Supporting Information

Synthesis and Kinetic Analysis of Two Conformationally Restricted Peptide Substrates of *Escherichia coli* Penicillin-binding Protein 5

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Figure S1. Models of the cyclic peptides **5** (structure on left) and **6** (structure on right) non-covalently bound at the active site of EcPBP6.

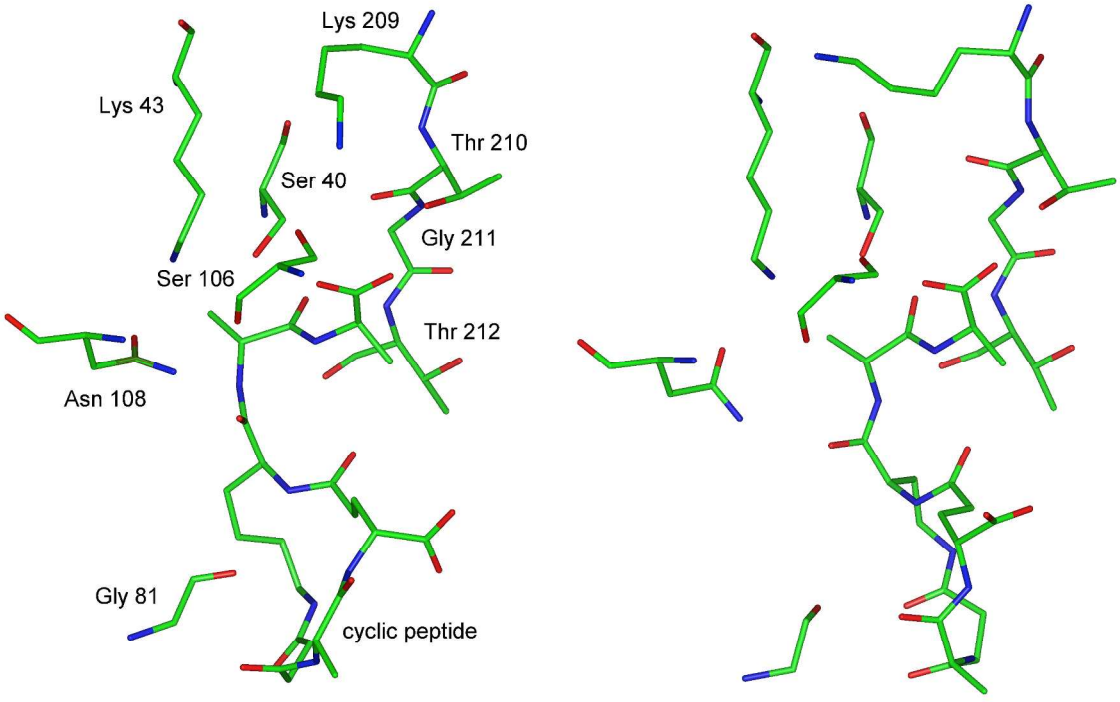


Figure S2. Superimposition of a model of **5** bound to EcPBP6 (thick bonds) on that of **5** bound to EcPBP6 (thin bonds). This diagram was achieved by superimposition of the α-C of the conserved active site residues (see main text, Figure 2). Ser 40 (44) and the cyclic peptides are shown in atomic colors.

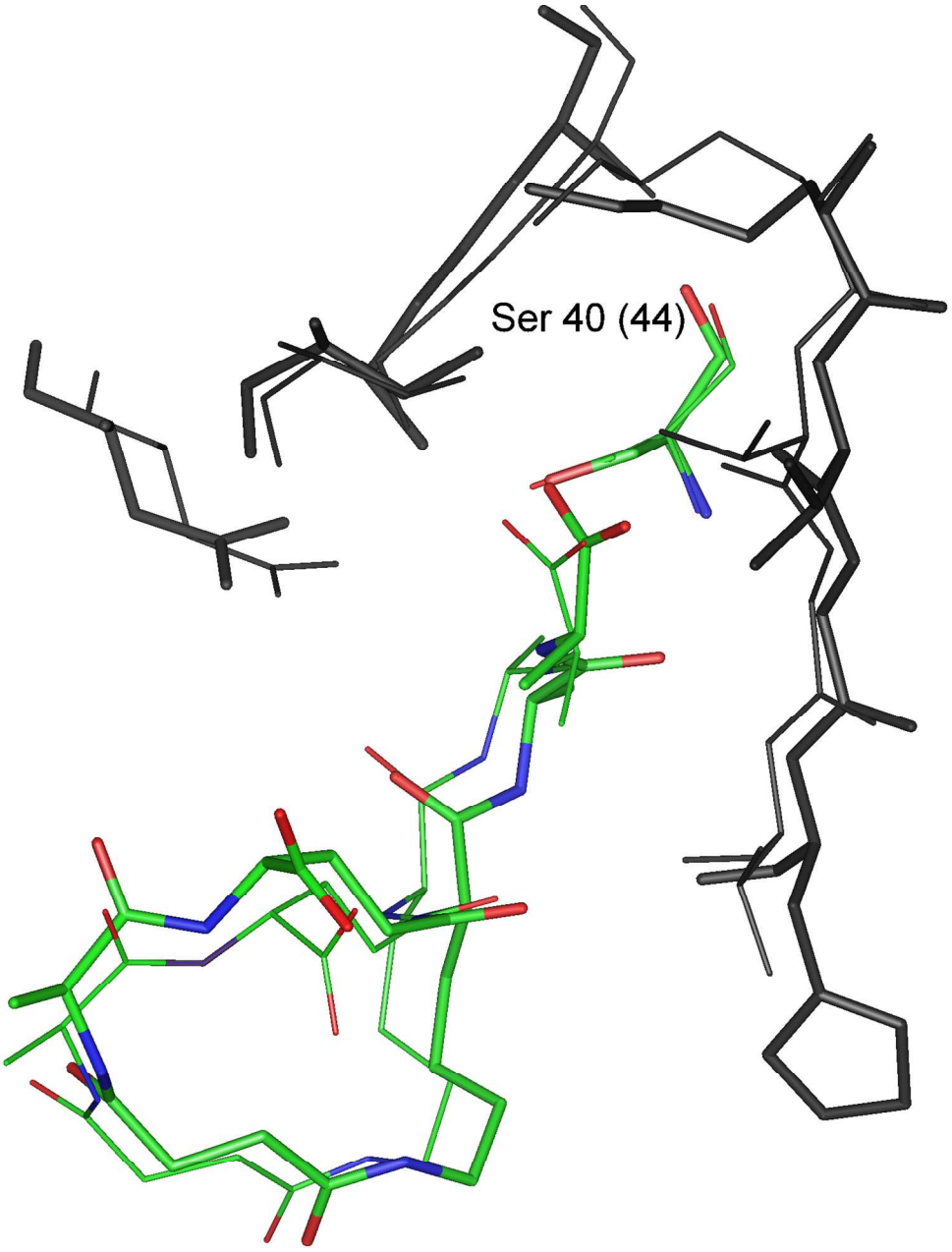


Figure S3a. NOESY spectrum of cyclic peptide **5** in 10% D_2O/H_2O v/v at 25° C. Correlations between the NH and α-CH regions are shown.

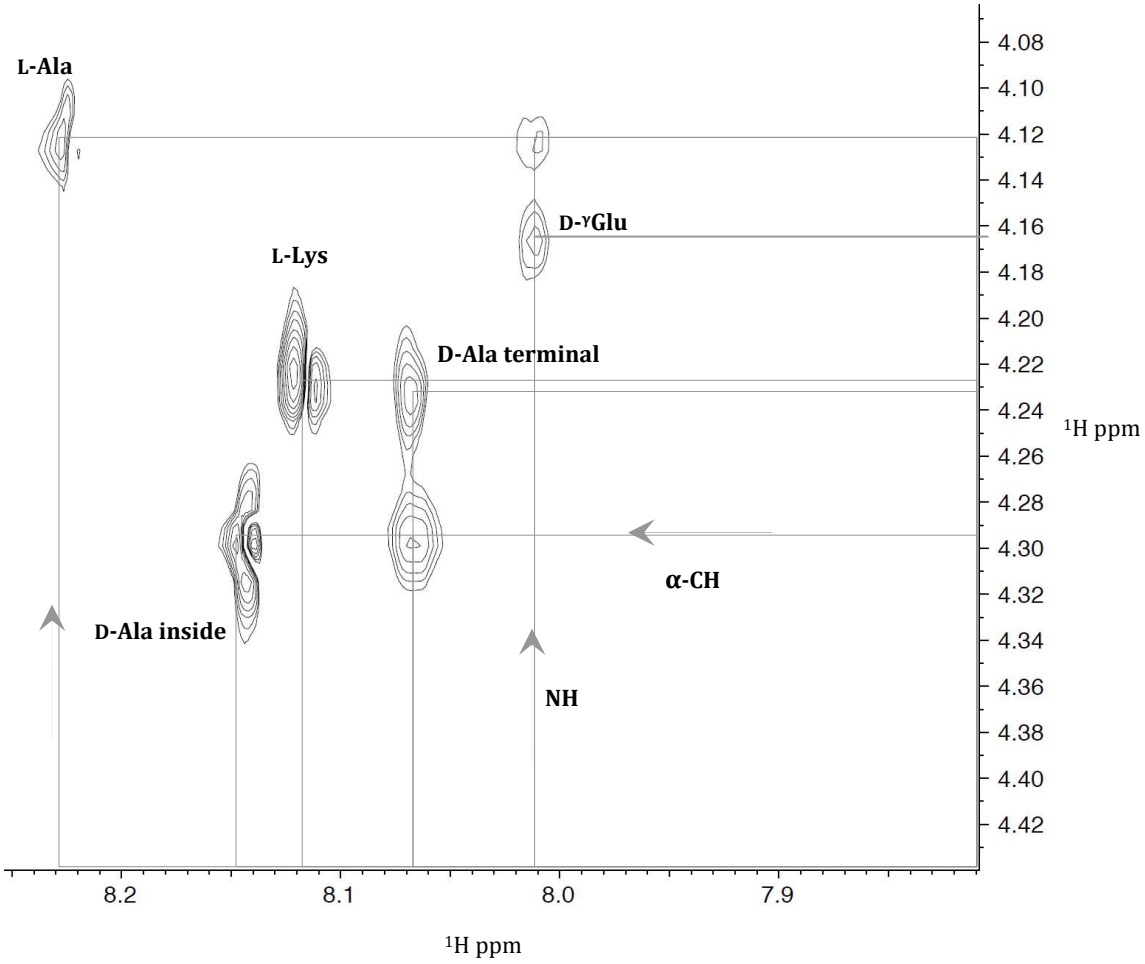


Figure S3b. NOESY spectrum of cyclic peptide **5** in 10% D_2O/H_2O v/v at 25^o C. Correlations between the NH and CH_2 regions are shown.

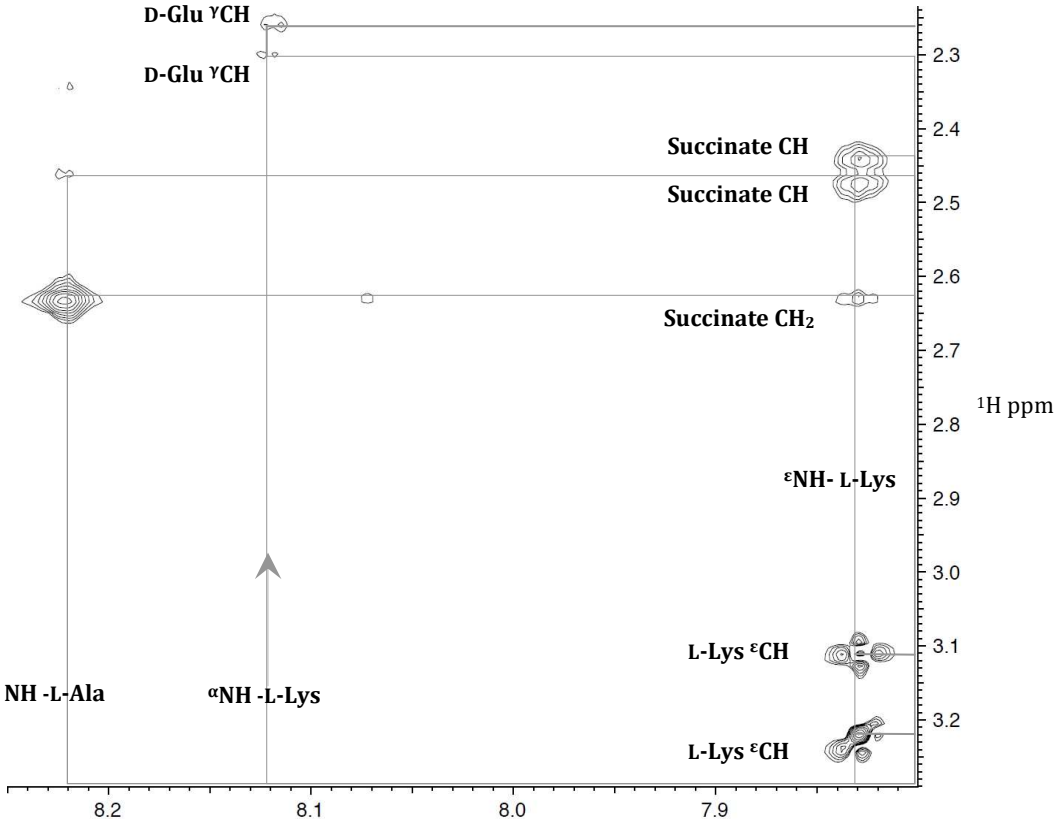


Figure S4a. NOESY spectrum of cyclic peptide **6** in 10% D_2O/H_2O v/v at 25° C. Correlations between the NH and α-CH regions are shown.

