Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Conformational Stability of Collagen Triple Helices Functionalized in the Yaa Position by Click Chemistry

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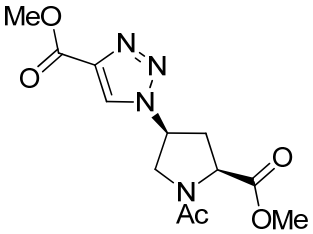
General aspects

Materials and reagents were of the highest commercially available grade and used without further purification. For solid phase peptide synthesis Rink Amide-ChemMatrix resin from pcas BioMatrix (Saint-Jean-sur-Richelieu, Canada) was used. Reactions were monitored by thin layer chromatography using Merck silica gel 60 F254 plates. Compounds were visualized by UV and ninhydrin. Flash chromatography was performed using Merck silica gel 60, particle size 40 - 63 µm. ^{1}H and ^{13}C NMR spectra were recorded on Bruker DPX 500 and DPX 400 spectrometers. Chemical shifts are reported in ppm using TMS as a reference. A Bruker Esquire 3000plus instrument was used for electrospray ionization (ESI) mass spectrometry measurements. High resolution mass spectra were recorded on a LTQ Orbitrap XL spectrometer from Thermo Fischer Scientific with a nanoelectrospray ion source at the University of Bern, Switzerland. Analytical HPLC was performed using a LiChrospher 100 RP-18e 5 µm (250 mm x 4 mm) column from Merck. Preparative HPLC was carried out on a LiChrospher RP-18e 5 µm (250 mm x 10 mm) column from Merck. For some peptides a Jupiter 4 µm Proteo 90 Å column (250 mm x 10.0 mm) was used. A Chirascan (Applied Photophysics Ltd, