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SUPPORTING INFORMATION

Synthesis of a Chelating Peptidomimetic Building Block

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Abbreviations.

ACH	α-cyano-4-hydroxycinnamic acid
ACN	acetonitrile
CTC	2-chlorotrityl chloride
DCM	dichloromethane
DIEA	*N,N*-diisopropylethylamine
DIC	*N,N*-diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
ESI-MS	electrospray-ionisation
EtOH	ethanol
Et_2O	diethylether
Fmoc	9-fluorenylmethyloxycarbonyl
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
MALDI-TOF	Matrix-assisted laser desorption/ionization – time of flight
MeOH	methanol
Oxyma	ethyl 2-cyano-2-(hydroxylimino)acetate
PDA	photodiode array
SPPS	solid phase peptide synthesis
TFA	trifluoroacetic acid
TIPS	triisopropylsilane
UPLC	Ultra High Performance Liquid Chromatography
Xaa	*L*-amino acid (three letter code)

General Information for the Synthesis of 10 and Peptides cP2

Reagents were purchased from commercial providers (Sigma Aldrich, Alfa Aesar, Iris Biotech). Solvents were obtained from dry and distilled sources.

Merck silica 60 aluminum backed plates pre-coated in silica gel 60 (F_{254}) were used for thin layer chromatography, TLC. Spots were visualized by UV fluorescence deletion (365 nm) or by staining with basic $KMnO_4$ followed by heating.

Reaction progresses and purities were followed using a Water2695 HPLC fitted with an Xbridge C18 (3.5µm; 4.6x100mm) column and connected to a waters 996 photodiode array detector. Data was managed with Empower 2 software. Linear gradients of ACN (+0.036% TFA) into H_2O (+0.045% TFA) were run at a flow rate of 1.0 mL/min over 8 min. UV detection was performed at 220 nm. HPLC gradients are expressed as percentage of ACN at the start and the end of the gradient ("30-100" gradient was run with 30% until 100% ACN over 8 minutes).

NMR spectra were recorded at 400 MHz (^{1}H, 400.123 MHz; ^{13}C, 100.611 MHz). Chemical shifts are reported in ppm relative to the TMS signal recorded at δ_H 0.00 ppm in $CDCl_3$ solvent. The ^{13}C values were referenced to the residual chloroform signal at δ_C 77.0 ppm in $CDCl_3$. ^{1}H NMR shift values are reported as chemical shift (δ), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br s, broad singlet; dd, doublet of doublets), coupling constant (*J* in Hz) and relative integral. The ^{13}C NMR shift values are reported as chemical shift (δ).

Mass spectrometry was carried out on a Waters2695 HPLC micromass ZQ fitted with an Xselect CSG C18 (3.5µm; 2.1x100mm) column and connected to a Waters2998 photodiode array detector.

For accurate mass spectra by nanoelectrospray, the capillary temperature was 180 ºC in positive ionization mode with a tube lens of 119 V.

MALDI-TOF mass spectra were recorded on a MALDI-TOF Applied Biosystem 4700 with a N_2 laser of 337 nm using ACH matrix (10 mg/mL of ACH in ACN-H_2O-TFA (1:1:0.1). Samples were prepared by mixing sample solution from the HPLC vial with ACH matrix 1:1 and drying on the MALDI plate in air.

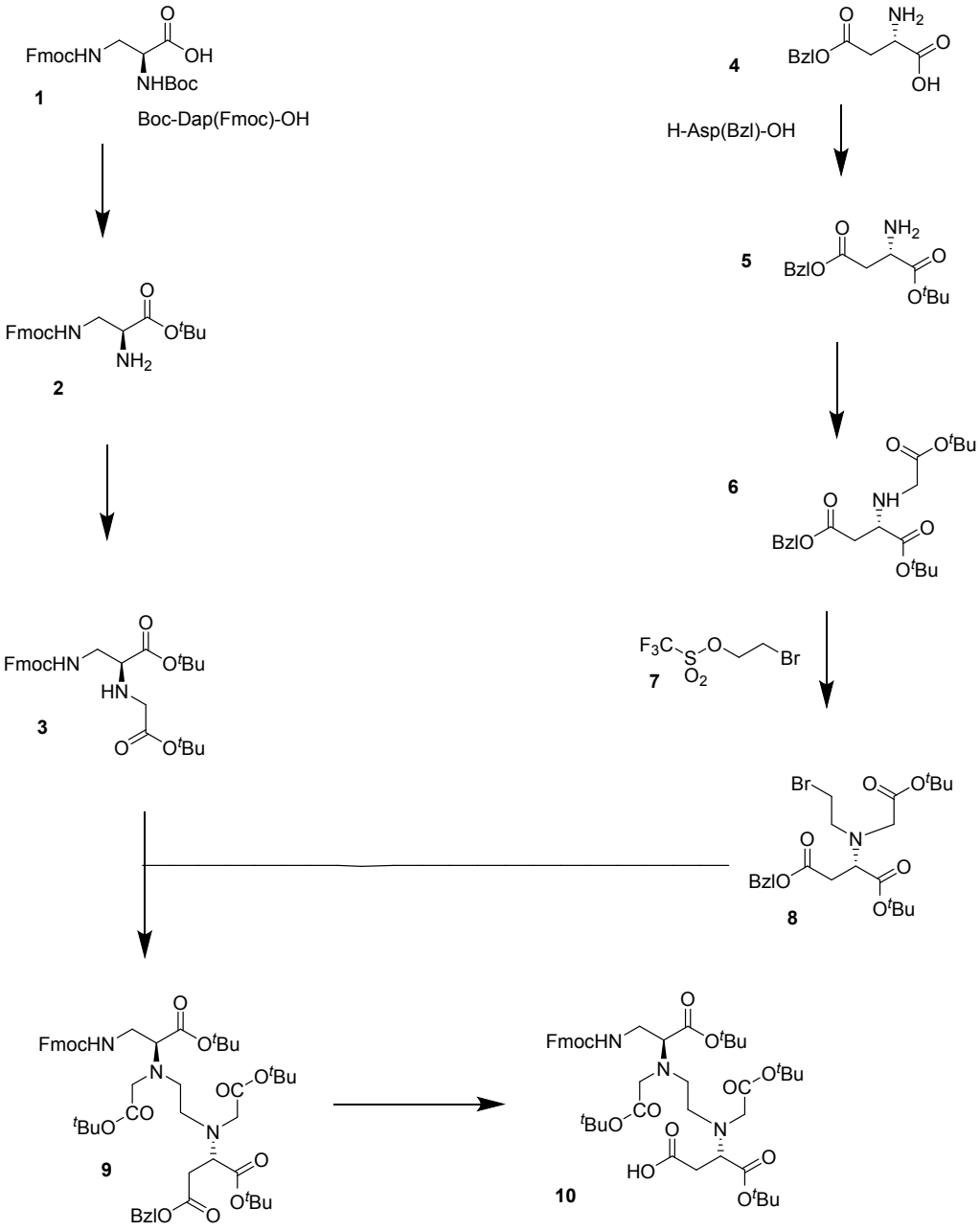
Melting points were detected on an electrothermal melting point apparatus and are uncorrected.

Optical rotations (in 10^{-1} deg cm^2 g^{-1}) were measured at 25 ºC using a Jasco P-2000 polarimeter at 598 nm.

Infrared spectroscopy (IR) was carried out on a Nexus FT-IR 470 machine using pressurized KBr solid plates. Absorption maxima are expressed in wavenumbers (cm^{-1})

Fmoc loading values were attained from a UV-2501PC connected to a UV-vis recording spectrophotometer.

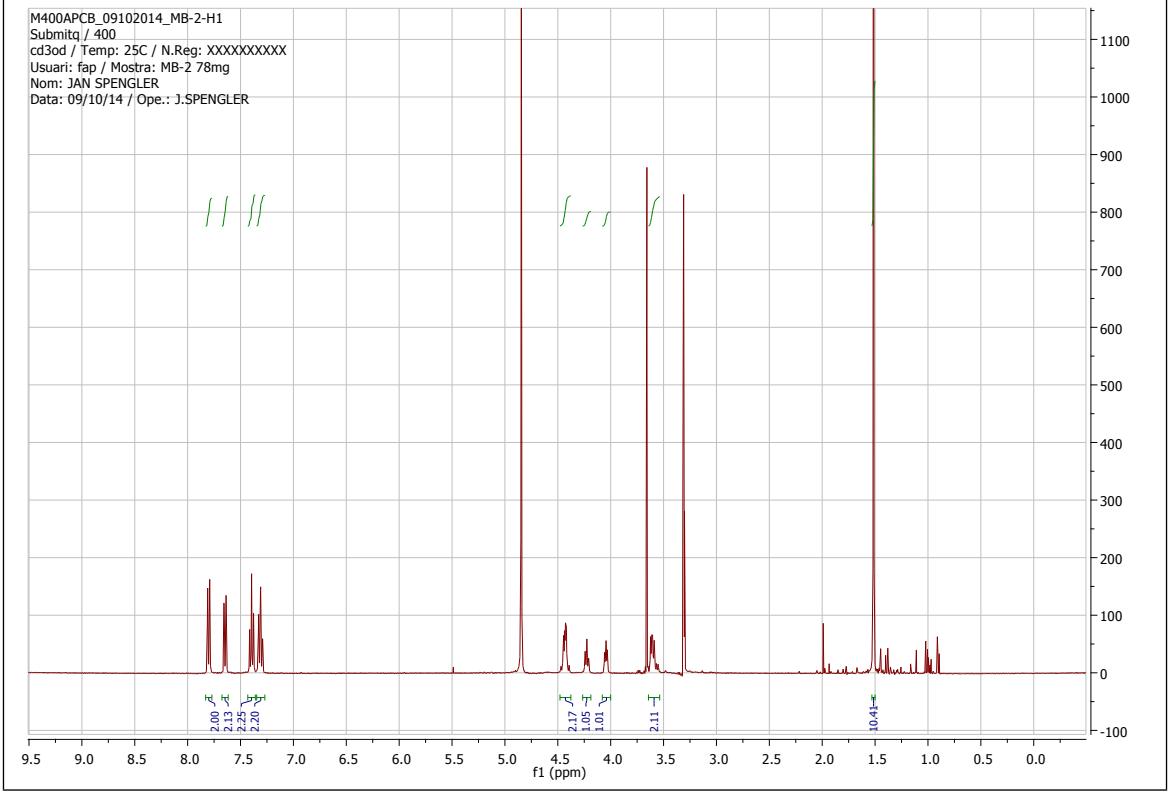
Synthesis of **Fmoc-[Dap-Asp]EDTA(O^{t}Bu)-OH**



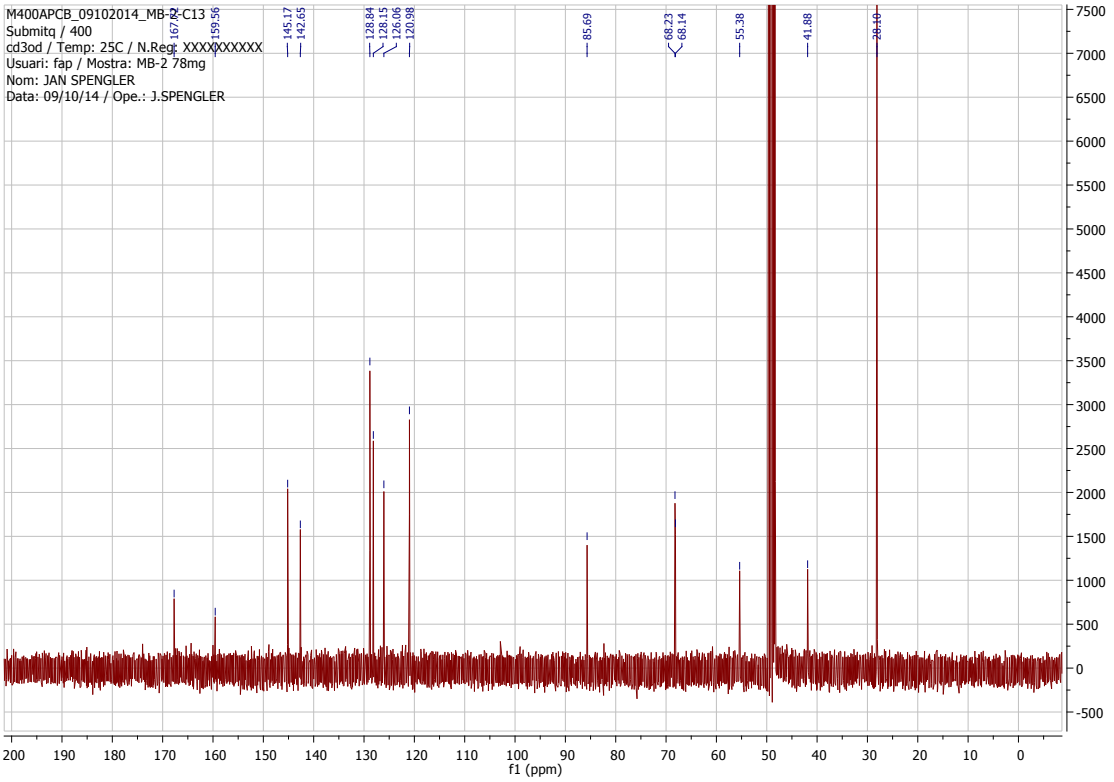
2: (*S*)-*Tert*.-butyl *N*-⍰-(9-fluorenylmethyloxycarbonyl)-2,3-diaminopropionate hydrochloride [H-Dap(Fmoc)-O^{t}Bu * HCl].