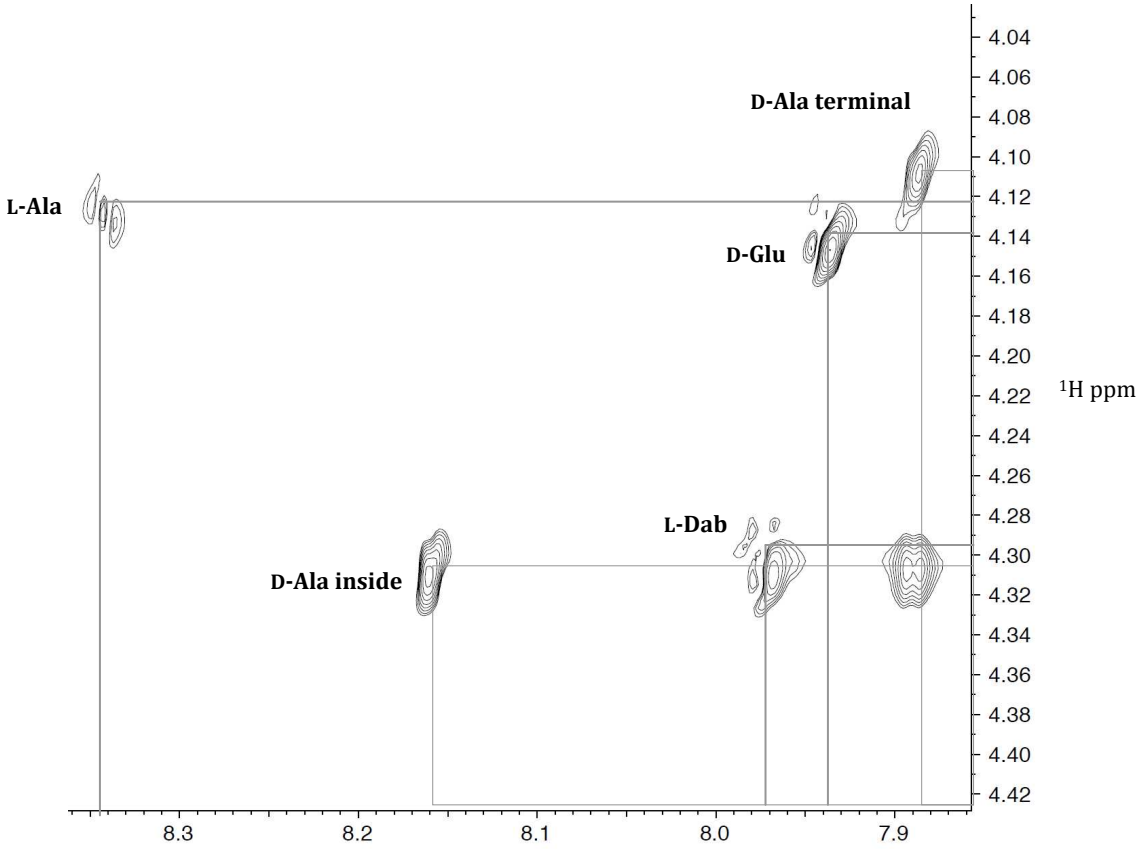


Figure S4b. NOESY spectrum of cyclic peptide **6** in 10% D_2O/H_2O v/v at 25° C. Correlation between the NH and CH_2 regions are shown.

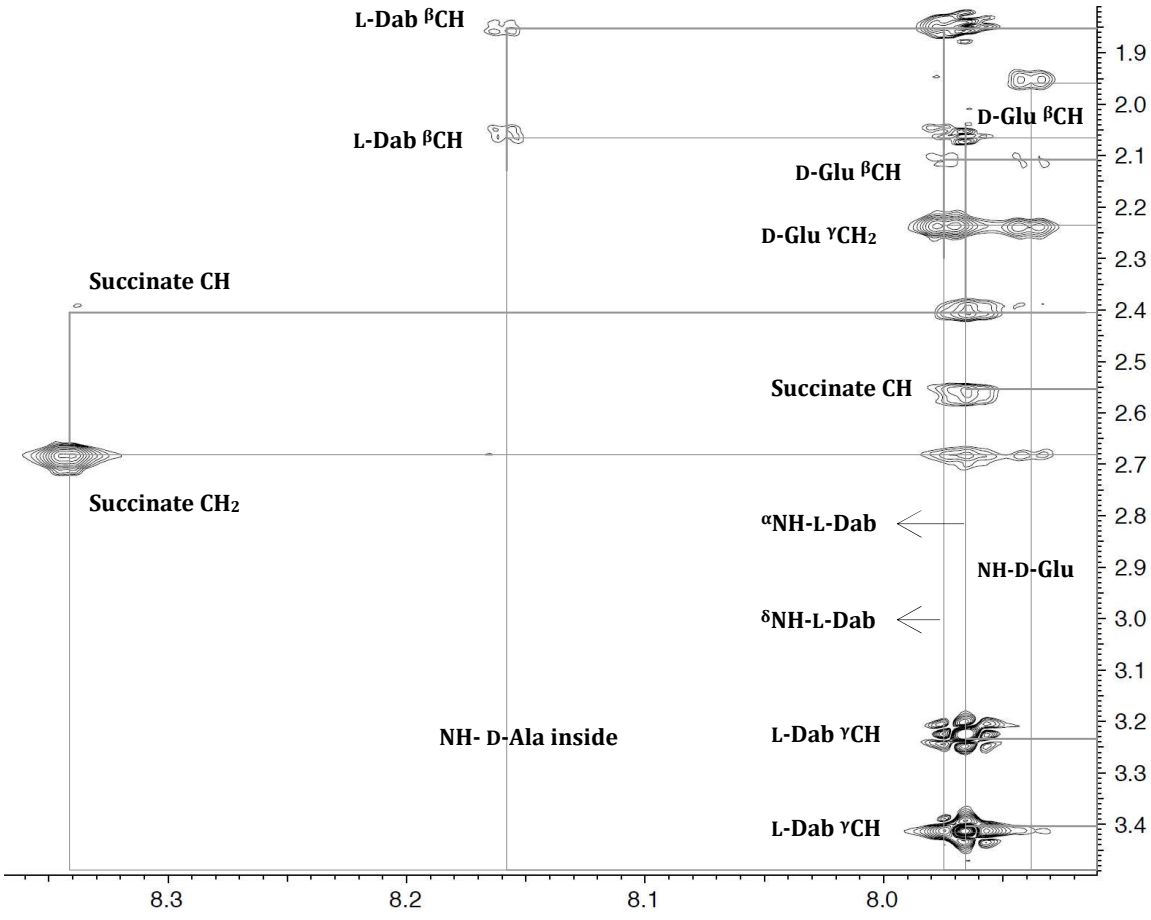


Figure S4c. NOESY spectrum of cyclic peptide **6** in 10% D_2O/H_2O v/v at 25° C. Correlations between the NH resonances are shown.

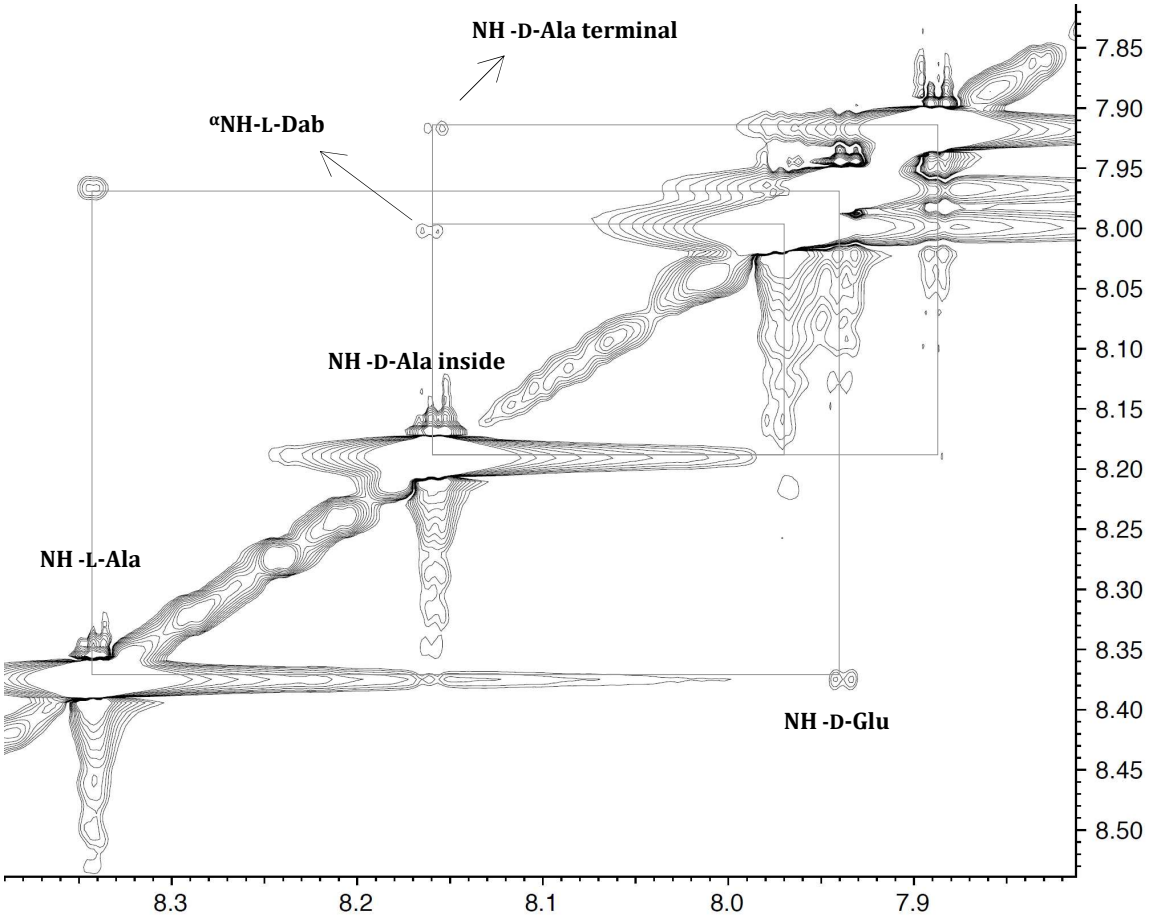


Figure S4d. TOCSY spectrum of cyclic peptide **6** in D_2O at 25° C. Colored lines represent intra-residue correlations.

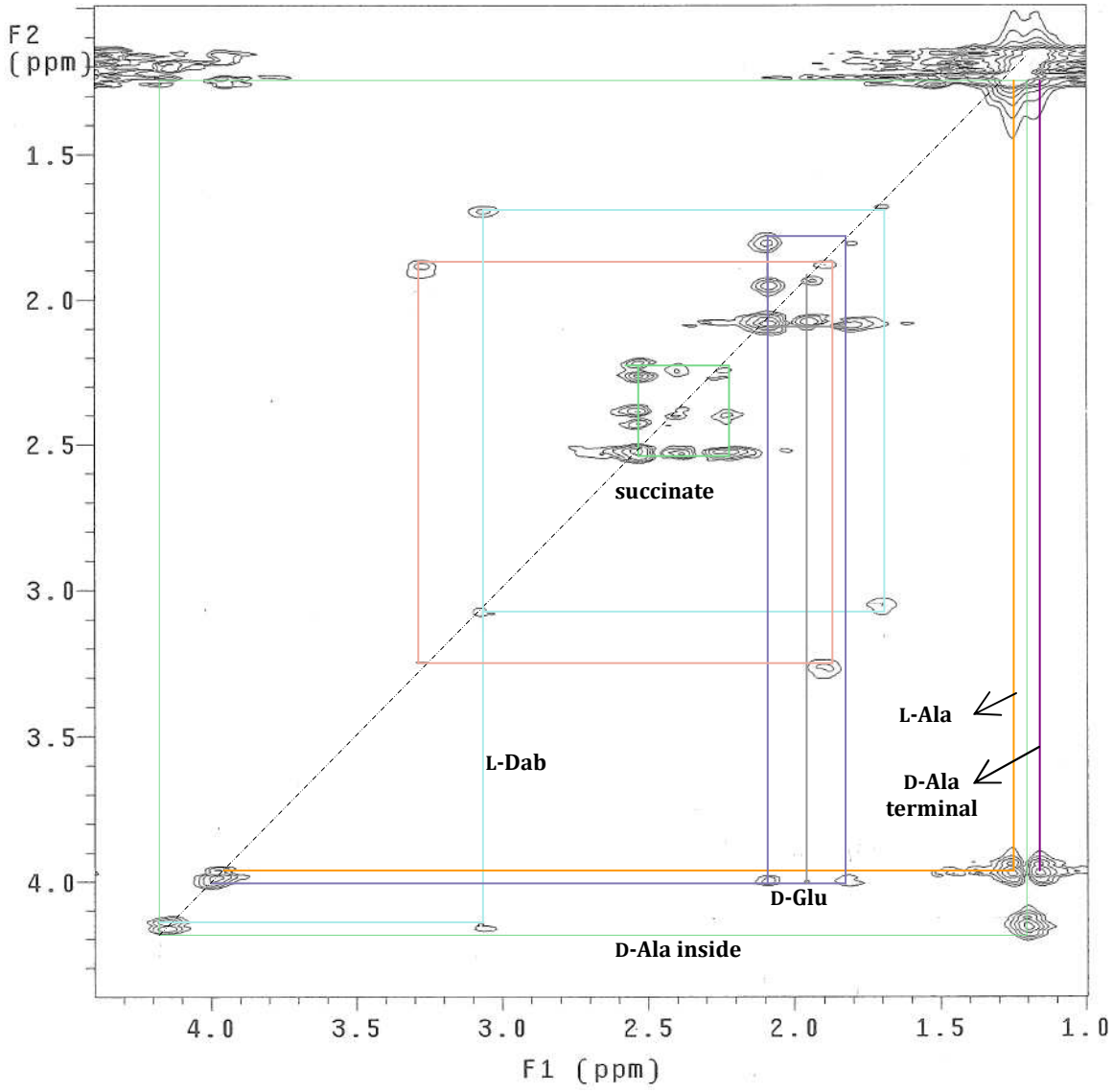


Figure S4e. COSY spectrum of **6** in 10% D_2O/H_2O v/v at 25° C. The aliphatic region is shown. Colored lines represent intra-residue correlations.

