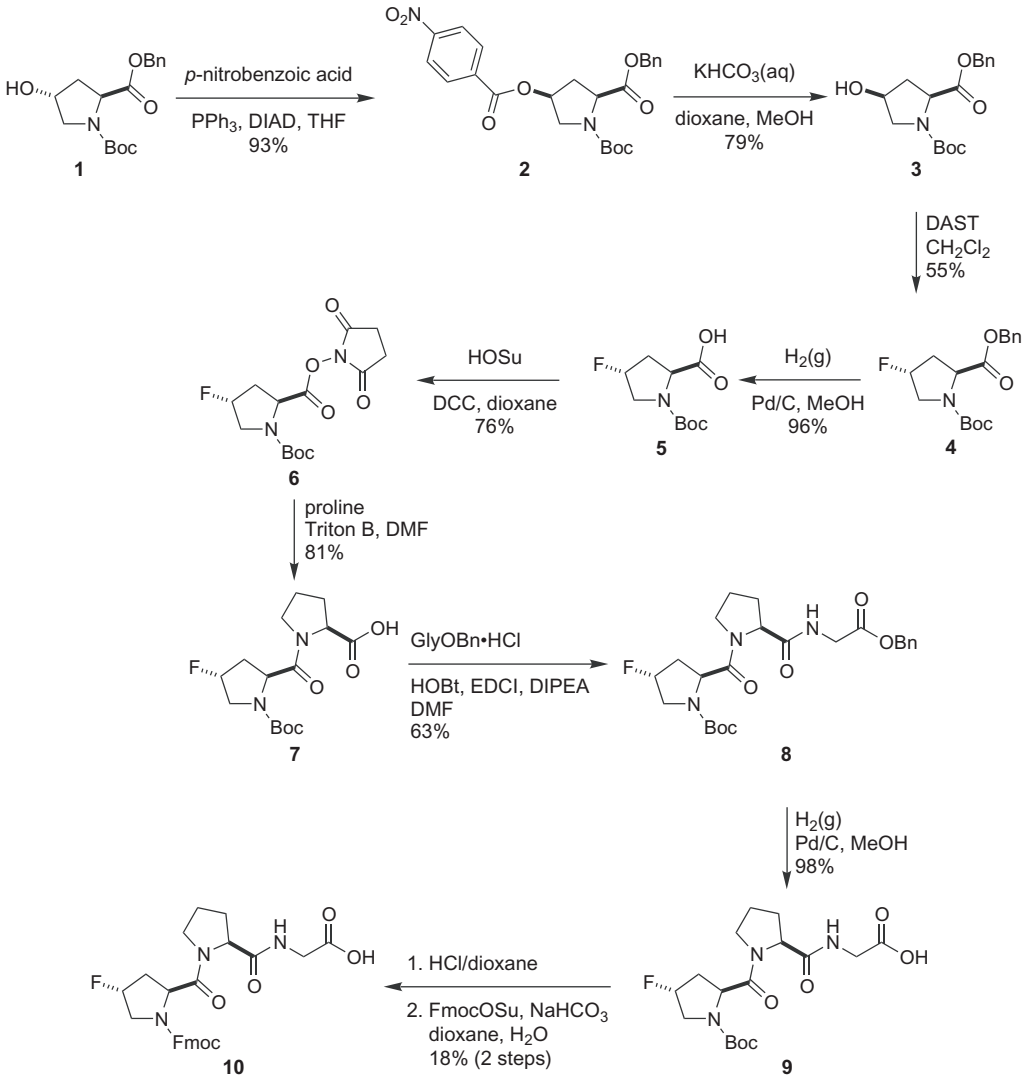
(S2) Applied Biosystems. *Determination of the Amino Acid Substitution Level* via *an Fmoc Assay*; Technical Note 123485 Rev 2; Documents on Demand–Applied Biosystems Web Page. http://docs.appliedbiosystems.com/search.taf (accesssed April 2003).

(S3) Bretscher, L. E.; Jenkins, C. L.; Taylor, K. M.; DeRider, M. L.; Raines, R. T. *J. Am. Chem. Soc.* **2001**, *123*, 777–778.