In a 250 mL flask, Boc-Dap(Fmoc)-OH **1** (2.5 g, 5.79 mmol) was stirred with *tert*.butylacetate (70 mL). On addition of 70% aqueous $HClO_4$ (0.35 mL) a clear solution was formed. Note: There was a slight gas evolution. It was stirred overnight. The reaction mixture was checked by HPLC. The product **2** as well as minor amounts of H-Dap(Fmoc)-OH were detected. To this solution were added a saturated solution of $NaHCO_3$ (200 mL) and AcOEt (100 mL). After the gas evolution has ceased it was filtered through loose cotton. The phases were separated. The organic phase was washed with brine (3 x 30 mL) and dried over $MgSO_4$. Filtration and concentration in vacuum yielded an oil which was dissolved in dioxane (40 mL). 4M HCl in dioxane (2 mL) were put to this solution. Concentration in vacuum yielded **2** as white solid (2.29 g, 95%). ^{1}H NMR (CD_3OD) δ 7.80 (d, J = 7.6 Hz, 2H), 7.65 (dd, J = 7.5, 0.8 Hz, 2H), 7.40 (t, J = 7.4 Hz, 2H), 7.31 (t, J = 7.4 Hz, 2H), 4.48 – 4.38 (m, 2H), 4.23 (t, J = 6.7 Hz, 1H), 4.08 – 4.00 (m, 1H), 3.64 – 3.54 (m, 2H), 1.51 (s, 9H); ^{13}C NMR (CD_3OD) δ 167.72, 159.56, 145.17, 142.65, 128.84, 128.15, 126.06, 120.98, 85.69, 68.23, 55.38, 41.88, 28.10; IR (KBr) 3357, 2963, 1745, 1702, 1538, 1262, 1162 cm^{-1}; ⍰$_D^{25}$ = -8.5 (c = 0.75, CH_3OH); accurate mass calc. $C_{22}H_{27}N_2O_4$ [M+H$^+$] requires 382.19653, found 383.19642.

2 [H-Dap(Fmoc)-O^{t}Bu * HCl]: ^{1}H NMR (CD_3OD) (residual dioxane at 3.66 ppm)



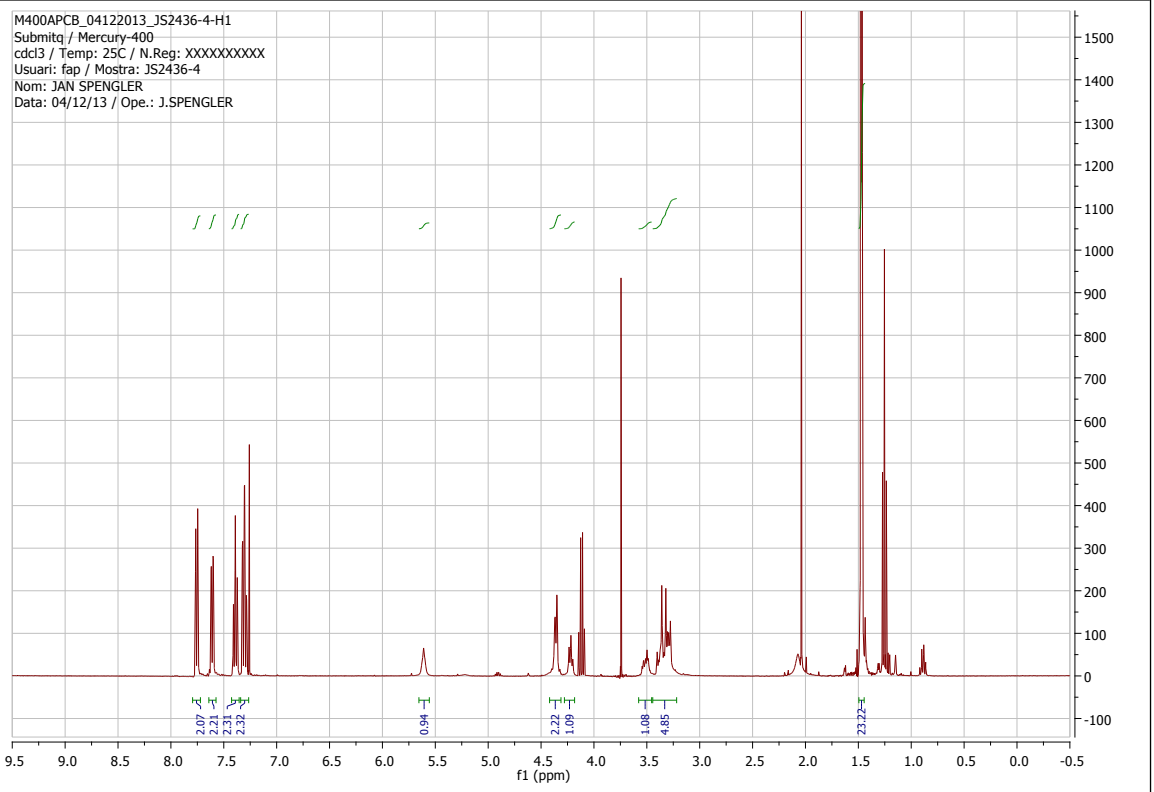
2 [H-Dap(Fmoc)-O^{t}Bu * HCl]: ^{13}C NMR (CD_3OD) (residual dioxane at 68.2 ppm)



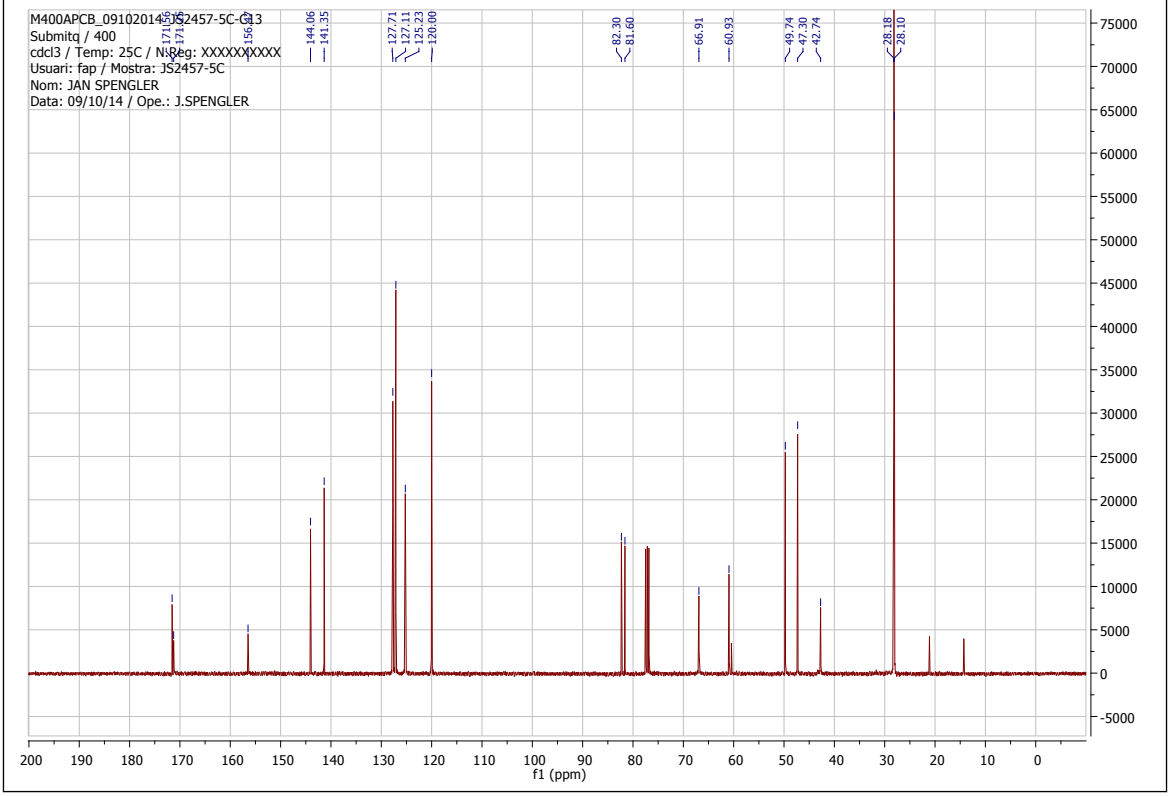
3: (*S*)-*Tert*.-butyl *N*-⍰-(*tert*.-butyloxycarbonylmethyl)-*N*-⍰-(9-fluorenylmethyloxycarbonyl)-2,3-diaminopropionate **[**(CH_2CO_2^{t}Bu)-Dap(Fmoc)-O^{t}Bu].

A solution of **2** (2.29 g, 5.48 mmol) and *tert*.-butyl bromoacetate (1.64 mL, 10.9 mmol) in ACN (100 mL) was stirred vigorously with 2M phosphate buffer (25 mL) overnight. The progress of the reaction was checked by HPLC. The conversion reaches to about 80% with formation di-substituted product. When conversion was lower, 2M phosphate buffer (25 mL) was added and it was stirred further 15 h. The solution was decanted from the solid, concentrated in vacuum, $CHCl_3$ (30 mL) was added to the remainder (both liquid and solid) and it was dried with $MgSO_4$. The $CHCl_3$-phase was subjected to column chromatography on silica (3 cm * 14 cm, hexane:ethyl acetate 1:1, R_f = 0.5 UV 254 nm) to give 2.2 g (81%) of **3** as oil. ^{1}H NMR ($CDCl_3$) δ 7.76 (d, J = 7.5 Hz, 2H), 7.61(m, 2H), 7.39 (t, J = 7.3 Hz, 2H), 7.30 (m, 2H), 5.61 (s, 1H), 4.42 – 4.31 (m, 2H), 4.22 (t, J = 7.1 Hz, 1H), 3.52 (dt, J = 10.4, 3.8 Hz, 1H), 3.44 – 3.22 (m, 4H), 1.49 – 1.44 (m, 18H); ^{13}C NMR ($CDCl_3$) δ 171.6, 171.3, 156.5, 144.1, 141.4, 127.7, 127.1, 125.2, 120.0, 82.3, 81.6, 66.9, 60.9, 49.7, 47.3, 42.7, 28.2, 28.1; IR (film) 3342, 2978, 2934, 1729, 1149 cm-1; ⍰$_D$25 = +4 (c=2, $CHCl_3$); accurate mass calc. $C_{28}H_{37}N_2O_6$ [M+H$^+$] requires 496.26461, found 497.26431.

3 [($CH_2CO_2{}^tBu$)-Dap(Fmoc)-O^{t}Bu]: ^{1}H NMR ($CDCl_3$) (AcOEt at 1.26, 2.05, 4.12 ppm)



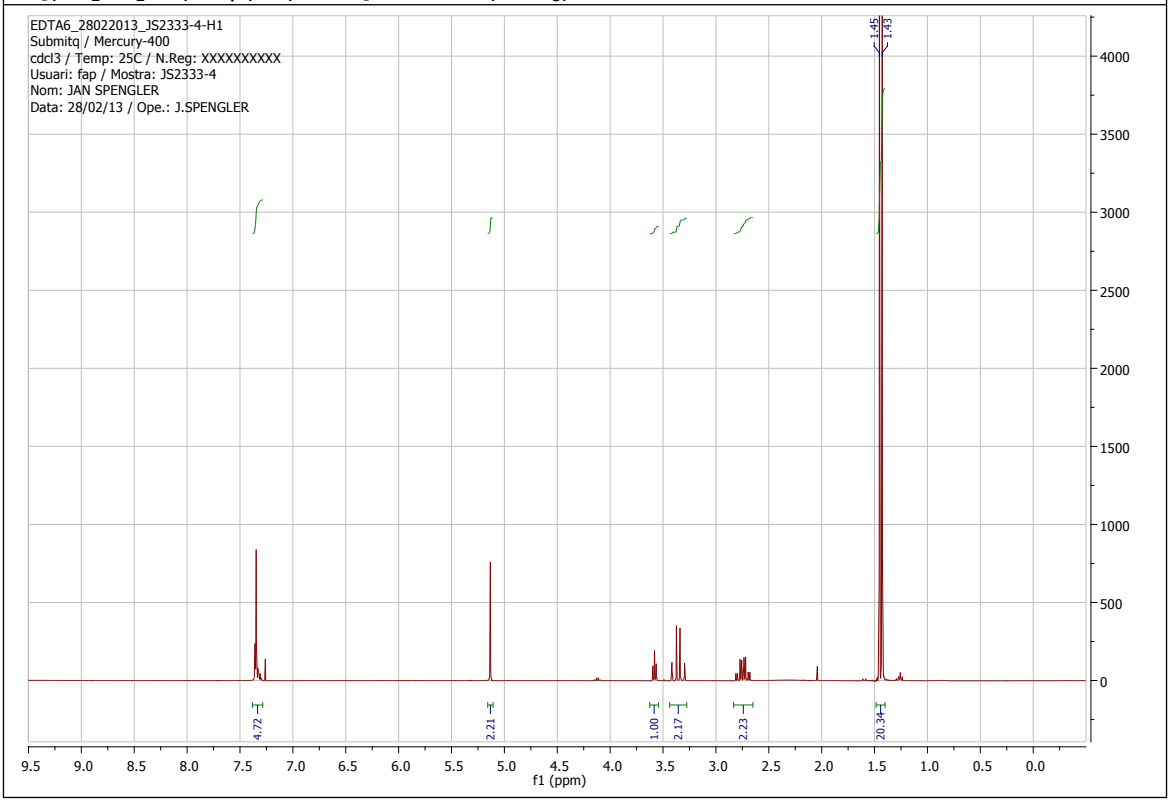
3 [($CH_2CO_2{}^tBu$)-Dap(Fmoc)-O^{t}Bu)]: ^{13}C NMR ($CDCl_3$) (AcOEt at 171.4, 60.5, 21.0 and 14.2 ppm)