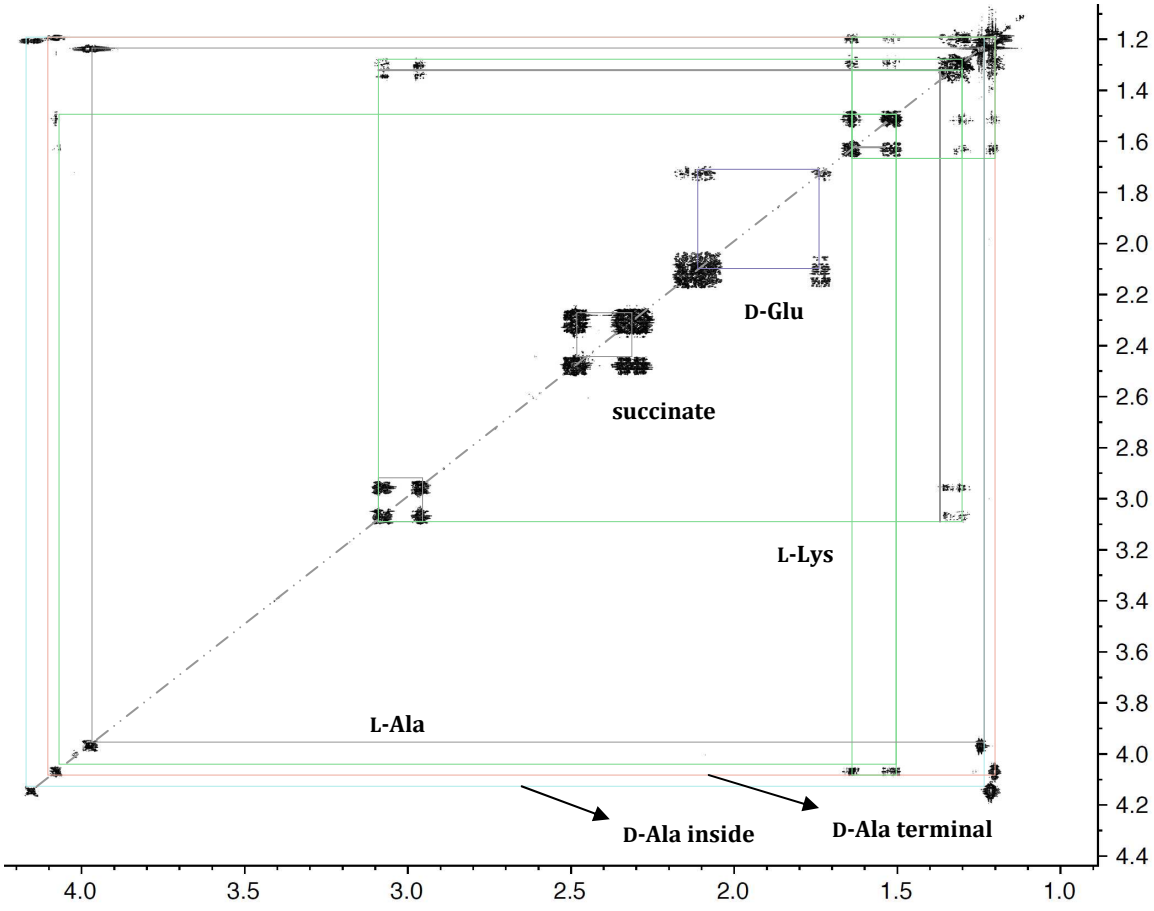


Figure S4f. COSY spectrum of **6** in 10% D_2O/H_2O v/v at 25° C. Correlations among NH and aliphatic CH instances are shown.

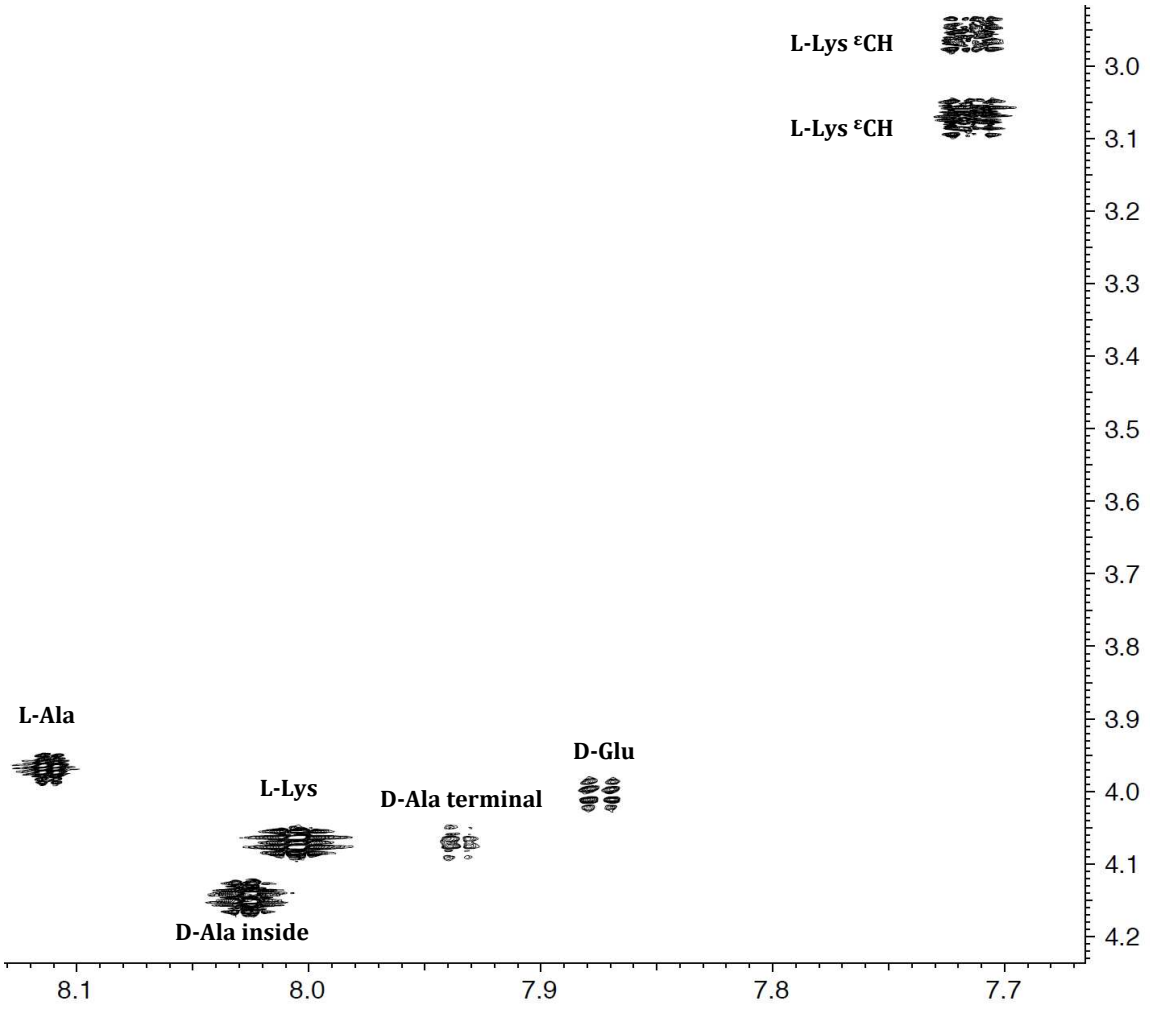


Figure S5. Progress curves for the hydrolysis of **5** (1 mM) catalyzed by EcPBP5 at 25° C, pH 8.5 (O) and pH 9.5 (O). The reactions were monitored spectrophotometrically at 230nm. The points shown are experimental and the solid lines from exponential fitting to obtain k_{cat}/K_m.

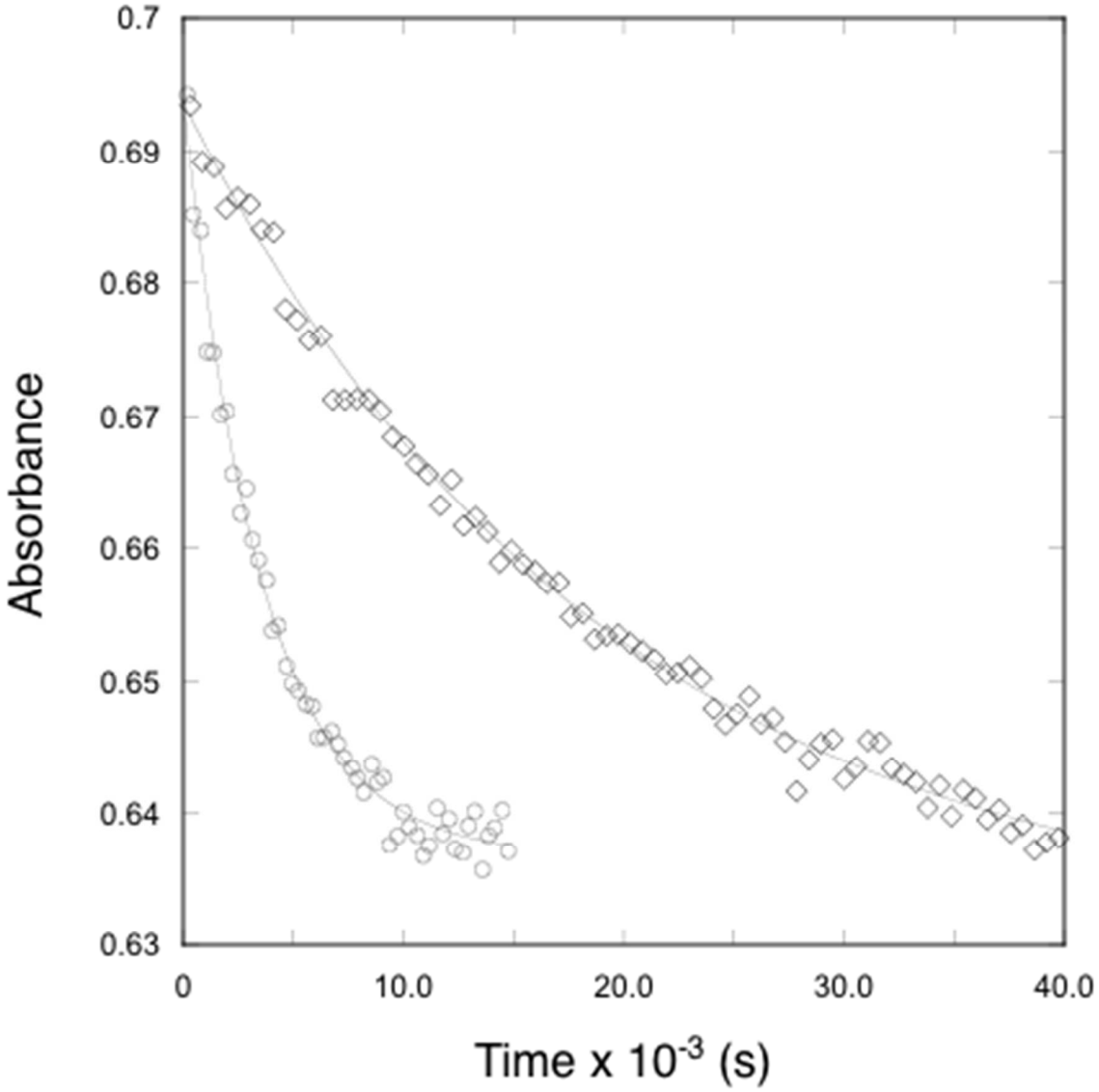
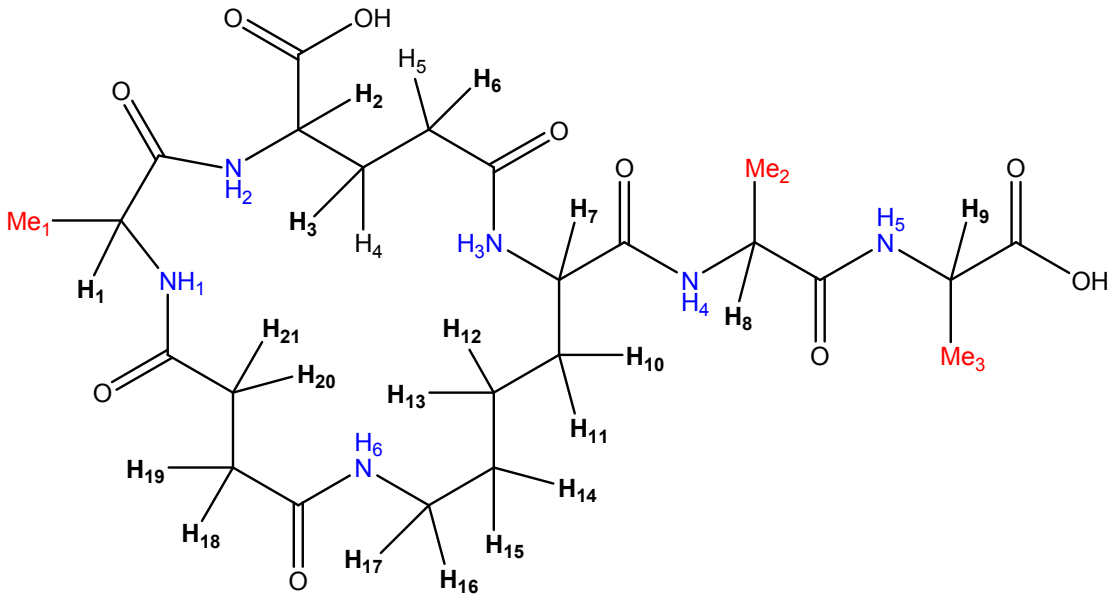