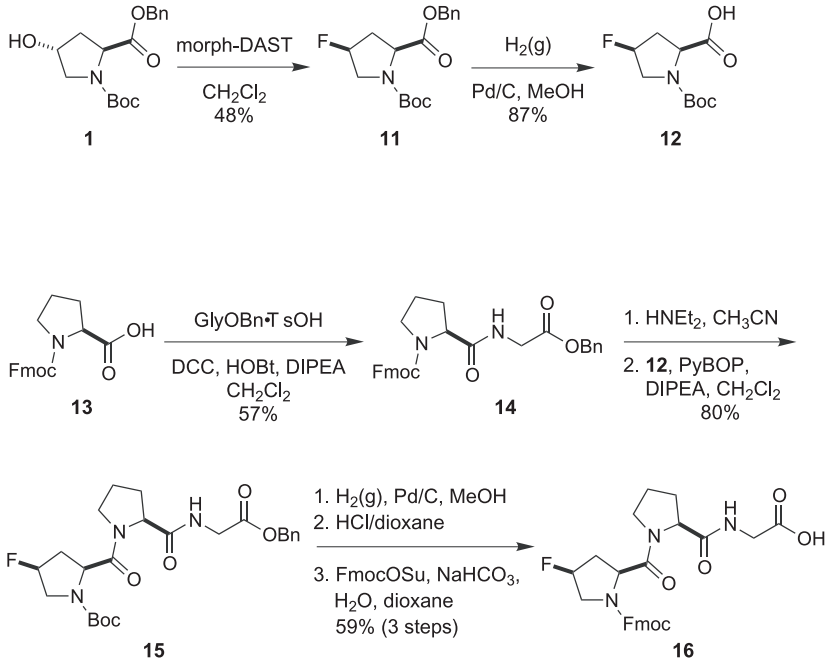
(S4) Reynolds, J. A.; McCaslin, D. R. *Methods Enzymol.* **1985**, *117*, 41–53.

(S5) Durchschlag, H.; Zipper, P. *Prog. Colloid Polymer Sci.* **1994**, *94*, 20–39.

Scheme S1


p-nitrobenzoic acid
PPh3, DIAD, THF
93%
KHCO3(aq)
dioxane, MeOH
79%
DAST
CH2Cl2
55%
H2(g)
Pd/C, MeOH
96%
HOSu
DCC, dioxane
76%
proline
Triton B, DMF
81%
GlyOBn•HCl
HOBt, EDCI, DIPEA
DMF
63%
H2(g)
Pd/C, MeOH
98%
1. HCl/dioxane
2. FmocOSu, NaHCO3
dioxane, H2O
18% (2 steps)
1
2
3
4
5
6
7
8
9
10

Scheme S2


HO
OBn
O
N
Boc
1
morph-DAST
CH2Cl2
48%
F
11
H2(g)
Pd/C, MeOH
87%
OH
12
Fmoc
13
GlyOBn•T sOH
DCC, HOBt, DIPEA
CH2Cl2
57%
H
14
1. HNEt2, CH3CN
2. 12, PyBOP,
DIPEA, CH2Cl2
80%
15
1. H2(g), Pd/C, MeOH
2. HCl/dioxane
3. FmocOSu, NaHCO3,
H2O, dioxane
59% (3 steps)
16

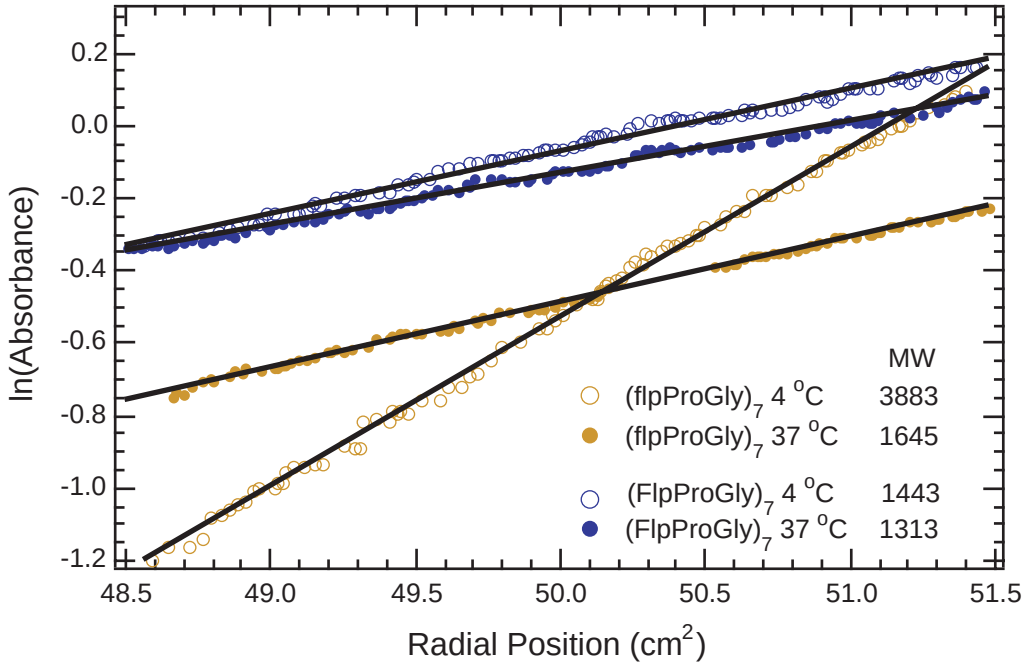


Figure S1. Sedimentation equilibrium data for $(flpProGly)_7$ (gold circles) and $(FlpProGly)_7$ (blue circles) at a rotor speed of 40,000 rpm. Equilibrium data were collected at 4 °C (open circles) and 37 °C (filled circles). Gradients were monitored at 220 nm. Apparent molecular weights determined from these data, assuming a partial specific volume of 0.684 cm^3/g, are shown.