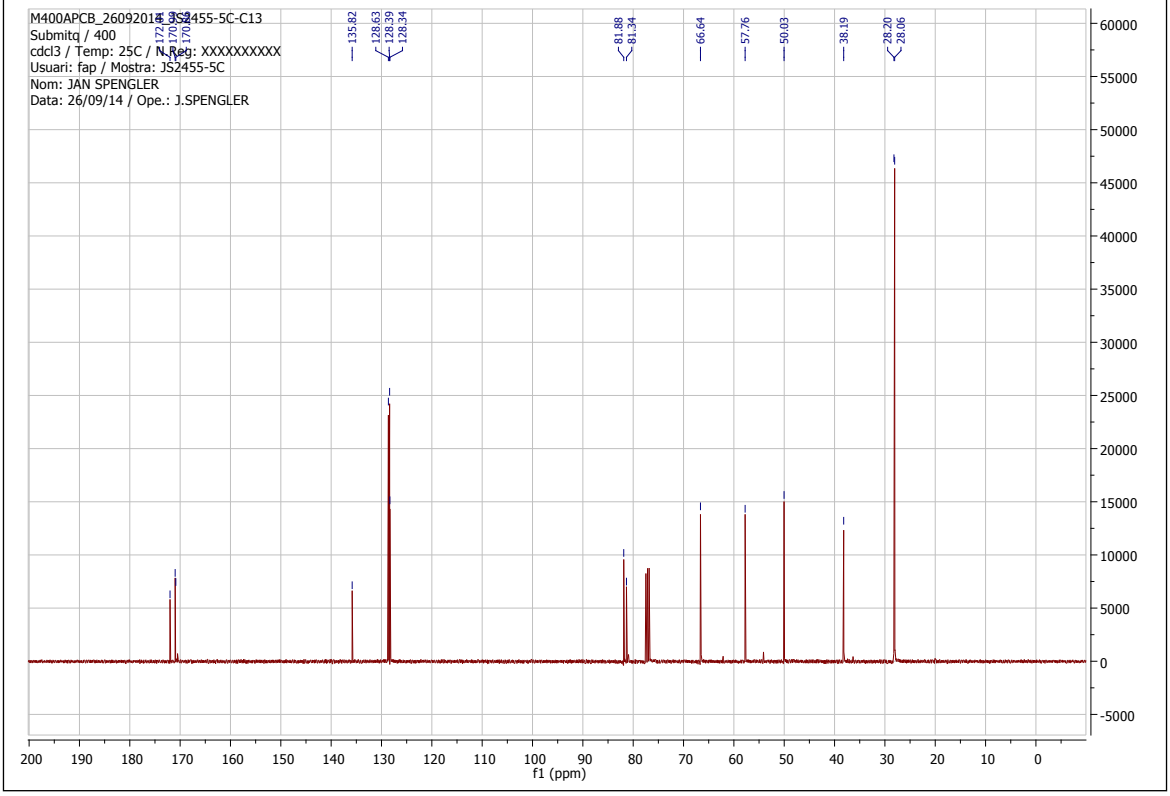
The reaction sequence **4** to **8** was performed as described in WO 2006/002873. The triflate **7** was prepared as described in *Angew. Chem. Int. Ed*. **2008**, 3784, SI.

6: *N*-(*tert*.-butyloxycarbonylmethyl)-*L*-aspartic acid-β-benzyl-α-*tert*.-butyl ester, ($CH_2CO_2{}^tBu$)-Asp(Bzl)-O^{t}Bu), was obtained from **5** (5.8 g, 21 mmol) as oil after column chromatography on silica (3 cm * 10 cm, hexane:ethyl acetate 3:1 + 1% triethylamine, R_f = 0.4 UV 254 nm) (4.7 g, 59%). ^{1}H NMR ($CDCl_3$) δ 7.38 – 7.28 (m, 5H), 5.13 (s, 2H), 3.58 (t, J = 6.3 Hz, 1H), 3.36 (q, J = 17.0 Hz, 2H), 2.75 (qd, J = 16.1, 6.3 Hz, 2H), 1.45 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 172.0, 170.0, 170.9, 135.8, 128.6, 128.4, 128.3, 81.9, 81.3, 66.6, 57.8, 50.0, 38.2, 28.2, 28.1; IR (film) 2978, 1737, 1368, 1151 cm-1; [α]$_D$25 = -6.5 (c=2, $CHCl_3$); accurate mass calc. $C_{21}H_{32}NO_6$ [M+H$^+$] requires 394.22241, found 394.2224.

6 [($CH_2CO_2{}^tBu$)-Asp(Bzl)-O^{t}Bu]: ^{1}H NMR ($CDCl_3$)

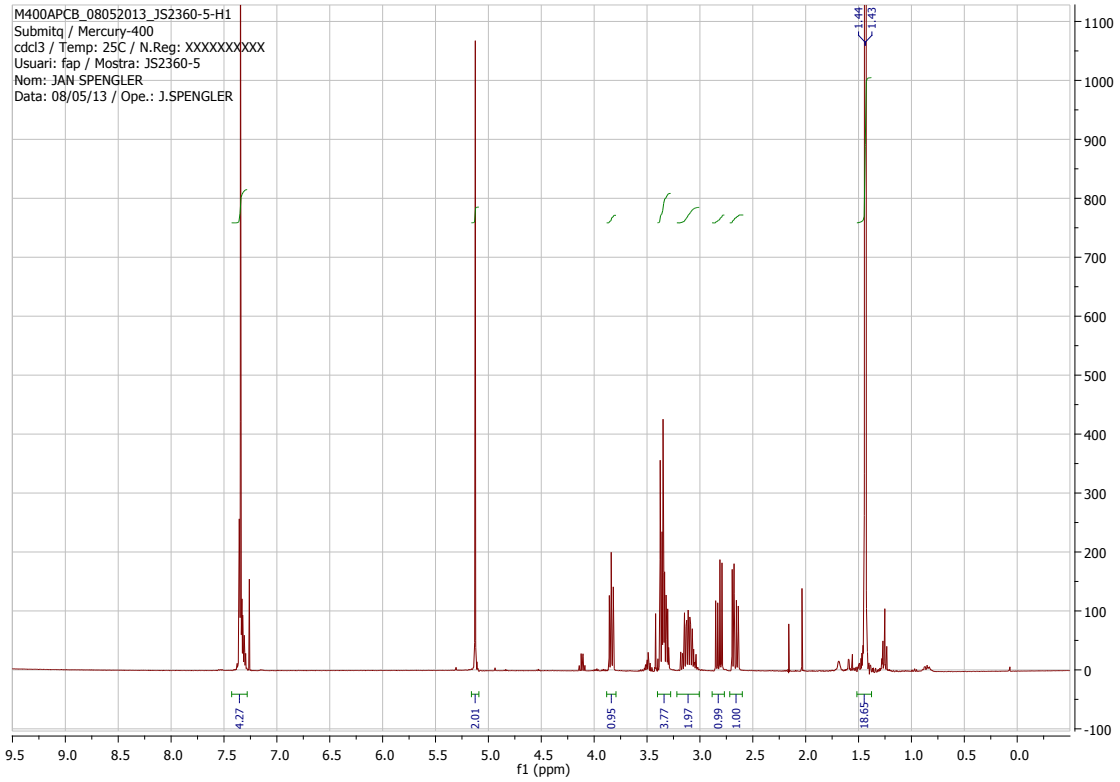


6 [$(CH_2CO_2{}^tBu)$-Asp(Bzl)-O^{t}Bu]: ^{13}C NMR ($CDCl_3$)

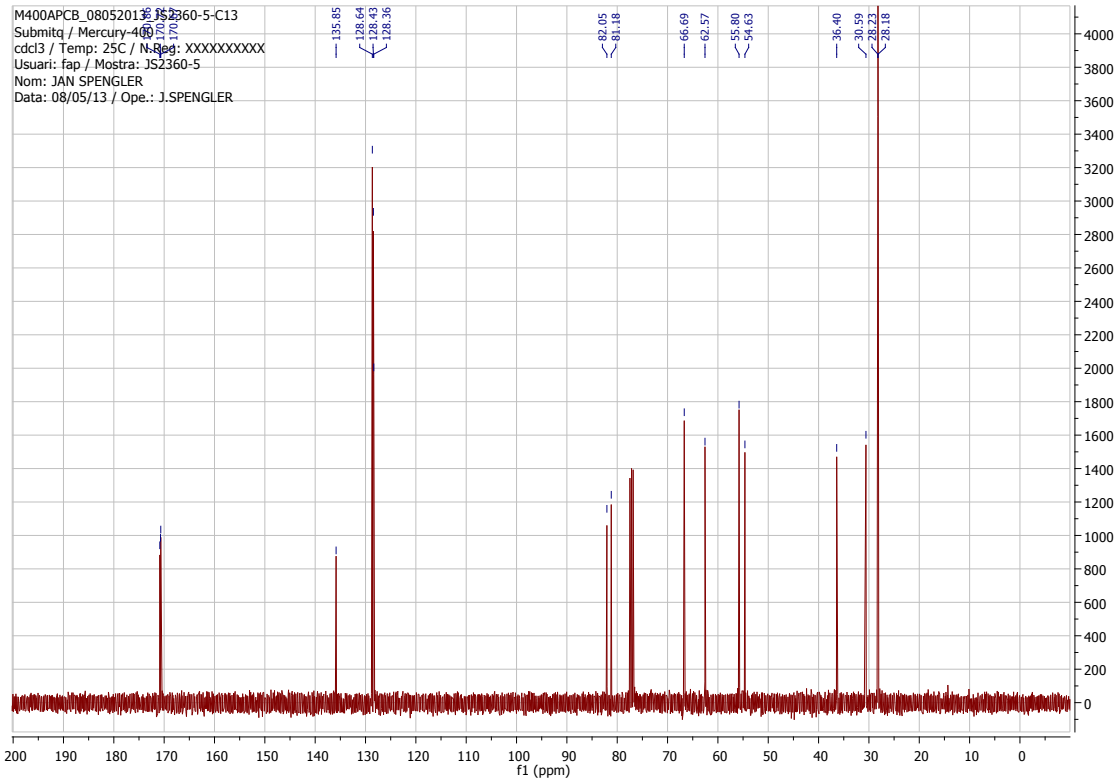


8: *N*-Bromoethyl-*N*-(*tert*.-butyloxycarbonylmethyl)-*L*-aspartic acid-β-benzyl-α-*tert*.-butyl ester, $(CH_2CH_2Br)(CH_2CO_2{}^tBu)$-Asp(Bzl)-O^{t}Bu, was obtained from **6** (3.0 g, 7.6 mmol) and **7** (5.4 g, 21 mmol) [Note: Slow reaction, approx. 3 days, lower excess of **7** lowers yield] as oil after column chromatography (2.1 g, 55%) (hexanes:AcOEt 4:1, R_f = 0.4). ^{1}H NMR ($CDCl_3$) δ 7.43 – 7.28 (m, 5H), 5.12 (s, 2H), 3.83 (m, 1H), 3.42– 3.28 (m, 4H), 3.22 – 3.01 (m, 2H), 2.82 (dd, J = 16.0, 7.8 Hz, 1H), 2.67 (dd, J = 16.0, 7.2 Hz, 1H), 1.44 (s, 9H), 1.43 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 170.9, 170.7, 170.7, 135.9, 128.6, 128.4, 128.4, 82.0, 81.2, 66.7, 62.6, 55.8, 54.6, 36.4, 30.6, 28.2, 28.2; IR (film) 2978, 1738, 1728, 1368, 1150 cm-1; [α]$_D$25 = -27 (c=2, $CHCl_3$); accurate mass calc. $C_{23}H_{35}BrNO_6$ [M+H$^+$] requires 500.16432, found 500.16445.

8 [$(CH_2CH_2Br)(CH_2CO_2{}^tBu)$-Asp(Bzl)-O^{t}Bu]: ^{1}H NMR ($CDCl_3$)



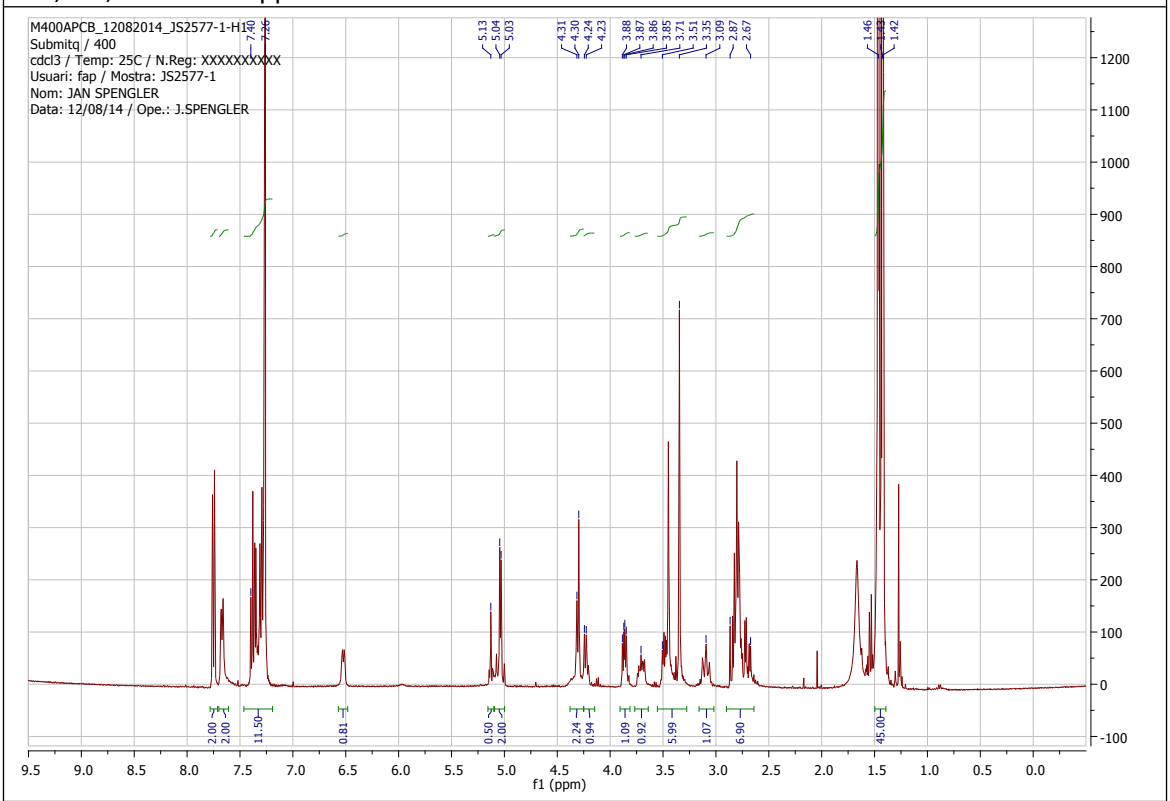
8 [$(CH_2CH_2Br)(CH_2CO_2{}^tBu)$-Asp(Bzl)-O^{t}Bu]: ^{13}C NMR ($CDCl_3$)



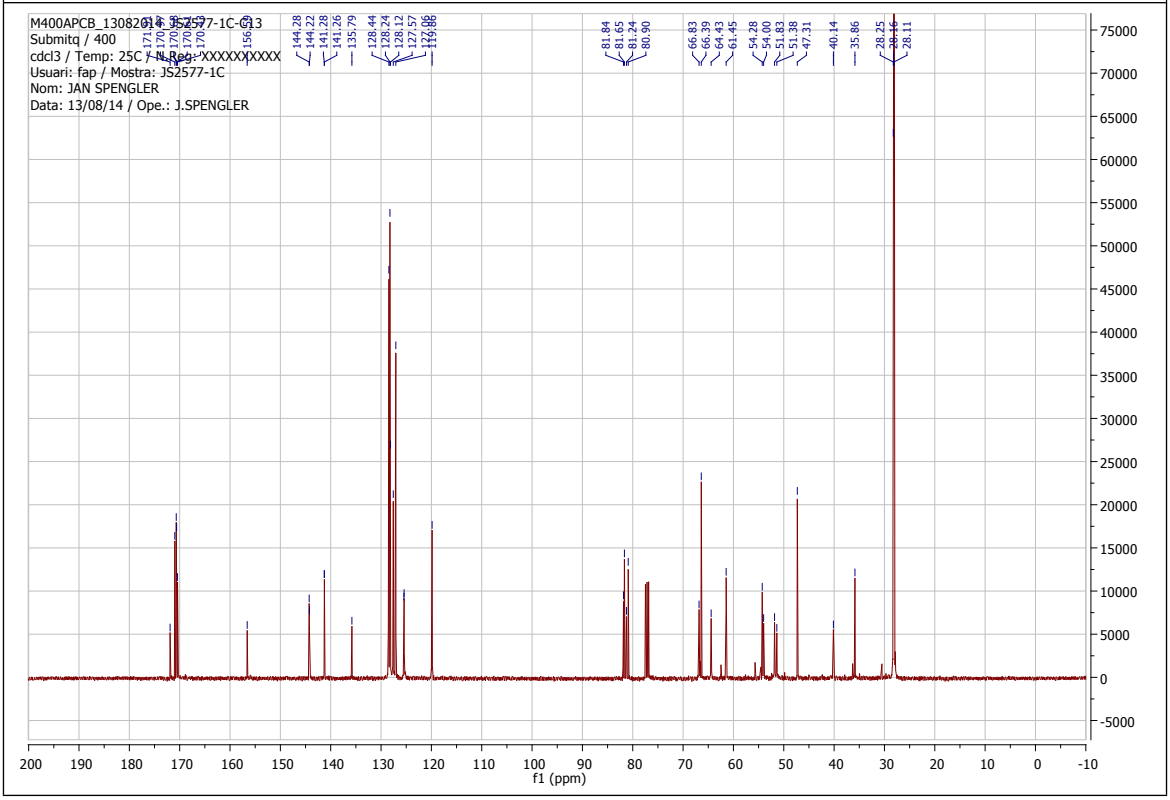
9: Fmoc-Dap/Asp-EDTA(^{t}Bu)-OBzl.

A solution of **3** (1.58 g, 3.19 mmol) and **8** (2.13 g, 4.26 mmol) in ACN (50 mL) was stirred vigorously with 2M phosphate buffer pH 8 (25 mL). The progress of the reaction was checked by HPLC. When **3** was converted (approx. after five days), the solution was decanted from the solid and concentrated in vacuum. $CHCl_3$ (30 mL) was added to the remainder (both liquid and solid) and it was dried with $MgSO_4$. The $CHCl_3$-phase was subjected to column chromatography on silica (3 cm * 10 cm, hexane:ethyl acetate 3:1, R_f = 0.25 UV 254 nm) to give **9** as oil (2.05 g, 70% resp. to **3**). The product was found to be inseparably contaminated with up to 25% of the side product **9b** (4-benzyl-1-(*tert*.-butyl)-(*S*)-2-(2-oxomorpholino)succinate) resulting from an intramolecular cyclization of **8** under basic conditions (additional signals in ^{1}H NMR and $[M-56+H]^+$ = 308 in HPLC-MS). ^{1}H NMR ($CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.67 (d, *J* = 6.9 Hz, 2H), 7.40-7.26 (m, 12H), 6.52 (d, *J* = 6.3 Hz, 1H), 5.13 (m, 0.5H), 5.04 (m, 2H), 3.11 (dd, *J* = 24.3, 10.7 Hz, 1H),4.31(m, 2H), 4.23 (m, 1H), 3.86 (m, 1H), 3.71 (m, 1H), 3.51-3.35 (m, 6H), 3.09 (m, 1H), 2.87-2.67 (m, 7H), 1.46-1.42 (m, 45H); ^{13}C NMR ($CDCl_3$) δ 171.9, 171.0, 170.7, 170.6, 170.4, 156.6, 144.3, 144.2, 141.3, 135.8, 128.4, 128.2, 128.1, 127.6, 127.1, 125.5, 125.4, 119.9, 81.8, 81.7, 81.2, 80.9, 66.8, 66.4, 64.4, 61.5, 54.3, 54.0, 51.8, 51.4, 47.3, 40.1, 35.9, 28.3, 28.2, 28.1; IR (film) 2978, 1724, 1369, 1151 cm^{-1}; $[\alpha]_D$25 = -43 (c=2, $CHCl_3$); accurate mass calc. $C_{51}H_{70}N_3O_{12}$ $[M+H^+]$ requires 916.49540, found 916.49633.

9 [Fmoc-Dap/Asp-EDTA(^{t}Bu)-OBzl]: ^{1}H NMR ($CDCl_3$) with additional signals of side product **9b** at 7.2, 5.1, 2.8 and 1.4 ppm



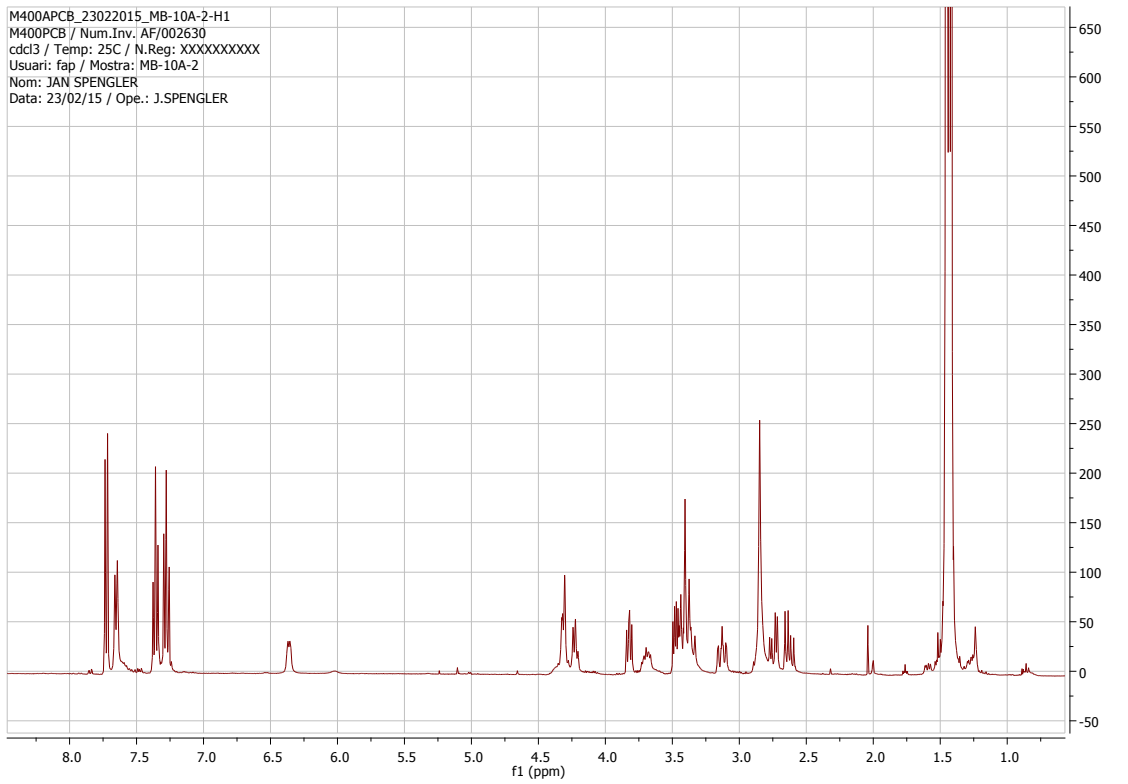
9 [Fmoc-Dap/Asp-EDTA(^{t}Bu)-OBzl]: ^{13}C NMR ($CDCl_3$)



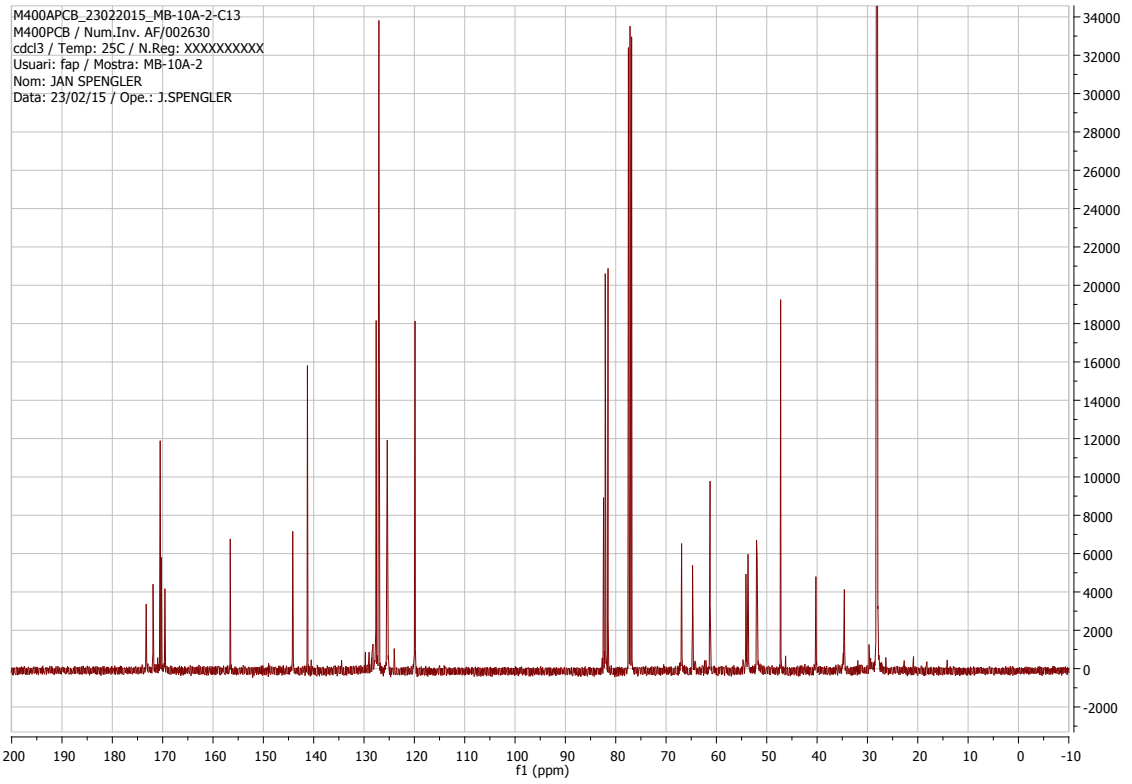
10: Fmoc-Dap/Asp-EDTA(^{t}Bu)-OH.