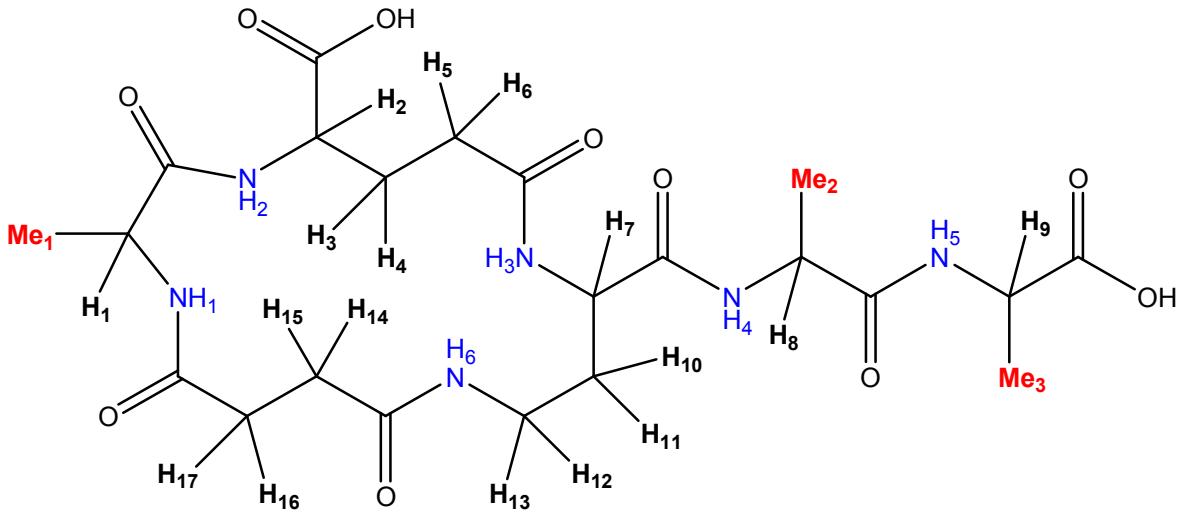
Table S1. ^{1}H NMR assignments for **5** in H2O: D2O (90:10).



Proton	Chemical shift, δ (ppm)	^{3}J-coupling (Hz)
NH_1	8.13, d	4.88
NH_2	7.89, d	7.94
NH_3	8.03, d	6.71
NH_4	7.96, d	6.10
NH_5	8.05, d	6.71
NH_6	7.73, t	8.00
H_1	4.00, m	-
H_2	4.03, m	-
H_3	1.75, m	-
H_4	2.08, m	-
H_5	2.18, m	-
H_6	1.75, m	-
H_7	4.08, m	-
H_8	4.18, m	-
H_9	4.10, m	-
H_{10}	1.55, m	-
H_{11}	1.65, m	-
H_{12}	1.37, m	-
H_{13}	1.32, m	-
H_{14}	1.32, m	-
H_{15}	1.20, m	-
H_{16}	3.00, m	-
H_{17}	3.10, m	-
H_{18}	2.50, m	-
H_{19}	2.50, m	-
H_{20}	2.30, m	-

H_{21}	2.35, m	-
CH_3^1	1.25, d	7.30
CH_3^2	1.21, m	-
CH_3^3	1.21, m	-

Table S2. ^{1}H NMR assignments for **6** in H_2O: D_2O (90:10).



Proton	Chemical shift, δ (ppm)	^{3}J-coupling (Hz)
NH_1	8.25, d	4.88
NH_2	7.84, d	7.82
NH_3	7.88, d	6.71
NH_4	7.96, d	6.10
NH_5	8.06, d	6.71
NH_6	7.86, m	
H_1	4.12, m	-
H_2	4.14, m	-
H_3	1.95, m	-
H_4	2.10, m	-
H_5	2.25, m	-
H_6	2.25, m	-
H_7	4.29, m	-
H_8	4.31, m	-
H_9	4.11, m	-
H_{10}	1.85, m	-
H_{11}	1.98, m	-
H_{12}	3.25, m	-
H_{13}	3.40, m	-
H_{14}	2.40, m	-
H_{15}	2.50, m	-
H_{16}	2.65, m	-
H_{17}	2.65, m	-
Me_1	1.26, d	7.20
Me_2	1.20, m	-
Me_3	1.19, m	-

Table S3. Dihedral angles (ϕ, degrees) from NMR Spectra and Calculated Energies for Energy-Minimized Cyclic Peptide Conformers

	Conformer	ϕ_1	ϕ_2	ϕ_3[a]	E (kcal/mol)
5	1	-66	155	-163	83.3
	2	-66	155	-76	78.5
	3	-66	88	-163	87.0
	4	-66	88	-76	90.2
	5	-174	155	-163	83.4
	6	-174	155	-76	85.6
	7	-174	88	-163	84.9
	8	-174	88	-76	82.1
					Avg. 84.4 ± 3.5
6	1	-66	157	-163	65.1
	2	-66	157	-76	66.8
	3	-66	90	-163	67.0
	4	-66	90	-76	68.8
	5	-174	157	-163	70.2
	6	-174	157	-76	62.3
	7	-174	90	-163	63.3
	8	-174	90	-76	68.8
					Avg. 66.5 ± 2.8

a. ϕ_4 and ϕ_5 values were fixed at 72 and 76 degrees, respectively

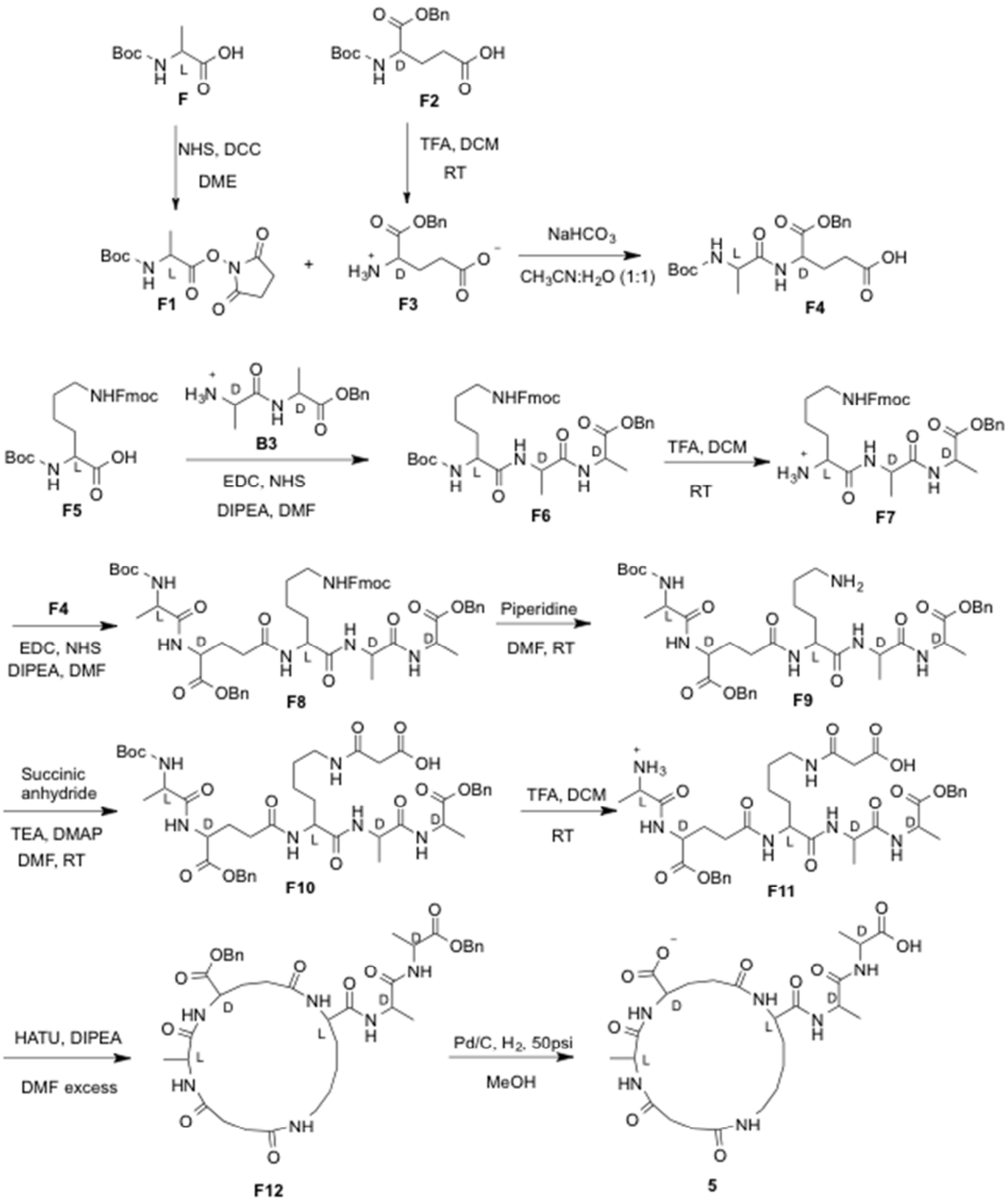
Table S4. NOESY Distances and those from computational conformers of **6**

Atoms	NOESY[a]	Conformer							
		1	2	3	4	5	6	7	8
		Distance (Å)							
L-Dab$^{\delta}$NH CH_2Suc	3.9 ± 0.6 (w)	4.5	3.7	3.7	3.7	4.4	4.4	3.7	3.8
L-Dab$^{\delta}$NH CHSuc	3.2 ± 0.5 (m)	2.9	2.6	2.8	2.8	2.1	2.1	2.8	2.7
L-Dab$^{\delta}$NH CHSuc	3.1 ± 0.5 (m)	3.0	3.5	3.5	3.5	2.9	2.9	3.5	3.5
L-AlaNH CH_2Suc	2.9 ± 0.4 (s)	2.8	2.7	3.1	3.1	2.6	2.8	3.2	2.5
L-Dab$^{\alpha}$NH D-Glu$^{\gamma}CH_2$	3.3 ± 0.5 (m)	3.4	3.1	3.1	3.2	2.3	2.3	2.3	2.3
L-Dab$^{\alpha}$NH D-Glu$^{\gamma}CH_2$	5.0 ± 0.7 (w)	4.0	3.5	3.5	3.5	3.4	3.4	3.5	3.5
L-GluNH D-Glu$^{\beta}$CH	3.7 ± 0.6 (m)	4.1	3.8	3.7	3.5	3.4	3.4	2.7	2.7

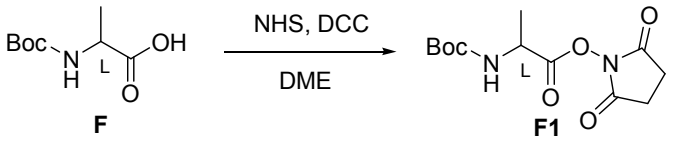
L-Glu$^{\delta}$NH D-Glu$^{\gamma}$CH	3.9 ± 0.6 (w)	4.5	3.2	4.2	4.1	3.8	3.8	3.6	3.6
L-AlaNH D-GluNH	3.4 ± 0.4 (m)	3.4	2.9	2.6	2.7	3.2	3.4	2.0	2.7
L-Ala$_4$NH L-Dab$^{\alpha}$NH	3.2 ± 0.4 (m)	3.2	3.0	3.0	3.0	3.0	3.2	2.1	2.1

[a] NOESY peak intensities are classified as weak (w), medium (m) or strong (s)

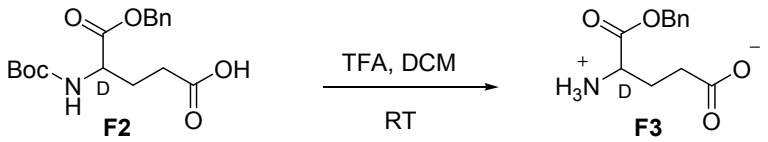
Scheme S1. Synthesis of cyclic peptide **5**



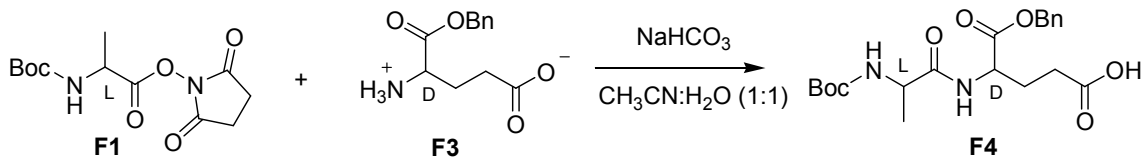
Synthesis of *Cyclo-[N^{α}(L-Ala-D-γ-Glu)-N^{ε}(succinyl)-L-Lys]-D-Ala-D-Ala* (5).



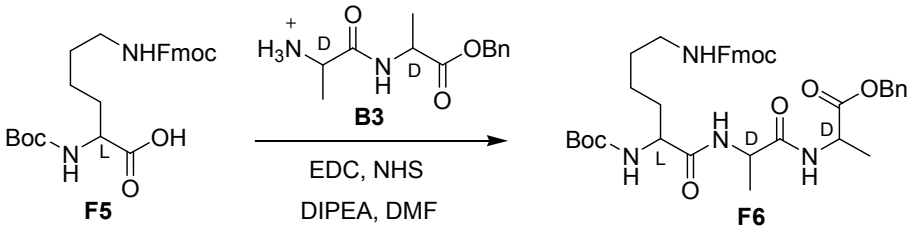
N-Boc-D-Ala-OSuc ester (**F1**). To a solution of N-Boc-D-alanine (**F**, 7.0 g, 37 mmol, 1.0 eq) and *N*-hydroxysuccinimide (4.26 g, 37 mmol, 1.0 eq) in dry dimethoxyethane (DME) (60 mL) at ice temperature was added *N,N'*-dicyclohexylcarbodiimide (DCC) (7.26 g, 37 mmol, 1.0 eq) and the mixture was allowed to stand at 5° C for 48 hours. The reaction mixture was then filtered and the filtrate evaporated to yield a colorless solid, which was dried by means of a vacuum pump for 24 hours. The crude material was recrystallized from isopropanol to afford **F1** as a white crystalline solid (9.0 g, 85%). ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.13 (d, J = 6.0 Hz, 3H), 1.36 (s, 9H), 2.58 (s, 4H), 4.40 (m, 1H), 6.86 (d, J = 7.0 Hz, 1H).



D-Glu α-benzyl ester (**F3**). TFA (13 mL) was added to a stirred solution of **F2** (5g, 15 mmol) in 50 mL dichloromethane (DCM) at ice temperature. The solution was stirred to room temperature over 7 h, after which TLC monitoring confirmed the disappearance of the starting material. DCM was removed by evaporation and the crude product was washed with toluene (2 x 10 mL). The remaining gum was dried under reduce pressure to give **F3** (5.15 g, 99 %), which was used without purification. ^{1}H NMR (D_6-DMSO, 300 MHz) δ 2.02 (m, 2H), 2.41 (m, 2H), 4.15 (m, 1H), 5.13 (AB q, J = 15 Hz, 2H), 7.42 (m, 5H), 8.49 (m, 2H).

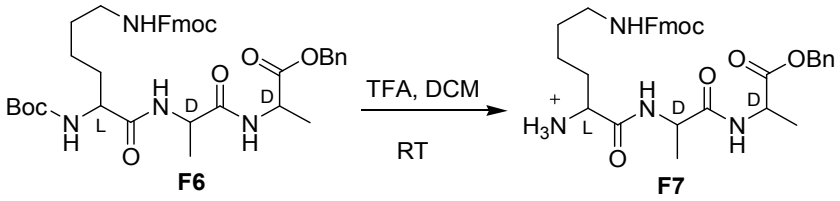


N-Boc-L-Ala-D-Glu α-benzyl ester (**F4**). Sodium bicarbonate (4.99 g, 59.4 mmol) was added to a mixture of **F3** (5.15 g, 14.7 mmol) and **F1** (4.25 g, 14.7 mmol) in 1:1 water: acetonitrile (200 mL) at ice temperature. The reaction mixture was then stirred to room temperature until TLC revealed the disappearance of **F3**. Acetonitrile was evaporated from the reaction mixture and the aqueous layer was acidified (1M HCl) and extracted with ethyl acetate (4 x 100 mL). The combined organic extracts were dried over Na_2SO_4 and the solvent removed by evaporation. The crude product was purified by column chromatography on silica gel (3:7 hexane: ethyl acetate) to yield **F4** as a colorless solid (3.74 g, 62% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.34 (d, J = 7.2 Hz, 3H), 1.43 (s, 9H), 2.04 (m, 2H), 2.30-2.45 (m, 2H), 4.23 (m, 1H), 4.65 (m, 1H), 5.16 (AB q, J = 15 Hz, 2H), 7.34 (m, 5H).



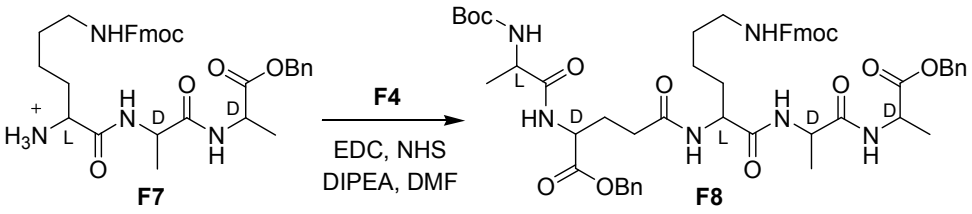
N$^{\alpha}$-Boc-N$^{\varepsilon}$-Fmoc-L-Lys-D-Ala-D-Ala benzyl ester (**F6**). To a solution of **F5** (2.35 g, 5 mmol) in dry DMF (10 mL) under inert atmosphere was added *N*-hydroxysuccinimide (0.72 g, 6.25 mmol) and 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC) (1.2 g, 6.25 mmol) at ice temperature. The reaction mixture was

brought to room temperature and stirred for 20 hours. To this mixture was added a solution of D-alanyl-D-alanine benzyl ester, **B3**[S1] (2.28 g, 6.25 mmol) in DMF (5 mL) followed by dropwise addition of DIPEA (2.23 mL, 12.7 mmol). The mixture was stirred overnight after which DMF was removed by evaporation under vacuum. The residue was dissolved in ethyl acetate and the solution washed with 1M HCl, $NaHCO_3$, and brine, and dried over Na_2SO_4. The crude product after the solvent removal by evaporation was purified by column chromatography on silica gel (3:7 hexane: ethyl acetate) to yield **F6** as a colorless solid (1.93 g, 54% yield). ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.01 (d, J = 7 Hz, 3H), 1.13 (d, J = 7 Hz, 3H), 1.32 (s, 9H), 1.53 (m, 6H), 2.92 (m, 2H), 3.82 (m, 1H), 4.18 (d, J = 5.1 Hz, 1H), 4.26 (d, J = 6.3 Hz, 2H), 5.07 (AB q, J = 15 Hz, 2H), 6.92 (d, J = 7.2 Hz, 1H), 7.35 (m, 9H), 7.65 (d, J = 6.9 Hz, 2H), 7.86 (d, J = 7.2 Hz, 2H), 7.98 (d, J = 8.4 Hz, 1H), 8.25 (d, J = 7.2 Hz, 1H); ESI/MS(+) m/z = 701.16 ($M + H^+$), 723.30 ($M + Na^+$).



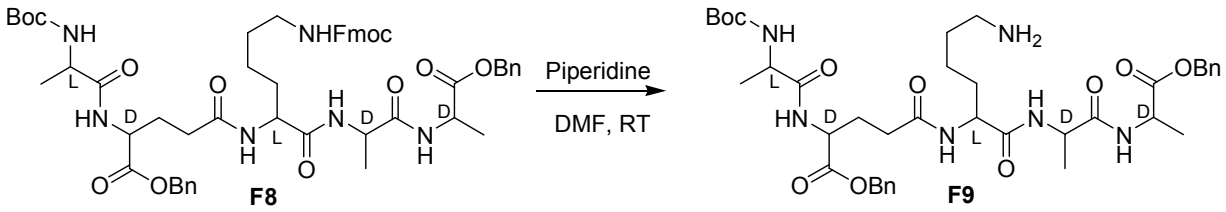
N$^{\varepsilon}$-Fmoc-L-Lys-D-Ala-D-Ala benzyl ester (**F7**). TFA (11 mL) was added to a stirred solution of **F6** (1.63 g, 2.3 mmol) in DCM (35 mL) at ice temperature. The solution was stirred to room temperature over 7 hours. TLC monitoring confirmed the disappearance of the starting material. DCM was removed by evaporation and the crude product was washed with toluene (2 x 10 mL). The remaining gum was dried under reduce pressure overnight to give **F7** (1.55 g, 93%), which was used without purification. ^{1}H NMR (D_6-

DMSO, 300 MHz) δ 1.15 (m, 6H), 1.53 (m, 6H), 2.92 (m, 2H), 3.82 (m, 1H), 4.18 (d, J = 5.1 Hz, 1H), 4.26 (d, J = 6.3 Hz, 2H), 5.06 (AB q, J = 15 Hz, 2H), 7.36 (m, 9H), 7.66 (d, J = 6.9 Hz, 2H), 7.86 (d, J = 7.2 Hz, 2H), 7.98 (d, J = 8.4 Hz, 1H), 8.25 (d, J = 7.2 Hz, 1H); ESI/MS(+) m/z = 601.69 ($M + H^+$), 623.51 ($M + Na^+$).

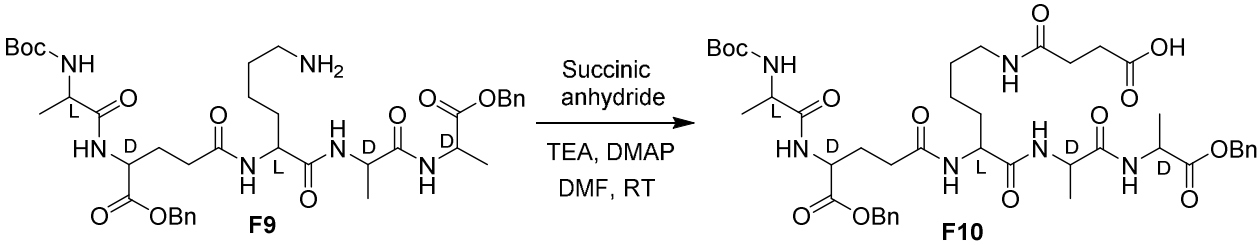


N^{α}-[Boc-L-Ala-D-γ-Glu(Bn)]-L-Lys(Fmoc)-D-Ala-D-Ala benzyl ester (**F8**). To a solution of **F4** (0.804 g, 1.97 mmol) in dry DMF (5 mL) under inert atmosphere was added *N*-hydroxysuccinimide (0.35 g, 3.06 mmol) and EDC (0.58 g, 3.06 mmol) at ice temperature. The reaction mixture was brought to room temperature and stirred for 20 hours. To this mixture was added a solution of **F7** (1.55 g, 2.16 mmol) in DMF (5 mL) followed by dropwise addition of DIPEA (0.94 mL, 5.39 mmol). The mixture was stirred overnight after which DMF was removed by evaporation under vacuum. The residue was dissolved in ethyl acetate and the solution washed with 1M HCl, $NaHCO_3$, and brine and dried over Na_2SO_4. The crude product after the solvent removal was purified by column chromatography on silica gel (0.3: 9.7 methanol : DCM) to yield **F8** as a colorless solid (1.2 g, 60% yield). ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.15 (d, J = 7.2 Hz, 6H), 1.29 (d, J = 7.2 Hz, 3H), 1.36 (s, 9H), 1.52 (m, 6H), 1.80 (m, 1H), 1.96 (m, 1H), 2.1 (m, 2H), 2.95 (m, 2H), 4.01 (m, 2H), 4.12 (m, 4H), 4.27 (d, J = 6.3 Hz, 2H), 5.08 (s, 4H), 6.84 (d, J = 7.2 Hz, 1H), 7.24 (m, 1H), 7.38 (m, 14H), 7.67 (d, J = 7.5 Hz, 2H), 7.88 (d, J = 7.8 Hz,

2H), 7.98 (d, *J* = 6.6 Hz, 1H), 8.16 (d, *J* = 7.2 Hz, 1H), 8.22 (d, *J* = 7.8 Hz, 1H), 8.25 (d, *J* = 6.90 Hz, 1H) ESI/MS(+) *m/z* = 1013.34 ($M + Na^+$).