To a solution of **9** (2.0 g, 2.18 mmol) in absolute ethanol (100 mL) was added Pd-C catalyst (10% Pd basis, 150 mg, wetted with a drop of water). The mixture was stirred thoroughly under a hydrogen atmosphere (1 atm). The progress of the reaction was checked by HPLC. After 1.5 h the reaction was stopped (Total conversion of the starting material. Longer reaction times cause hydrogenolytic cleavage of the Fmoc group). It was filtered through Celite, evaporated and purified by flash chromatography on silica (DCM:MeOH:HOAc 15:1:0.07) to give an oil (R_f = 0.7). Solid **10** was obtained after lyophilization from ACN/water (1.45 g, 81%). ^{1}H NMR ($CDCl_3$) δ 7.73 (d, *J* = 7.5 Hz, 2H), 7.65 (d, *J* = 7.3 Hz, 2H), 7.36 (t, *J* = 7.4 Hz, 2H), 7.27 (t, *J* = 7.3 Hz, 2H), 6.36 (br d, *J* = 6.4 Hz, 1H), 4.30 (m, 2H), 4.22 (m, 1H), 3.82 (dd, *J* = 9.1, 6.4 Hz, 1H), 3.70 (m, 1H), 3.50-3.33 (m, 5H), 3.13 (m, 1H), 2.85 (s, 4H), 2.74 (dd, *J* = 16.7, 6.3 Hz, 1H), 2.63 (dd, *J* = 16.6, 9.2 Hz, 1H), 1.46-1.42 (m, 36 H); ^{13}C NMR ($CDCl_3$) δ 173.3, 171.9, 170.5, 170.3, 169.6, 156.6, 144.2, 144.1, 141.3, 127.6, 127.0, 125.4, 119.9, 82.4, 82.1, 82.0, 81.5, 66.9, 64.7, 61.3, 54.1, 53.7, 52.0, 51.9, 47.2, 40.2, 34.6, 28.2, 28.11, 28.08, 28.0; IR (KBr) 3343, 2978, 2934, 1729, 1527, 1368, 1245, 1149 cm-1; $[\alpha]_D^{25}$ = -15 (c=2, $CHCl_3$); accurate mass calc. $C_{44}H_{64}N_3O_{12}$ [$M+H^+$] requires 826.44845, found 826.44833.

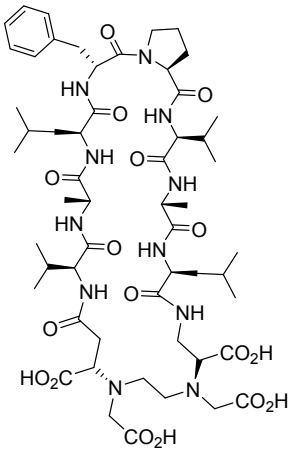
10 [Fmoc-Dap/Asp-EDTA(^{t}Bu)-OH]: ^{1}H NMR ($CDCl_3$)



10 [Fmoc-Dap/Asp-EDTA(^{t}Bu)-OH]: ^{13}C NMR ($CDCl_3$)



Peptide Synthesis



Molecular Weight: 1172,34

cP

Synthesis of peptide cP

Solid-phase reactions were performed in polystyrene syringes equipped with a porous polystyrene filter plate. Resins were stirred occasionally. Solvents were removed by vacuum-suction.

CTC resin (1.0 g) was swollen DCM/DMF 3:1 for 30 min. The solvents were removed and a mixture of **10** (290 mg, 0.35 mmol) and DIEA (600 µL, 3.5 mmol) in DCM was added and left for 2 h to react with the resin. MeOH was then added. After 10 min, the resin was washed (DCM).

Fmoc-groups were cleaved by two consecutive 10-min treatments of the resin (previously swollen in DCM) with 20% piperidine in DMF, then the resin was washed with DMF and DCM. Resin functionalization was determined by quantification of the UV-absorbance of dibenzofulvene-piperidide at 290 nm and was found to be 0.29 mmol/g.

Coupling reactions were performed in two consecutive 30 min-treatments with 1 mmol portions of Fmoc-Xaa activated with Oxyma/DIC in DCM/DMF (approx. 6 equiv. resp. to resin-loading). The completeness of the reaction was checked with the ninhydrine test (Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. Anal. Biochem. 1970, 34, 595). All unreacted material was washed from the resin with DMF followed by DCM (3 x 2 min).

For cleavage of the linear precursor, the resins were washed and swollen in DCM were treated five times with 10 mL-portions of 2% TFA in DCM, which were pooled and evaporated. The residue was lyophilized in ACN/H_2O to give the crude linear precursor.

Cyclizations of the linear precursor (100 mg, 0.071 mmol) were performed in DCM (70 mL, resulting peptide concentration 1 mM). A mixture of PyBOP (111 mg, 0.213 mmol) and HOBt*H_2O (32 mg, 0.213 mmol) in DMF (approx 0.5 mL) was added to the DCM-solution followed by DIEA (0.073 mL, 0.426 mmol). After stirring overnight, the solution was thoroughly washed with 1N HCl (2x30 mL), 10% $NaHCO_3$ (2x30 mL), and brine (2x30 mL), and was dried

over $MgSO_4$. After DCM removal the residue was dissolved in ACN (6 mL) and subjected to semi-preparaive HPLC. A gradient of 70 to 100% ACN in 10 min was used. The peptide eluted as a broad hedgehog-shaped peaks (with face left) over several fractions. Most of the ACN was removed from pooled fractions with a rotary evaporator. Precipitated peptide was re-dissolved by addition of ACN. Lyophilization gave cyclic and protected **cP** peptide as white flocks.

For final deprotection, the cyclic and protected **cP** peptide were dissolved in TFA/H_2O/TIPS (2 mL, 95:2.5:2.5). It was stirred for 15 h (the deprotection is slow). The volatiles were removed by a N_2-stream. The remainders were dissolved in H_2O/ACN and lyophilized to give pure c**P**.

cP: cyclo(Val-Ala-Leu-DPhe-Pro-Val-Ala-Leu-EDTA).

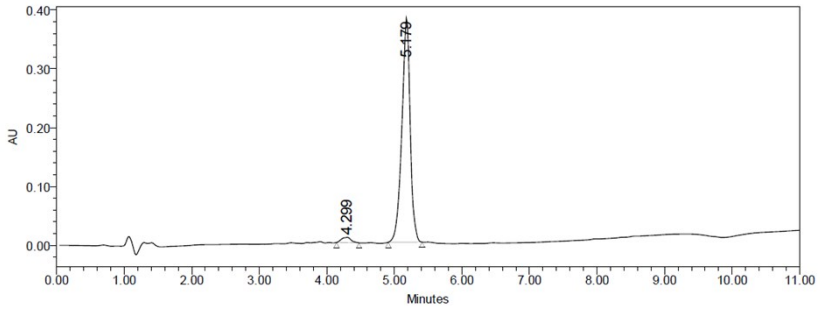
0.5 g CTC resin (f = 0.29 mmol/g) gave crude linear precursor (250 mg, 76% HPLC). HPLC (10-100): 7.8 min.; MALDI: 1415.1 $[M+H]^+$, 1437.2 $[M+Na]^+$.

Cyclization of 100 mg yielded after HPLC-purification **protected cP** (36 mg). HPLC (30-100): 8.5 min.

Deprotection (39 mg) yielded **cP** as white powder (lyophilized for 3 days, 19 mg, 97% HPLC). HPLC (30-100): 5.2 min.; MALDI: 1172.5 $[M+H]^+$, 1194.5 $[M+Na]^+$.

cP HPLC

Sample Name:	JS2688P2a	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	12/4/2015 11:22:11 AM
Vial:	44	Acq. Method Set:	A30100t8T25
Injection #:	1	Date Processed:	12/4/2015 11:52:11 AM
Injection Volume:	5.00 ul	Processing Method:	defaulc
Run Time:	14.0 Minutes	Channel Name:	Wvln Ch1
Sample Set Name:	031215	Proc. Chnl. Descr.:	PDA 220.0 nm



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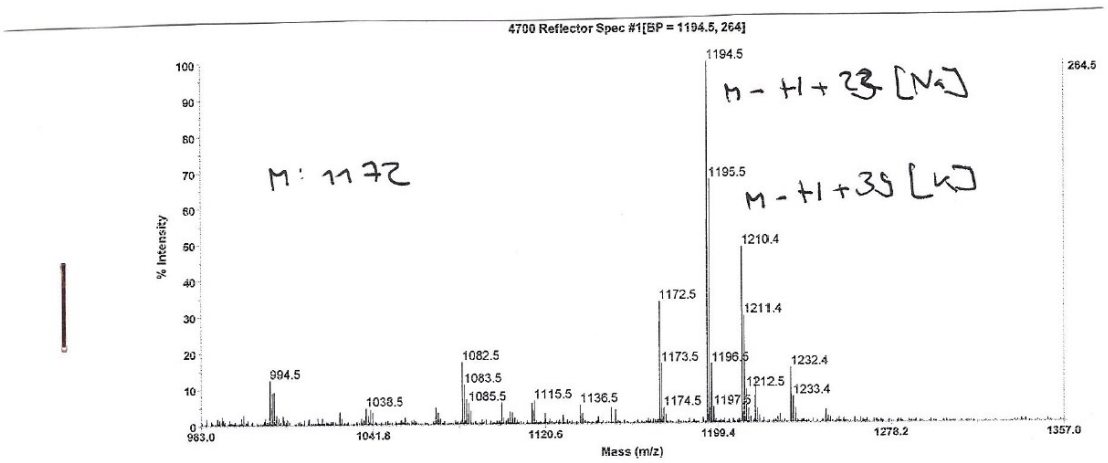
cP MALDI



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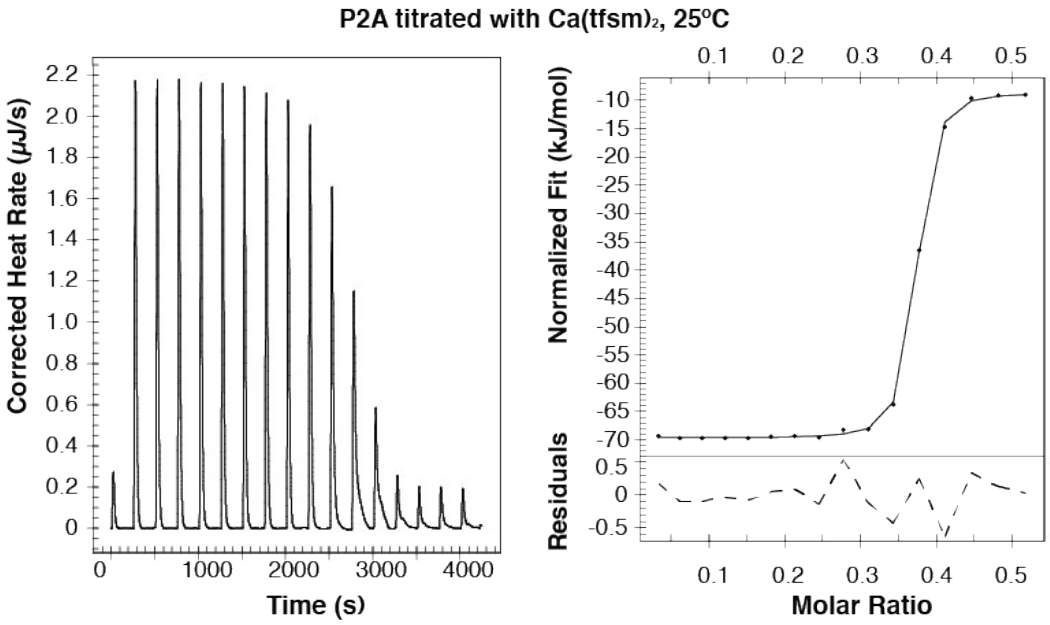
CHARACTERIZATION OF THE PEPTIDE (cP) – CA^{2+} INTERACTION

Isothermal Titration Calorimetry (ITC): ITC binding experiments were performed in a nano ITC calorimeter (TA Instruments) at 25 ºC and 37 ºC, respectively. All compounds were weighed with a high precision balance and dissolved in 20 mM Tris-HCl pH 7.2. Peptide solutions were heated to 70 ºC and filtered. All samples were degassed and centrifuged prior to the experiments. For binding experiments, sigmoidal curves were optimized adding 48 µL of the ligand solution to the 190 µL of peptide and data was collected while stirring at 220 revolutions per minute (rpm) to facilitate the binding reaction. Depending on the affinity, a 1.1 to 3.2 fold ligand excess was added to the peptide (140 µM) in 32 steps of 1.5 µL with a delay of 3 minutes between injections. The NanoAnalyze software (TA Instruments) was used to analyze the binding isotherms assuming a single binding site in each molecule. Baseline controls were acquired with buffer and pure ligand solutions. All measurements have been repeated at least twice. **ITC Results: cP** peptide with Ca(tfms): @25ºC K_D = 41.6 ± 3.3 nM; n = 0.359 ± 0.001; @37ºC K_D = 68.9 ± 4.8 nM, n = 0.137 ± 0.001.



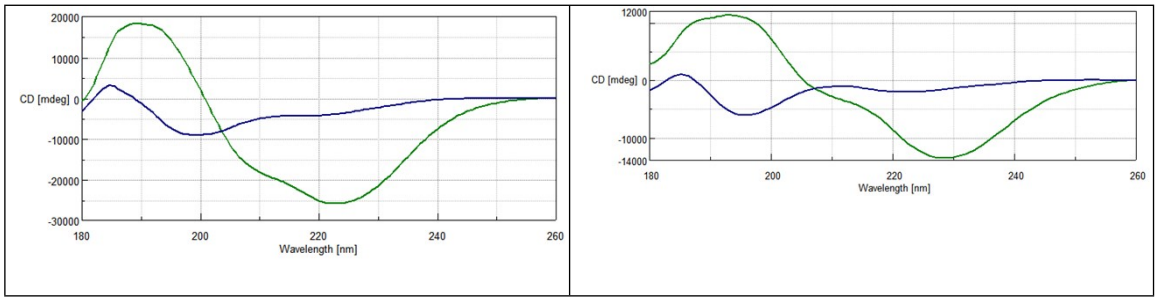
Titration of **cP** with $Ca(tfms)_2$ using Isothermal Calorimetry. The data was fitted using a two independent site models. The residuals of the fitting are also included.

Circular Dichroism (CD): CD measurements were performed with a Spectropolarimeter JASCO model 815, under nitrogen flux. Spectra were recorded in a 2 mm-cuvette at ambient

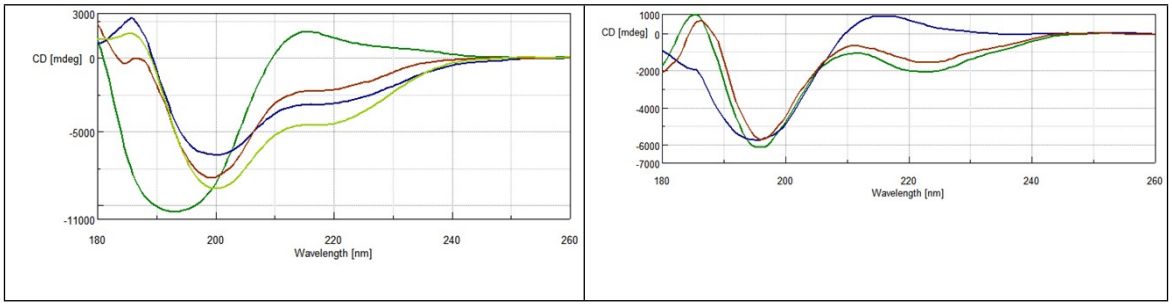
temperature with following parameters: sensibilty: standard; D.I.T. 2 sec; band width: 1 nm; range 260-180 nm; data pitch: 0.5 nm; scan rate: 50 nm/min, three scans were performed.

Raw data were normalized by multiplication with a factor {(M [g*mol^{-1}]/number amide bonds)/c [mg*mL^{-1}]*d [cm]*10} and put to 0 at 260 nm. For smoothening, the Savitzky-Golay factor 21 was applied.

Samples were prepared from peptide solutions [2 mM in TFE/water 1:1, adjusted to pH 8.5) and calciumtrifluoromethanesulfonate (10 mM in TFE/water 1:1, adjusted to pH 10.5)] by dilution in sodium phosphate buffer (pH 7.2) to the indicated concentrations.



Peptides **1** and **2** (0.1 mM) in 10 mM phosphate buffer pH 7.2. Left: **P1a** (green) *vs* **P2a** (blue).



Peptides **2** (0.1 mM) in 10 mM phosphate buffer pH 7.2. Left: **P2a** (light green); **P2a**+1 equiv Ca (dark green); =**P2a**+1 equiv Ca+$HClO_4$ to pH1 (blue); **P2a**+1 equiv Ca+EDTA (red). Right: **P2b** (green); **P2b** + 1 equiv Ca (blue); **P2b** + 1 equiv Ca+EDTA (red).

Nuclear Magnetic Resonance (NMR):

NMR experiments were carried out on a Bruker Avance III 600 MHz spectrometer equipped with a cryoprobe. Samples were prepared in 20 mM Tris buffer (pH 7.0), 10% D_2O at 1.3 mM peptide concentration. Residue specific assignments were obtained from bidimensional COSY, TOCSY and ROESY experiments. The TOCSY and ROESY mixing times were 70 and 200 ms, respectively. ^{13}C resonances were assigned from 2D ^{1}H-^{13}C HSQC spectra. Bidimensional experiments were performed at 25 °C except ROESY spectra that were acquired at two temperatures (15 and 25 °C). For the determination of the temperature coefficients of the

amide protons, the ^{N}H chemical shifts were plotted versus the acquisition temperature (20, 25, 30, 35 and 40 °C) and the data points were adjusted to a linear fit.

In the absence of Ca^{2+}, a major species was observed for peptide **cP**. The configuration of the D-Phe-Pro bond is *trans*, as confirmed by the ^{13}Cβ - ^{13}Cγ chemical shift difference observed (Δδ = 5.3 ppm). The chemical shifts are listed in Tables S1 and S2.

The relatively low dispersion of Hα and NH amide proton signals suggests backbone flexibility (Figure S1). The NH resonances of Phe4, Ala7 and Leu8 that appeared as broad signals at 25 °C were sharpened when the temperature was increased. This behavior indicates exchange broadening effects due to inter-conversion between distinct conformations, which are observable under these experimental conditions. In agreement with this observation, the temperature coefficients for the amide hydrogens showed intermediate values (Table S5). Remarkably, the D-Phe4 NH group exhibited the lowest temperature dependence ($\Delta\delta/\Delta T$ = -3.5 ppb K^{-1}) perhaps due to its orientation towards the interior of the β-turn-like structure. This orientation is favored by the trans configuration of the DPhe-Pro bond [1].

The difference between observed and random coil ^{13}Cα chemical shifts showed negative values for Leu3, Phe4, Val6 and Ala7 suggesting the presence of conformations populating β–sheet structures for this particular turn (Figure S5). The magnitude of $^{3}J_{\alpha NH}$ coupling constants is also sensitive to the local conformation of the polypeptide backbone. However, due to broadening and/or signal overlap only the $^{3}J_{\alpha NH}$ values of three residues could be measured: Ala2 (6.0 Hz), Leu3 (9.0 Hz) and Val6 (7.8 Hz) (Table S5). The relative high $^{3}J_{\alpha NH}$ values for Leu3 (9.0 Hz) and Val6 (7.8 Hz) also support the presence of beta-turn stabilized conformations.

The ROESY spectra of peptide **cP** (Figure S3) exhibited strong intra-residue and sequential dαN (i, i+1) NOE connectivities, characteristic of peptides displaying extended conformations (strands and beta-turns), with dαN (i, i+1) distances of ~2.2 Å and strong dαN (i, i+1) ROE cross-peaks.. Non-sequential inter-residue ROEs were not observed either at 15 or 25 °C.

[1] Schubert, M., Labudde, D., Oschkinat, H. & Schmieder, P. A software tool for the prediction of Xaa-Pro peptide bond conformations in proteins based on 13C chemical shift statistics. Journal of Biomolecular NMR 24, 149-154 (2002).

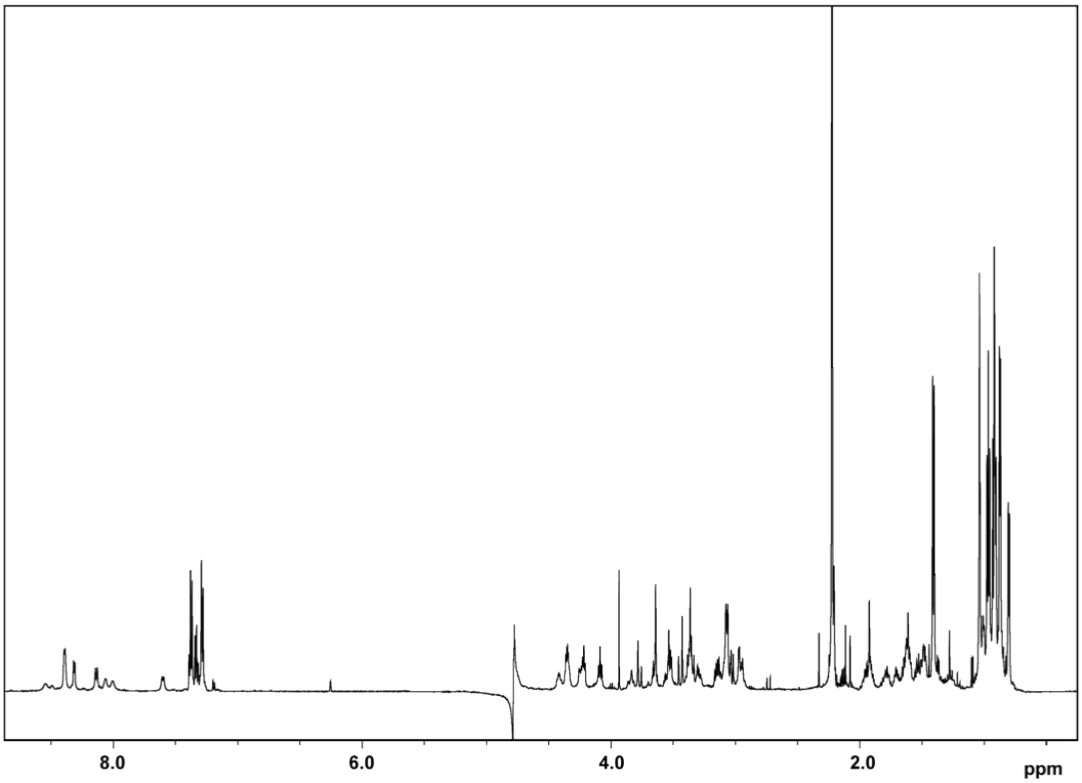


Figure S1. 1D ^{1}H NMR spectrum at 25°C of peptide **cP** (1.3 mM) in the absence of Ca^{2+} in 20 mM Tris (pH = 7.0), 10% D_2O.

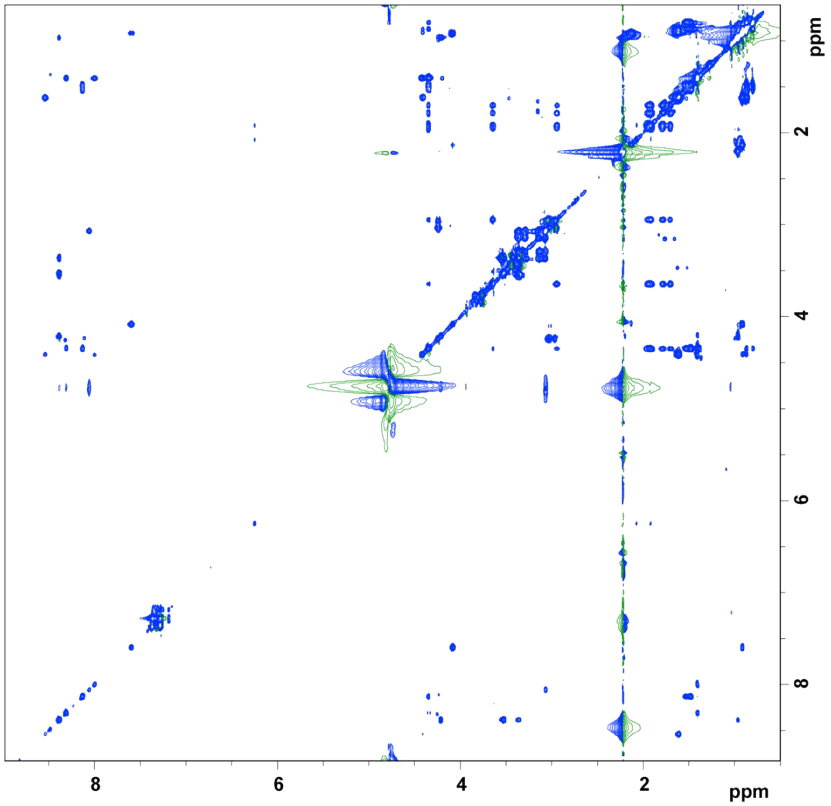


Figure S2. 2D TOCSY spectrum at 25°C of peptide **cP** (1.3 mM) in the absence of Ca^{2+} in 20 mM Tris (pH = 7.0), 10% D_2O.