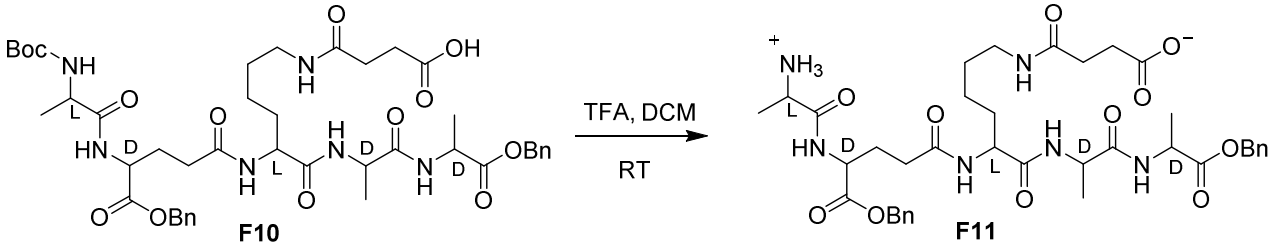


N^{α}-[Boc-L-Ala-D-γ-Glu(Bn)])-L-Lys-D-Ala-D-Ala benzyl ester (**F9**). To a solution of **F8** (0.7 g, 0.7 mmol) in DMF (4 mL) was added piperidine (0.42 mL, 4.24 mmol) at room temperature and the mixture was stirred for 5 hours until TLC revealed the disappearance of **F8**. The solvent was evaporated and the crude solid was washed with diethyl ether (5 mL). After the solid was dried under reduced pressure, it was recrystallized from isopropanol to yield **F9** as a colorless solid (0.465 g, 86%). ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.15 (d, *J* = 7.2 Hz, 6H), 1.29 (d, *J* = 7.2 Hz, 3H), 1.38 (s, 9H), 1.52 (m, 6H), 1.80 (m, 1H), 1.96 (m, 1H), 2.1 (m, 2H), 2.95 (m, 2H), 4.01 (m, 2H), 4.12 (m, 3H), 5.08 (s, 4H), 6.84 (d, *J* = 7.2 Hz, 1H), 7.24 (m, 1H), 7.38 (m, 10H), 7.98 (d, *J* = 6.6 Hz, 1H), 8.16 (d, *J* = 7.2 Hz, 1H), 8.22 (d, *J* = 7.8 Hz, 1H), 8.25 (d, *J* = 6.90 Hz, 1H).



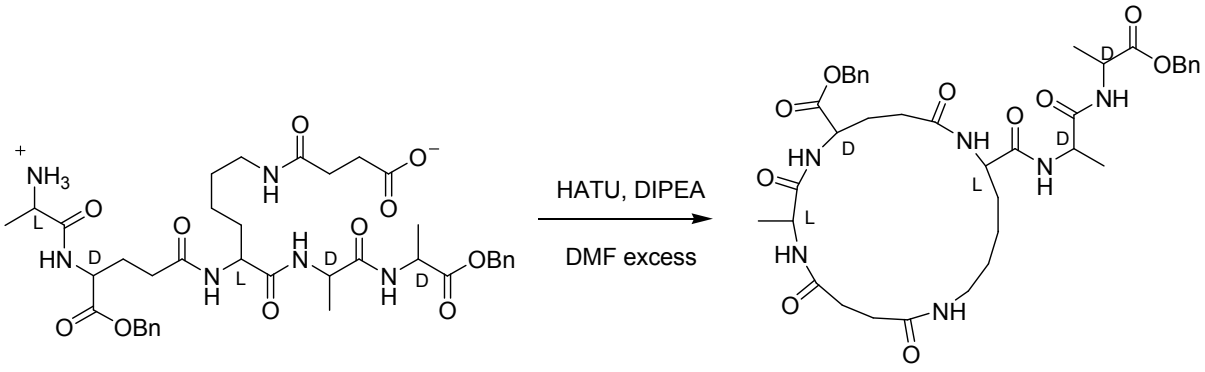
N^{α}-[Boc-L-Ala-D-γ-Glu(Bn)]-N^{ε}(succinyl)-L-Lys-D-Ala-D-Ala benzyl ester (**F10**). To a solution of **F9** (0.47 g, 0.6 mmol) in dry DMF (7 mL) was added succinic anhydride (0.18 g, 1.81 mmol), DMAP (17 mg, 0.01 mmol) and triethylamine (0.11 mL, 0.75

mmol) at room temperature and the mixture was stirred overnight when TLC revealed the disappearance of **F9**. DMF was removed by evaporation under vacuum. The crude product was purified by column chromatography on silica gel (0.4: 9.6 methanol: DCM) to yield **F10** as a colorless solid (0.275 g, 54% yield). ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.16 (d, J = 7.2 Hz, 6H), 1.30 (d, J = 7.2 Hz, 3H), 1.36 (s, 9H), 1.52 (m, 6H), 1.82 (m, 1H), 1.96 (m, 1H), 2.16 (m, 2H), 2.28 (m, 2H), 2.40 (m, 2H), 2.97 (m, 2H), 4.0 (m, 1H), 4.14 (m, 1H), 4.28 (m, 3H), 5.09 (s, 4H), 6.84 (d, J = 7.2 Hz, 1H), 7.39 (m, 10H), 7.79 (m, 1H), 7.98 (d, J = 6.6 Hz, 1H), 8.18 (d, J = 7.2 Hz, 1H), 8.22 (d, J = 7.8 Hz, 1H), 8.25 (d, J = 6.90 Hz, 1H).



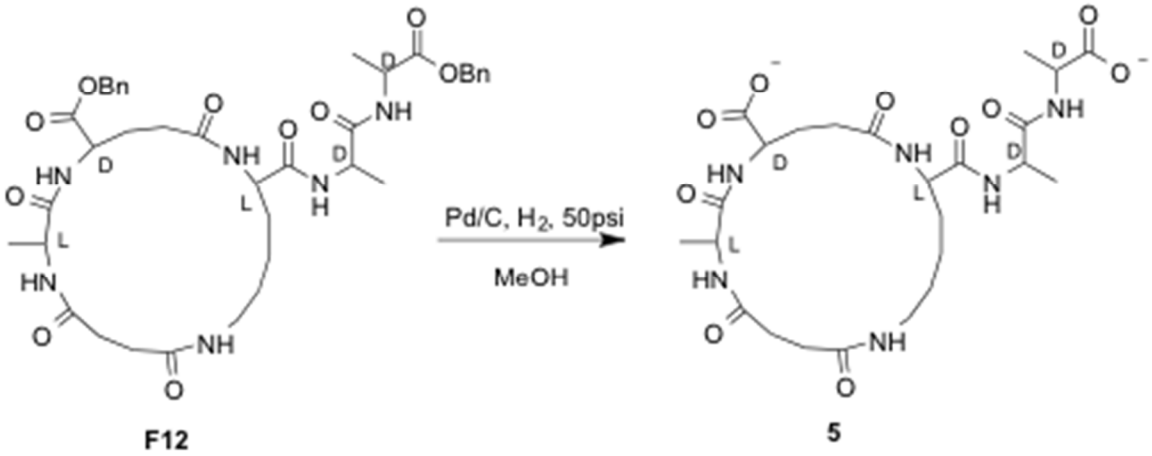
N$^{\alpha}$-[L-Ala-D-γ-Glu(Bn)]-N$^{\epsilon}$(succinyl)-L-Lys-D-Ala-D-Ala benzyl ester (**F11**). TFA (4 mL) was added to a stirred solution of **F10** (0.27 g, 0.31 mmol) in 15 mL DCM at ice temperature. The solution was stirred to room temperature over 7 hours. TLC monitoring confirmed the disappearance of the starting material. DCM was removed by evaporation and the crude product was washed with toluene (2 x 10 mL). The remaining gum was dried under reduce pressure overnight to give **F11** (0.24 g, 89%), which was used without purification. ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.17 (d, J = 7.2 Hz, 6H), 1.30 (d, J = 7.2 Hz, 3H), 1.6 (m, 6H), 1.82 (m, 1H), 1.96 (m, 1H), 2.16 (m, 2H), 2.28 (m, 2H), 2.40 (m, 2H), 2.97 (m, 2H), 4.0 (m, 1H), 4.2 (m, 1H), 4.28 (m, 3H), 5.09 (s, 4H), 7.38 (m, 10H),

7.79 (m, 1H), 7.98 (d, *J* = 6.6 Hz, 1H), 8.18 (d, *J* = 7.2 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.25 (d, *J* = 6.90 Hz, 1H).



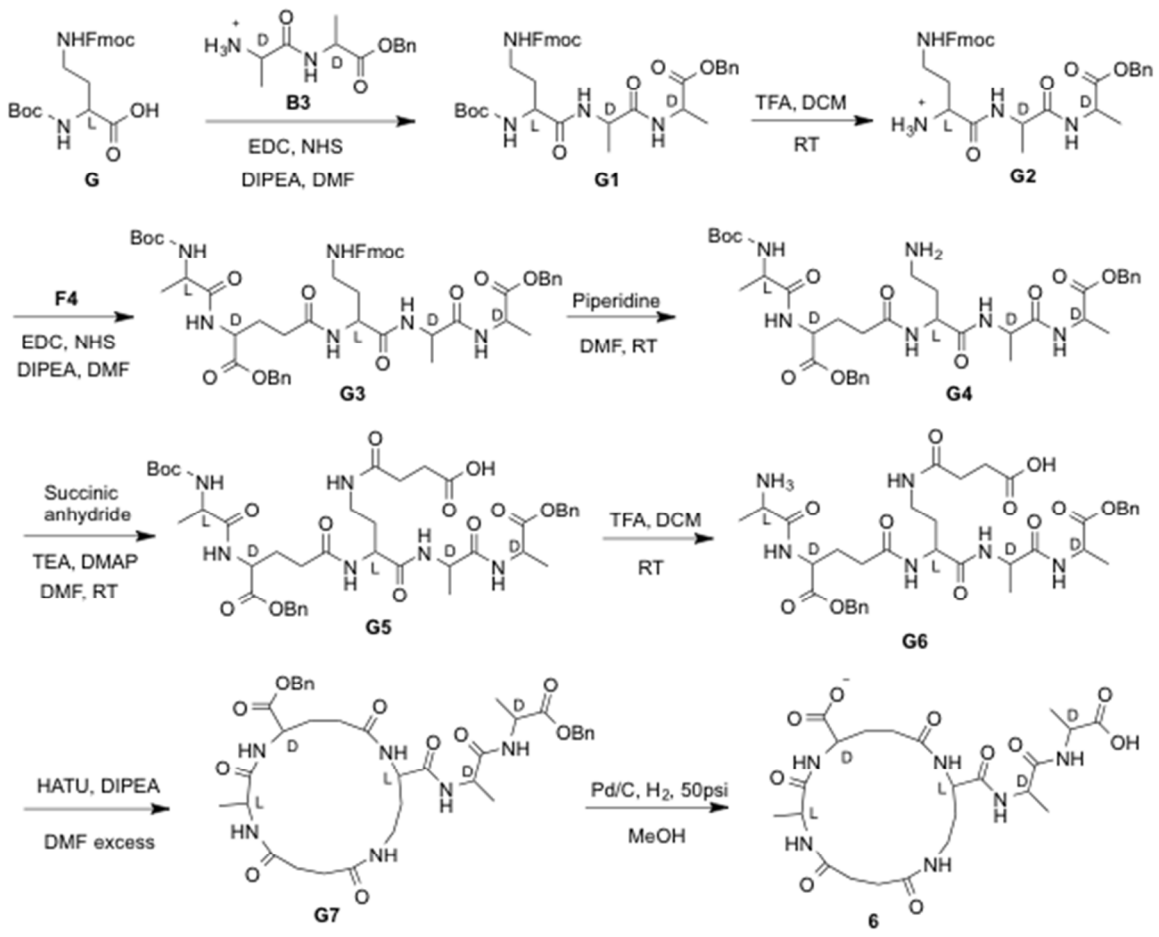
F11 **F12**

Cyclo-{N$^{\alpha}$[L-Ala-D-γ-Glu(Bn)]-N$^{\varepsilon}$(succinyl)-L-Lys}-D-Ala-D-Ala benzyl ester (**F12**). To a solution of HATU (0.155 g, 0.4 mmol) in dry DMF (100 mL) under an inert atmosphere was added a solution of **F11** (0.24 g, 0.272 mmol) in 20 mL DMF at ice temperature. DIPEA (0.142 mL, 0.81 mmol) was added dropwise to this mixture after which the reaction mixture was stirred at room temperature for 48 hours. DMF was removed by evaporation under vacuum. The residue was dissolved in ethyl acetate and the solution washed with 1M HCl and brine and was dried over Na_2SO_4. The crude product after the solvent removal by evaporation was purified by column chromatography on silica gel (0.5: 9.5 methanol: DCM) to yield **F12** as a colorless solid (0.1 g, 60% yield). ^{1}H NMR (D_6-DMSO, 300 MHz) δ 1.18 (m, 6H), 1.30 (d, *J* = 7.2 Hz, 3H), 1.4 (m, 4H), 1.56 (m, 2H), 2.05 (m, 2H), 2.28 (m, 2H), 2.22 (m, 2H), 2.35 (m, 2H), 2.94 (m, 1H), 3.15 (m, 1H), 3.98 (m, 2H), 4.28 (m, 3H), 5.08 (s, 4H), 7.33 (m, 10H), 7.80 (m, 1H), 7.98 (d, *J* = 6.6 Hz, 1H), 8.22 (m, 4H). ESI/MS (+) *m/z* = 751.03 (M + H$^+$), 773.41 (M + Na$^+$).