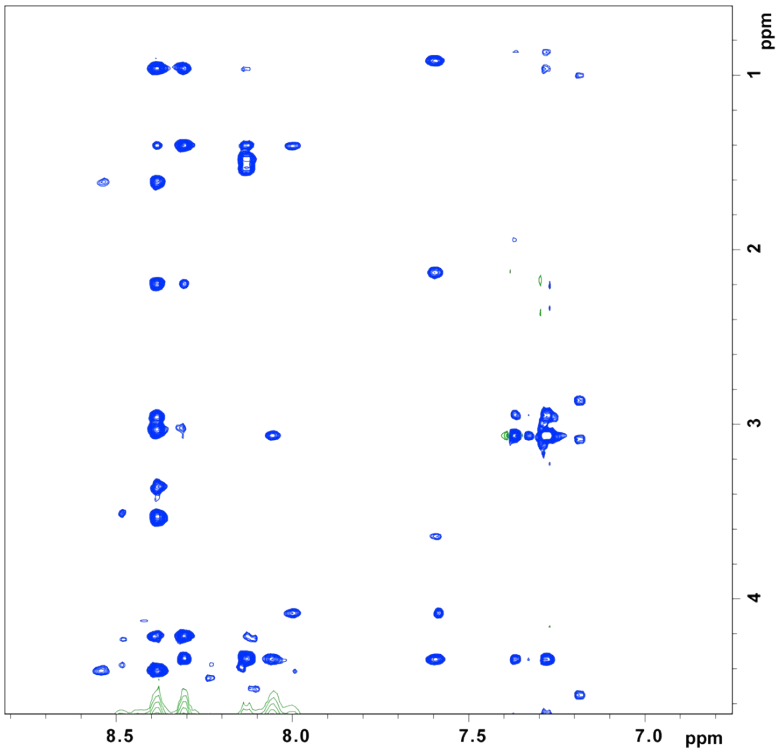


Figure S3. Portion of the 2D ROESY spectrum at 25°C of peptide **cP** (1.3 mM) in the absence of Ca^{2+} in 20 mM Tris (pH = 7.0), 10% D_2O.

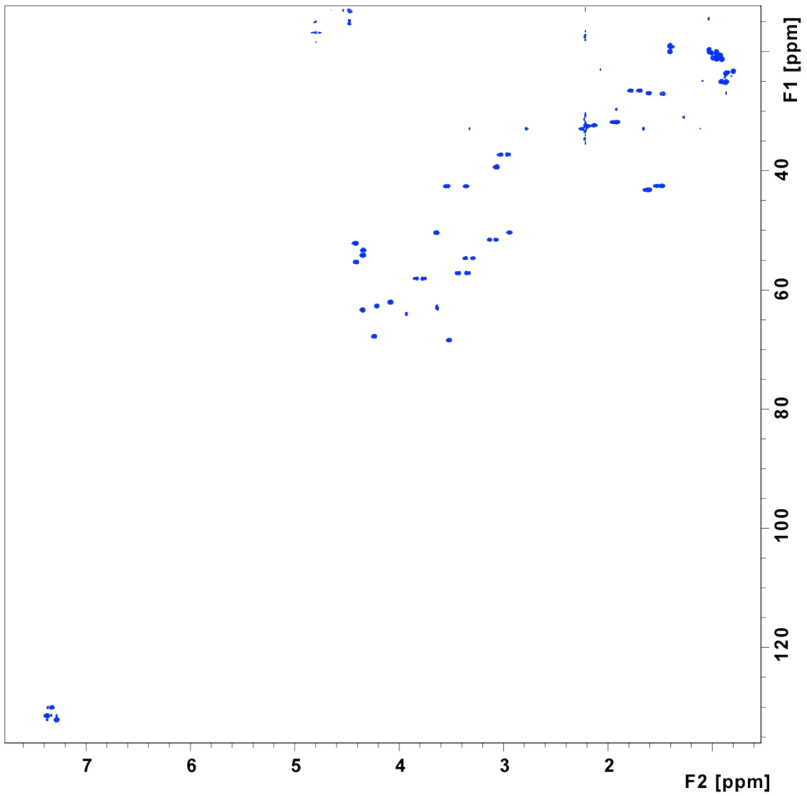


Figure S4. 2D ^{1}H ^{13}C HSQC spectrum of peptide **cP** (1.3 mM) at 25°C, in the absence of Ca^{2+} in 20 mM Tris (pH = 7.0), 10% D_2O.

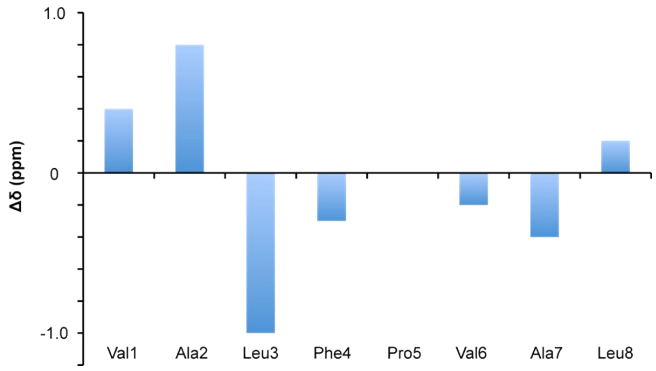


Figure S5. Difference between observed $^{13}C\alpha$ chemical shifts of peptide **cP** in the absence of Ca^{2+} and random coil values (Wishart *et al.* J Biomol NMR, 6(2):135-140).

We acquired 1D ^{1}H NMR spectra of the peptide in the presence of increasing amounts of Ca^{2+}. As illustrated in Figure S6, at substoichiometric amounts of Ca^{2+}, resonances characteristic for the Ca^{2+}-free state dissappeared with incremental increases of Ca^{2+} with the simultaneous appeareance of a second set of resonances. This behavior is characteristic of slow exchange in the NMR time scale between the Ca^{2+}-free and Ca^{2+}-bound forms of the peptide. When the concentration of Ca^{2+} exceeded that of peptide only minor changes in chemical shifts were observed.

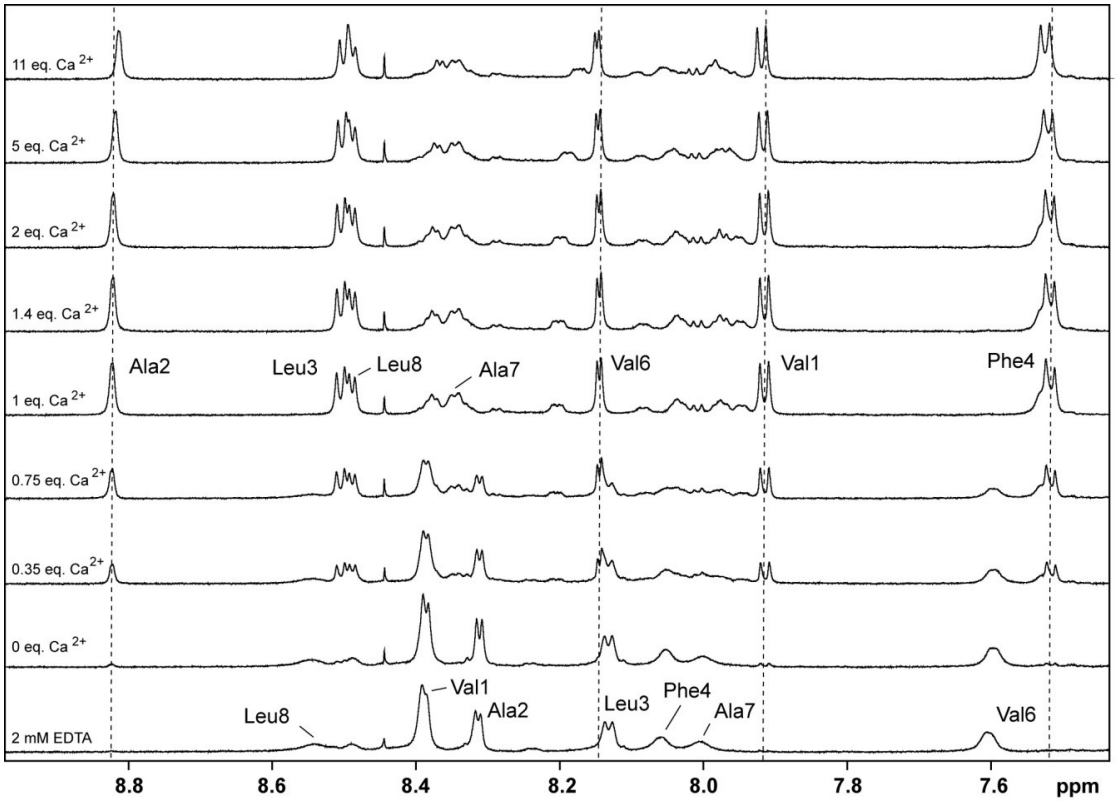


Figure S6. Effects of Ca^{2+} in the amide region of peptide **cP**.

To characterize the peptide Ca^{2+}-bound state, we performed 2D homonuclear TOCSY, COSY, and ROESY and heteronuclear ^{1}H ^{13}C HSQC NMR experiments in the presence of equimolar amounts of Ca^{2+}. A subset of minor NMR signals coexisting with those of the major Ca^{2+}-bound species was present in the spectra of **cP**. The chemical shifts of the major species are listed in tables S3 and S4. The configuration of the Phe-Pro bond is trans, as confirmed by the $^{13}C\beta$ - $^{13}C\gamma$ chemical shift difference observed ($\Delta\delta$ = 5.1 ppm) and the observation of a sequential ROE correlation between the Hα proton of Phe4 and the Hδ proton of Pro5 .

As shown in Figure S11, the addition of Ca^{2+} induced significant ^{13}C chemical shift changes. The differences between observed and random coil $^{13}C\alpha$ chemical shifts are showed in Figure S12. In the presence of Ca^{2+}, the $^{13}C\alpha$ chemical shifts of Leu3, Phe4, Pro5, and Ala7 exhibited negative deviations from random coil values whereas Val6 showed the opposite deviation (Figure S12)[2]. Interestingly, as seen in Table S5, the value of the $^{3}J_{\alpha NH}$ coupling of Val6 in the Ca^{2+}-bound state (4.5 Hz) is remarkable lower than that of the free state (7.8 Hz) or the typical value (>8.0 Hz) expected for a well-defined β–strand conformation. Taking together, these observations suggest that Ca^{2+}-binding may induce a distortion in the β-turn-like structure of **cP**.

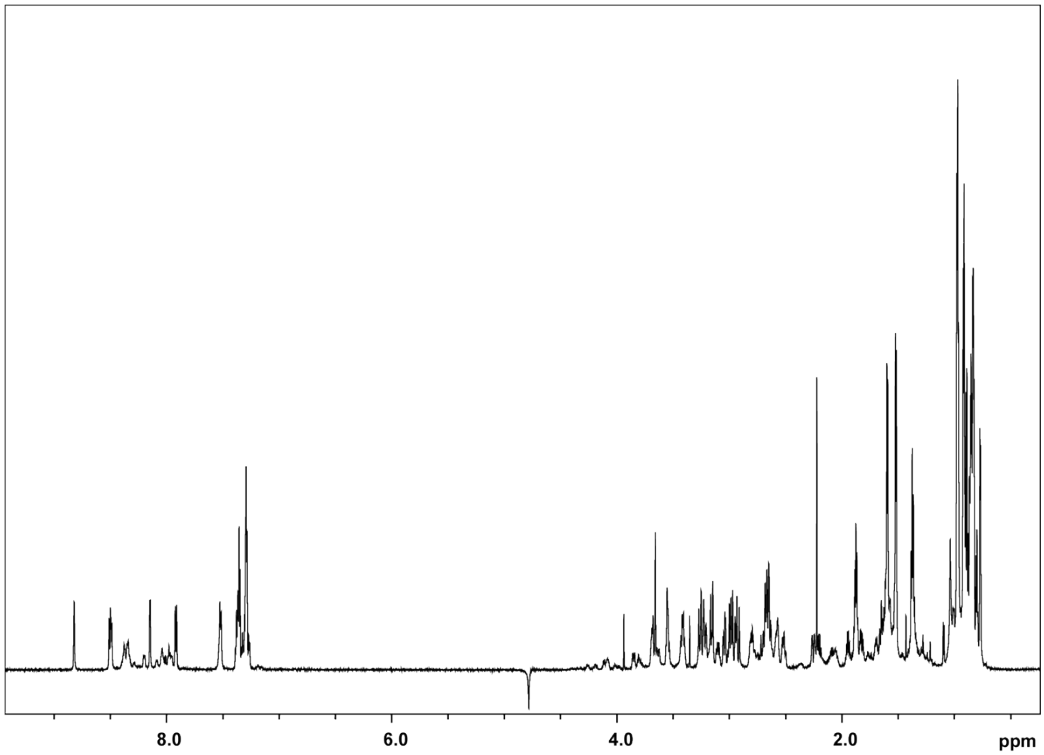


Figure S7. 1D ^{1}H NMR spectrum at 25°C of peptide **cP** (1.3 mM) in 20 mM Tris (pH = 7.0), 10% D_2O in the presence of equimolar amounts of Ca^{2+}.

[2] Schubert, M., Labudde, D., Oschkinat, H. & Schmieder, P. A software tool for the prediction of Xaa-Pro peptide bond conformations in proteins based on 13C chemical shift statistics. Journal of Biomolecular NMR 24, 149-154 (2002).