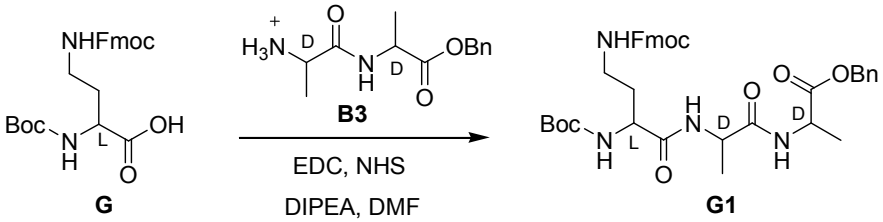


Cyclo-[N$^{\alpha}$(L-Ala-D-γ-Glu)-N$^{\varepsilon}$(succinyl)-L-Lys]-D-Ala-D-Ala (**5**). To a solution of **F12** (40 mg, 0.05 mmol) in methanol (20 mL) was added 10% Pd/C (10 mg). The hydrogenation reaction was conducted at 40 psi H_2 at room temperature for 6 h. Pd/C was removed by filtration through a Celite pad. The filtrate was evaporated leaving an off-white solid which was purified by elution in water from a Sephadex G-10 column to afford **5** as a colorless solid (18 mg, 60% yield): ^{1}H NMR (D_2O, 400 MHz) δ 1.22 (m, 6H), 1.26 (d, J = 7.2 Hz, 3H), 1.36 (m, 5H), 1.56 (m, 1H), 1.63 (m, 1H), 1.81 (m, 1H), 2.19 (m, 4H), 2.33 (m, 2H), 2.48 (t, J = 6 Hz, 2H), 2.97 (m, 1H), 3.07 (m, 1H), 3.98 (q J = 7.2 Hz, 1H), 4.07 (m, 2H), 4.16 (m, 2H). HRMS (ESI+) Found m/z 571.2725 (M + H^+), calcd 571.2728 for $C_{24}H_{39}N_6O_{10}$.

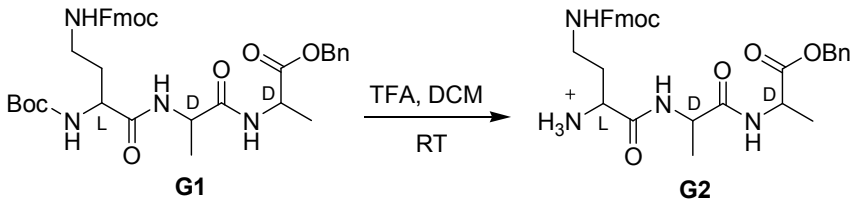
Scheme S2. Synthesis of cyclic peptide **6**



Synthesis of *Cyclo-[N^{α}-(L-Ala-D-γ-Glu)-N^{ε}(succinyl)-L-Dab]-D-Ala-D-Ala* (6).



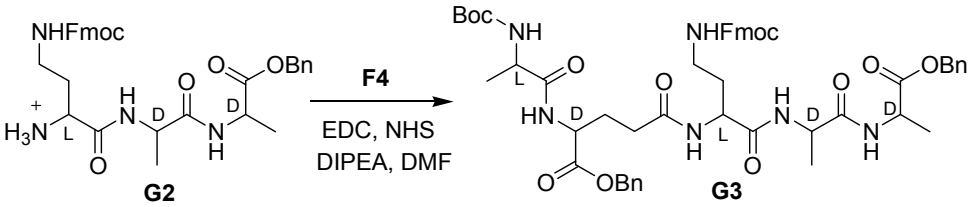
N^{α}-Boc-N^{ϵ}-Fmoc-L-Dab-D-Ala-D-Ala benzyl ester (**G1**). To a solution of **G** (3.0 g, 6.4 mmol) in dry DMF (10 mL) under an inert atmosphere was added *N*-hydroxysuccinimide (0.92 g, 8.0 mmol) and EDC (1.53 g, 8.0 mmol) at ice temperature. The reaction mixture was brought to room temperature and stirred for 20 hours. To this mixture was added a solution of D-alanyl-D-alanine benzyl ester, **B3**[S1] (2.79 g, 7.6 mmol) in DMF (5.0 mL) followed by dropwise addition of DIPEA (2.68 mL, 15.3 mmol). The mixture was stirred overnight, after which DMF was removed by evaporation under vacuum. The residue was dissolved in ethyl acetate and the solution washed with 1M HCl, $NaHCO_3$, and brine and dried over Na_2SO_4. The crude product, after solvent removal by evaporation, was purified by column chromatography on silica gel (0.2:9.8 methanol: dichloromethane) to yield **G1** as a colorless solid (2.0 g, 50% yield). ^{1}H NMR (D_6- DMSO, 400 MHz) δ 1.13 (d, J = 7.2 Hz, 3H), 1.30 (d, J = 7.4 Hz, 3H), 1.33 (s, 9H), 1.59 (m, 1H), 1.70 (m, 1H), 2.98 (m, 2H), 3.93 (m, 1H), 4.18 (q, J = 6.8 Hz, 1H), 4.25-4.27 (d, J = 6.3 Hz, 2H), 4.31 (m, 2H), 5.08 (AB q, J = 15 Hz, 2H), 7.0 (d, J = 7.2 Hz, 1H), 7.22 (m, 1H), 7.33 (m, 8H), 7.4 (m, 2H), 7.65 (d, J = 7.8 Hz, 2H), 7.85-7.88 (d, J = 7.2 Hz, 2H), 7.97-8.0 (d, J = 8.4 Hz, 1H), 8.3 (d, J = 7.2 Hz, 1H).



G1 **G2**

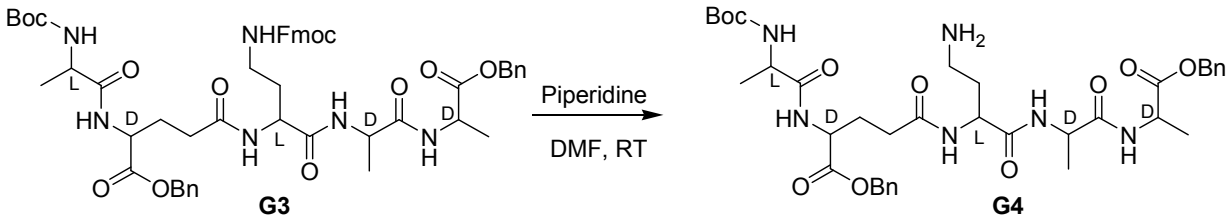
N^{ϵ}-Fmoc-L-Dab-D-Ala-D-Ala benzyl ester (**G2**). TFA (8.0 mL) was added to a stirred solution of **G1** (1.52 g, 2.2 mmol) in DCM (35 mL) at ice temperature. The solution was stirred to room temperature over 7 hours. TLC monitoring confirmed the disappearance of the starting material. DCM was removed by evaporation and the crude product was

washed with toluene (2 x 10 mL). The remaining gum was dried under reduce pressure overnight to give **G2** (1.34 g, 93%), which was used without purification. ^{1}H NMR (D_6-DMSO, 400 MHz) δ 1.13 (d, J = 7.2 Hz, 3H), 1.30 (d, J = 7.4 Hz, 3H), 1.6 (m, 1H), 1.71 (m, 1H), 2.98 (m, 2H), 3.93 (m, 1H), 4.18 (q, J = 6.8 Hz, 1H), 4.26 (d, J = 6.3 Hz, 2H), 4.31 (m, 2H), 5.08 (AB q, J = 15 Hz, 2H), 7.22 (m, 1H), 7.33 (m, 8H), 7.4 (m, 2H), 7.65 (d, J = 7.8 Hz, 2H), 7.87 (d, J = 7.2 Hz, 2H), 8.0 (d, J = 8.4 Hz, 1H), 8.3 (d, J = 7.2 Hz, 1H).

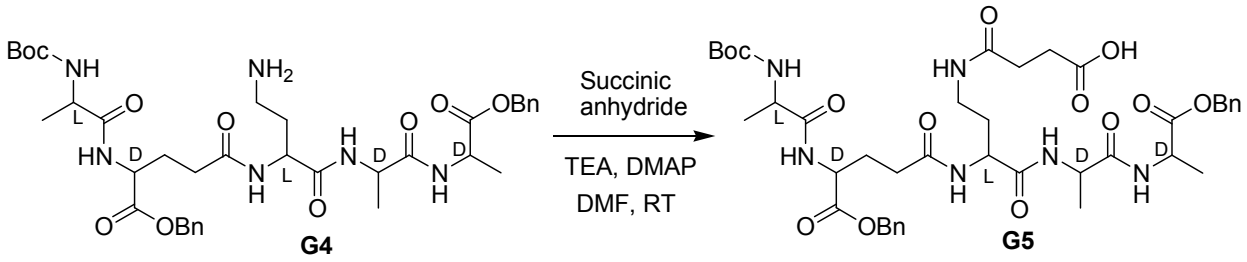


N$^{\alpha}$-[Boc-L-Ala-D-γ-Glu(Bn)]-L-Dab(Fmoc)-D-Ala-D-Ala benzyl ester (**G3**). To a solution of **F4** (0.80 g, 1.96 mmol) in dry DMF (7 mL) under inert atmosphere was added *N*-hydroxysuccinimide (0.28 g, 2.45 mmol) and EDC (0.47 g, 2.45 mmol) at ice temperature. The reaction mixture was brought to room temperature and stirred for 20 hours. To this mixture was added a solution of **G2** (1.35 g, 1.96 mmol) in DMF (7 mL) followed by dropwise addition of DIPEA (0.82 mL, 4.71 mmol). The mixture was stirred overnight after which DMF was removed by evaporation under vacuum. The residue was dissolved in ethyl acetate and the solution washed with 1M HCl, $NaHCO_3$ and brine, and dried over Na_2SO_4. After solvent removal by evaporation, the crude product was purified by column chromatography on silica gel (0.2: 9.8 methanol: DCM) to yield **G3** as a colorless solid (1.0 g, 58% yield). ^{1}H NMR (D_6-DMSO, 400 MHz) δ 1.14 (d, J = 7.4 Hz, 3H), 1.17 (d, J = 6.8 Hz), 1.27 (d, J = 6.8 Hz, 3H), 1.33 (s, 9H), 1.61 (m, 1H), 1.72 (m, 1H), 1.89 (m, 1H), 1.92 (m, 1H), 2.12 (m, 2H), 2.95 (m, 2H), 4.01 (m, 3H), 4.12 (m, 3H),

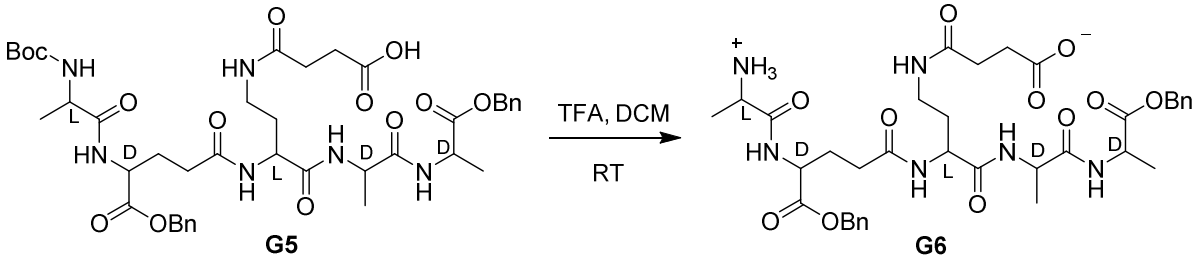
4.27 (d, *J* = 6.3 Hz, 2H), 5.07 (AB q, *J* = 15 Hz, 4H), 6.83 (d, *J* = 7.2 Hz, 1H), 7.24 (m, 1H), 7.31 (m, 14H), 7.4 (m, 2H), 7.67 (d, *J* = 7.5 Hz, 2H), 7.84 (d, *J* = 7.8 Hz, 2H), 8.02 (d, *J* = 6.6 Hz, 1H), 8.16 (d, *J* = 7.2 Hz, 1H), 8.22 (d, *J* = 7.8 Hz, 1H), 8.3 (d, *J* = 6.90 Hz, 1H).



N$^{\alpha}$*-[Boc-L-Ala-D-γ-Glu(Bn)]-L-Dab-D-Ala-D-Ala benzyl ester* (**G4**). To a solution of **G3** (0.765 g, 0.79 mmol) in dry DMF (8.0 mL) was added piperidine (0.47 mL, 4.77 mmol) at room temperature and the mixture was stirred for 5 hours until TLC revealed the disappearance of **G3.** The solvent was removed by evaporation and the crude solid washed with diethyl ether (5 mL). After the solid was dried under reduced pressure, it was recrystallized from isopropanol to yield **G4** as a colorless solid (0.456 g, 79%). ^{1}H NMR (D_6-DMSO, 400 MHz) δ 1.14 (d, *J* = 7.4 Hz, 3H), 1.17 (d, *J* = 6.8 Hz), 1.27 (d, *J* = 6.8 Hz, 3H), 1.33 (s, 9H), 1.61 (m, 1H), 1.72 (m, 1H), 1.89 (m, 1H), 1.92 (m, 1H), 2.12 (m, 2H), 2.91 (m, 2H), 4.01 (m, 3H), 4.12 (m, 3H), 4.27 (d, *J* = 6.3 Hz, 2H), 5.07 (AB q, *J* = 15 Hz, 4H), 6.84 (d, *J* = 7.2 Hz, 1H), 7.24 (m, 1H), 7.32-7.43 (m, 10H), 7.98 (d, *J* = 6.6 Hz, 1H), 8.16 (d, *J* = 7.2 Hz, 1H), 8.21 (d, *J* = 7.8 Hz, 1H), 8.25 (d, *J* = 6.90 Hz, 1H).

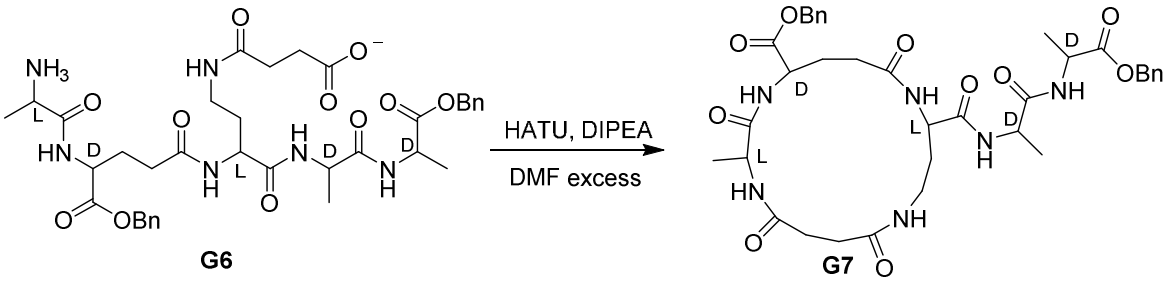


N^{α}-[Boc-L-Ala-D-γ-Glu(Bn)]-N^{ε}(succinyl)-L-Dab-D-Ala-D-Ala benzyl ester (**G5**). To a solution of **G4** (0.407 g, 0.55 mmol) in DMF (5 mL) was added succinic anhydride (0.165 g, 1.62 mmol), DMAP (10 mg, 0.08 mmol) and triethylamine (0.1 mL, 0.69 mmol) at room temperature and the mixture was stirred overnight when TLC revealed the disappearance of **G4**. DMF was removed by evaporation under vacuum. The crude product was purified by column chromatography on silica gel (0.4: 9.6 methanol: DCM) to yield **G5** as a colorless solid (0.3 g, 66% yield). ^{1}H NMR (D_6-DMSO, 400 MHz) δ 1.13 (m, 6H), 1.28 (d, J = 7.2 Hz, 3H), 1.34 (s, 9H), 1.58 (m, 1H), 1.69 (m, 1H), 1.80 (m, 1H), 1.93 (m, 1H), 2.14 (m, 2H), 2.28 (m, 2H), 2.40 (m, 2H), 2.97 (m, 2H), 4.14 (m, 1H), 4.28 (m, 4H), 5.07 (AB q, J = 15 Hz, 4H), 6.84 (d, J = 7.6 Hz, 1H), 7.32 (m, 10H), 7.75 (m, 1H), 8.04 (d, J = 6.8 Hz, 1H), 8.14 (d, J = 7.6 Hz, 1H), 8.21 (d, J = 8 Hz, 1H), 8.29 (d, J = 7.2 Hz, 1H).



N^{α}-[L-Ala-D-γ-Glu(Bn)]-N^{ε}(succinyl)-L-Dab-D-Ala-D-Ala benzyl ester (**G6**). TFA (5 mL) was added to a stirred solution of **G5** (0.3 g, 0.35 mmol) in DCM (15 mL) at ice

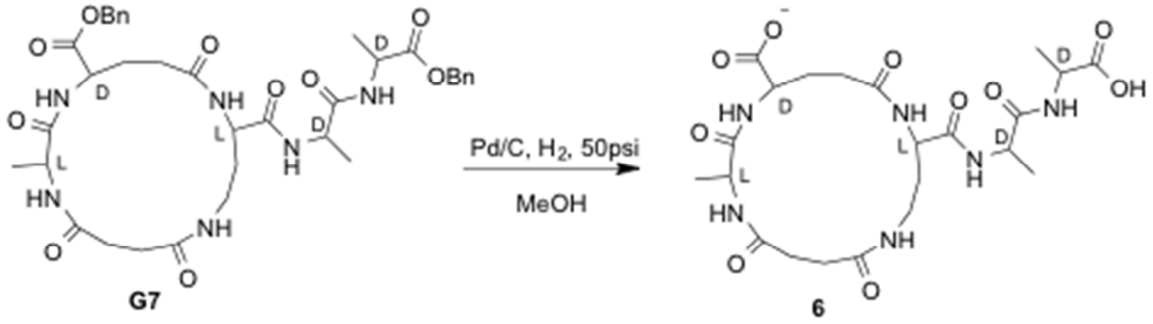
temperature. The solution was stirred to room temperature over 11 hours. TLC monitoring confirmed the disappearance of the starting material. DCM was removed by evaporation and the crude product was washed with toluene (2 x 10 mL). The remaining gum was dried under reduce pressure overnight to give **G6** (0.3 g, 99%) which was used without purification. ^{1}H NMR (D_6-DMSO, 400 MHz) δ 1.13 (m, 6H), 1.28 (d, J = 7.2 Hz, 3H), 1.58 (m, 1H), 1.69 (m, 1H), 1.80 (m, 1H), 1.93 (m, 1H), 2.14 (m, 2H), 2.28 (m, 2H), 2.40 (m, 2H), 2.97 (m, 2H), 4.12 (m, 1H), 4.28 (m, 4H), 5.07 (AB q, J = 15 Hz, 4H), 7.32 (m, 10H), 7.75 (m, 1H), 8.04 (d, J = 6.8 Hz, 1H), 8.14 (d, J = 7.6 Hz, 1H), 8.21 (d, J = 8 Hz, 1H), 8.29 (d, J = 7.2 Hz, 1H).



G6 G7

Cyclo-{N^{α}-[L-Ala-D-γ-Glu(Bn)]-N^{ε}-(succinyl)-L-Dab}-D-Ala-D-Ala benzyl ester (**G7**). To a solution of HATU (0.13 g, 0.17 mmol) in dry DMF (65 mL) under an inert atmosphere was added a solution of **G6** (0.103 g, 0.272 mmol) in DMF (20 mL) at ice temperature. DIPEA (0.1 mL, 0.52 mmol) was added dropwise to this mixture after which the reaction mixture was stirred at room temperature for 52 hours. Excess DMF was removed by evaporation under vacuum. The residue was dissolved in ethyl acetate and the solution washed with 1M HCl and brine and dried over Na_2SO_4. The crude product after solvent removal by evaporation was purified by column chromatography on silica gel (0.8: 9.2 methanol: DCM) to yield **G7** as a colorless solid (95 mg, 95% yield). ^{1}H NMR (CD_3OD,

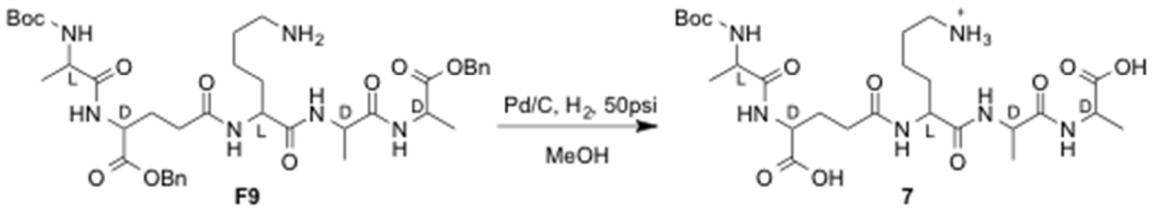
400 MHz) δ 1.3 (d, J = 7.2 Hz, 3H), 1.32 (d, J = 7.2 Hz, 3H), 1.38 (d, J = 7.2 Hz, 3H), 1.77 (m, 1H), 2.0 (m, 1H), 2.1 (m, 1H), 2.21 (m, 1H), 2.35 (m, 2H), 2.46 (m, 1H), 2.67 (m, 1H), 3.15 (m, 1H), 3.36 (m, 1H), 4.22 (q, J = 6.4 Hz, 1H), 4.37 (m, 3H), 4.41 (q, J = 7.2 Hz, 1H), 5.08 (q, J = 15 Hz, 4H), 7.33 (m, 10H). ESI/MS (+) m/z = 723.03 ($M + H^+$), 746.41 ($M + Na^+$).



Cyclo-[N^{α}-(L-Ala-D-γ-Glu)-N^{ε}(succinyl)-L-Dab]-D-Ala-D-Ala (**6**). To a solution of **G7** (40 mg, 0.05 mmol) in methanol (20 mL) was added 10% Pd/C (10 mg). The hydrogenation reaction was conducted at 40 psi H_2 at room temperature for 6 h. Pd/C was removed by filtration through a celite pad. The filtrate was evaporated leaving an off-white solid that was purified by BioRad P2-gel (45-90 μm) column chromatography to afford **44** as a colorless solid (10 mg, 33% yield). ^{1}H NMR (D_2O, 400 MHz) δ 1.17 (d, J = 7.6 Hz, 3H), 1.20 (d, J = 7.2 Hz, 3H), 1.25 (d, J = 7.2Hz, 3H), 1.70 (m, 1H), 1.82 (m, 1H), 1.88 (m, 1H), 1.94 (m, 1H), 2.09 (t, J = 8Hz, 2H), 2.27 (m, 1H), 2.40 (m, 1H), 2.53(m, 2H), 3.08 (m, 1H), 3.26 (m, 1H), 3.96 (m, 3H), 4.16 (m, 2H). ESI/MS (-) m/z = 541.23 (M - 1). HRMS (ESI+) m/z 543.2419 ($M + H^+$), calcd 543.2415 for $C_{22}H_{35}N_6O_{10}$.

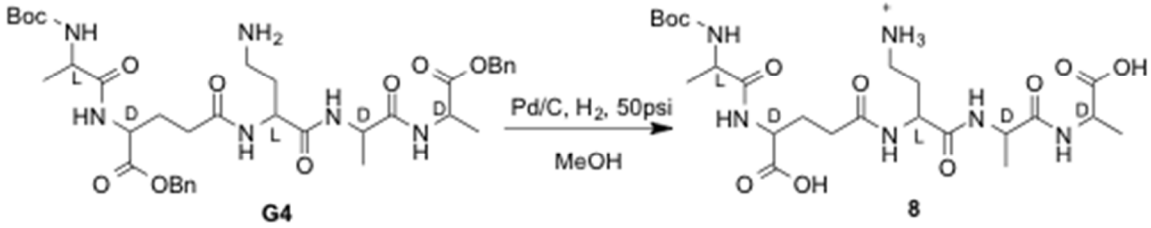
Synthesis of acyclic analogues 7 and 8

Synthesis of N-*Boc-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala* (7)



To a solution of **F9** (40 mg, 0.05 mmol) in methanol (20 mL) was added 10% Pd/C (10 mg). The hydrogenation reaction was conducted at 40 psi H_2 at room temperature for 6 h. Pd/C was removed by filtration through a Celite pad. The filtrate was evaporated leaving an off-white solid that was purified by Sephadex G-10 column chromatography to furnish **7** as a colorless solid (16 mg, 57% yield). ^{1}H NMR (D_2O, 300 MHz) δ 1.2 (m, 9H), 1.24 (s, 9H), 1.3 (m, 2H), 1.48 (m, 2H), 1.53 (m, 2H), 1.76 (m, 1H), 1.99 (m, 1H), 2.17 (m, 4H), 2.81 (t, J = 7.2 Hz, 2H), 3.86 (q, J = 7.5 Hz, 1H), 4.05 (q, J = 7.2 Hz, 3H), 4.16 (q, J = 7.2 Hz, 1H) ESI/MS (-) m/z = 587.27 ($M - H^+$).

Synthesis of *N^{α}-(Boc-L-Ala-D-γ-Glu)-L-Dab-D-Ala-D-Ala* (8)



N^{α}-(Boc-L-Ala-D-γ-Glu)-L-Dab-D-Ala-D-Ala (**8**). To a solution of **G4** (48 mg, 0.07 mmol) in methanol (20 mL) was added 10% Pd/C (12 mg). The hydrogenation reaction was conducted at 40 psi H_2 at room temperature for 6 h. Pd/C was removed by filtration

through a Celite pad. The filtrate was evaporated leaving an off-white solid which was purified by elution in water from a BioRad P2 gel (45-90 μm) column to furnish **8** as a colorless solid (15 mg, 57% yield). ^{1}H NMR (D_2O, 400 MHz) δ 1.17 (m, 6H), 1.22 (d, J = 6.8 Hz, 3H), 1.26 (s, 9H), 1.61 (m, 1H), 1.71 (m, 1H), 1.92 (m, 1H), 2.02 (m, 1H), 2.17 (m, 2H), 2.90 (m, 2H), 3.87 (q, J = 7 Hz, 1H), 3.98 (m, 2H), 4.15 (m, 2H). ESI/MS (+) m/z = 561.21($M + H^+$), 583.13 ($M + Na^+$)