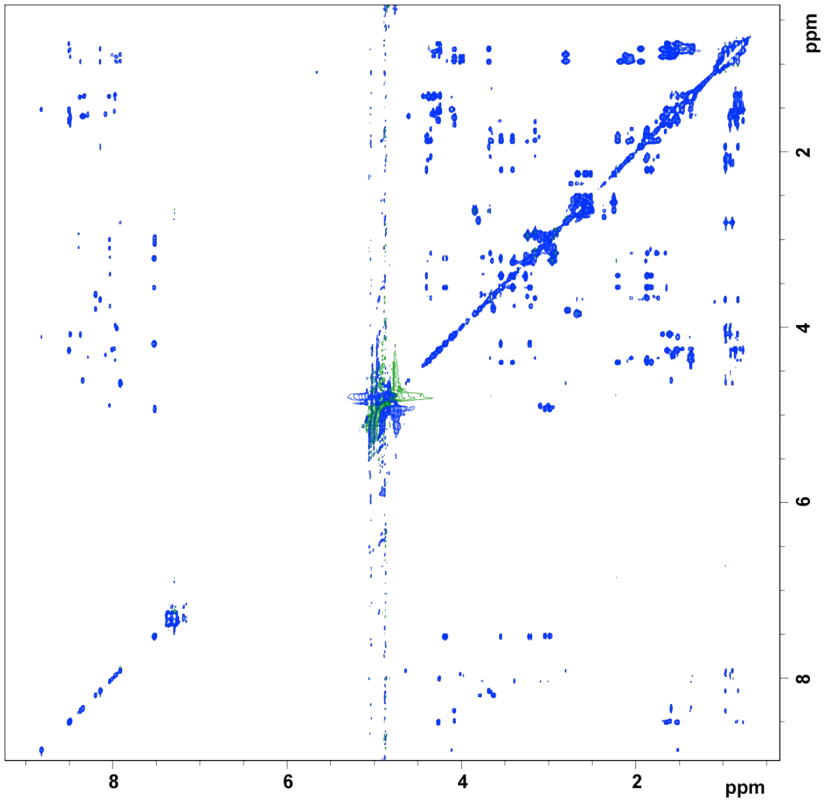


Figure S8. 2D TOCSY spectrum at 25°C of peptide **cP** (1.3 mM) in 20 mM Tris (pH = 7.0), 10% D_2O in the presence of equimolar amounts of Ca^{2+}.

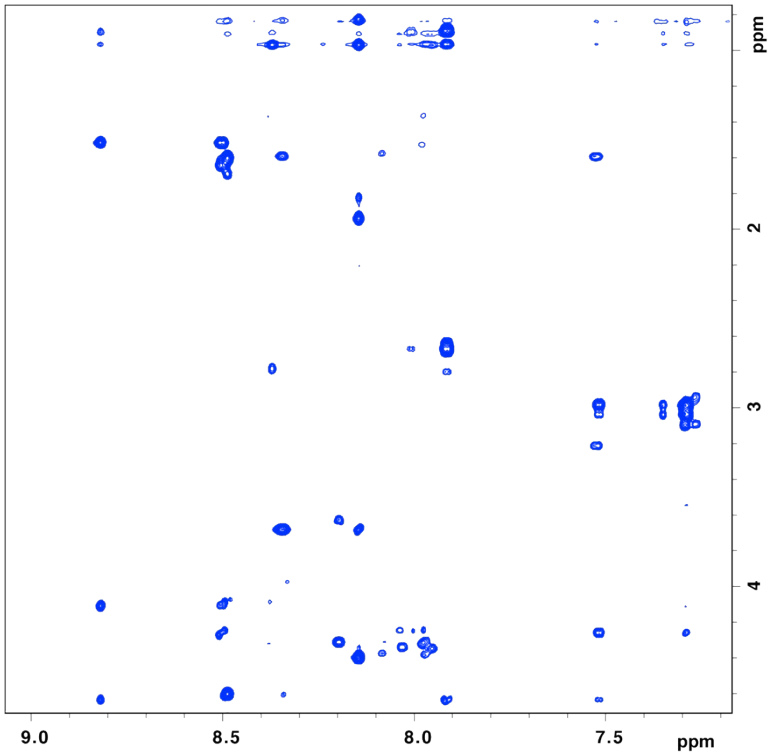


Figure S9. Portion of the 2D ROESY spectrum at 25°C of peptide **cP** (1.3 mM) in 20 mM Tris (pH = 7.0), 10% D_2O in the presence of equimolar amounts of Ca^{2+}.

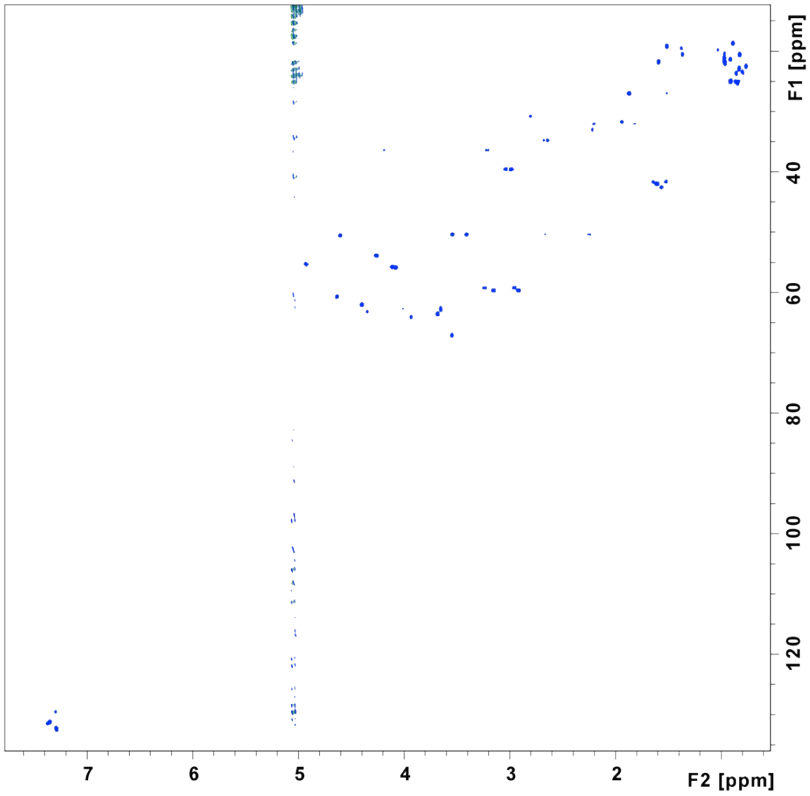


Figure S10. 2D ^{1}H ^{13}C HSQC spectrum at 25°C of peptide **cP** (1.3 mM) in 20 mM Tris (pH = 7.0), 10% D_2O in the presence of equimolar amounts of Ca^{2+}.

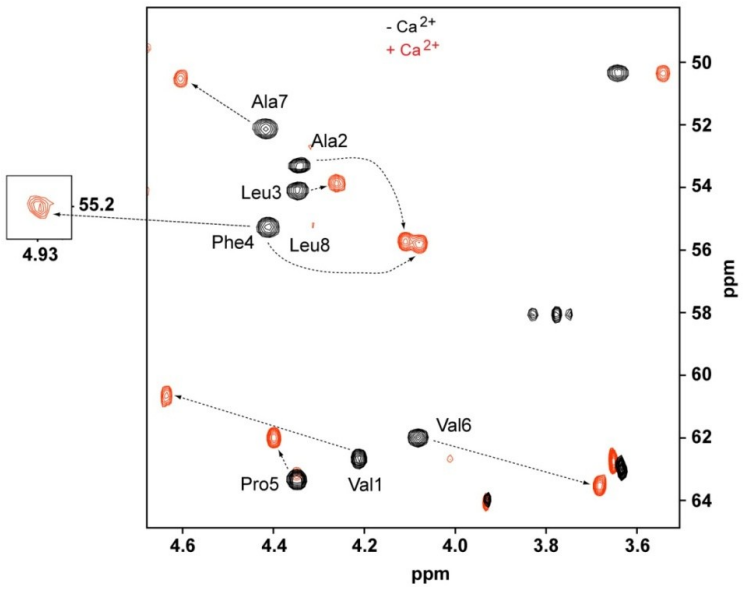


Figure S11. Comparison of the ^{1}H ^{13}C HSQC spectra (only the alpha carbon region) at 25°C of peptide **cP** in the presence (red) or absence of Ca2+ (black), showing the changes in Cα chemical shifts induced by the presence of equimolar amounts of Ca^{2+}.

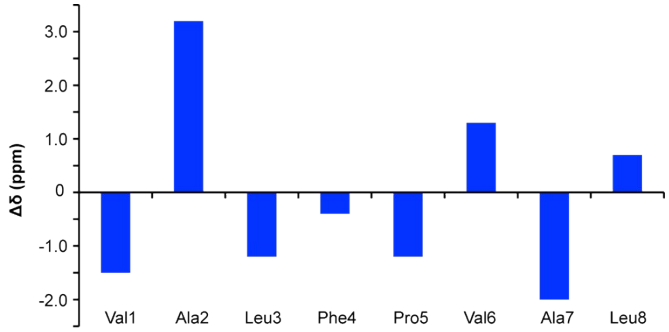


Figure S12. Difference between observed $^{13}C\alpha$ chemical shifts of peptide **cP** in the presence of Ca^{2+} and random coil values (Wishart *et al.* J Biomol NMR, 6(2):135-140).

Table S1. ^{1}H chemical shifts (ppm) of peptide **cP** in the absence of Ca^{2+}

Residue	NH	α	β	others
Val1	8.39	4.21	2.21	γ 0.97
Ala2	8.31	4.34	1.40	-----
Leu3	8.14	4.35	1.52, 1.48	γ 1.48 δ 0.87, 0.80
Phe4	8.06	4.41	3.07	δ 7.28 ε 7.37 ζ 7.33
Pro5	---	4.35	1.95, 1.91	γ 1.78, 1.70 δ 3.64, 2.94
Val6	7.60	4.08	2.13	γ 0.92
Ala7	8.00	4.42	1.41	-----
Leu8	8.54	4.41	1.62	γ 1.61 δ 0.91, 0.86

All chemical shifts were measured at 25 °C and referenced relative to internal DSS (0 ppm).

Table S2. ^{13}C chemical shifts (ppm) of peptide **cP** in the absence of Ca^{2+}

Residue	α	β	others
Val1	62.6	32.4	γ 21.0, 20.1
Ala2	53.3	19.1 or 19.9	------
Leu3	54.1	42.5	γ 27.1 δ 23.6, 23.2
Phe4	55.3	39.3	δ 132.0 ε 131.4 ζ 130.0
Pro5	63.3	31.8	γ 26.5 δ 50.3
Val6	62.0	32.3	γ 21.2, 20.7
Ala7	52.1	19.1 or 19.9	-------
Leu8	55.3	43.2	γ 27.0 δ 25.1, 25.0

Table S3. ^{1}H chemical shifts (ppm) of peptide **cP** in the presence of Ca^{2+}

Residue	NH	α	β	others
Val1	7.92	4.60	2.81	γ 0.96, 0.89
Ala2	8.82	4.11	1.52	-----
Leu3	8.50	4.27	1.64, 1.52	γ 1.51 δ 0.85, 0.77
Phe4	7.52	4.93	3.04, 2.98	δ 7.29 ε 7.35 ζ 7.30
Pro5	---	4.40	2.21, 1.82	γ 1.87 δ 3.54, 3.41
Val6	8.15	3.69	1.95	γ 0.97, 0.82
Ala7	8.35	4.60	1.59	-----
Leu8	8.49	4.08	1.60	γ 1.69 δ 0.91, 0.83

All chemical shifts were measured at 25 °C and referenced relative to internal DSS (0 ppm).

Table S4. ^{13}C chemical shifts (ppm) of peptide **cP** in the presence of Ca^{2+}

Residue	α	β	others
Val1	60.7	30.9	γ 21.8, 18.6
Ala2	55.7	19.2	------
Leu3	53.9	41.6	γ 27.0 δ 23.6, 22.5
Phe4	55.2	39.6	δ 132.3 ε 131.3 ζ 129.5
Pro5	62.1	32.0	γ 26.9 δ 50.4
Val6	63.5	31.7	γ 21.6, 20.5
Ala7	50.5	21.7	-------
Leu8	55.8	40.9	γ 27.1 δ 25.0, 22.9

Table S5. $^{3}J_{HNH}\alpha$ coupling constants and temperature coefficients of NH amide resonances of peptide **cP** in the absence and in the presence of Ca^{2+}. Some $^{3}J_{HNH}\alpha$ values could not be determined because the resonances were broad (b) or due to signal overlap (ov).

	- Ca^{2+}		+ Ca^{2+}	
Residue	$^{3}J_{HNH}\alpha$ (Hz)	$d\delta/dT$ (ppb K^{-1})	$^{3}J_{HNH}\alpha$ (Hz)	$d\delta/dT$ (ppb K^{-1})
Val1	ov	-4.9	9.5	-1.8
Ala2	6.0	-4.8	b	-10.2
Leu3	9.0	-4.9	8.0	-6.8
Phe4	b	-3.5	9.1	2.0
Pro5	--	--	--	--
Val6	7.8	-7.7	4.5	-10.3
Ala7	b	-6.6	ov	-5.2
Leu8	b	-6.0	7.0	-10.0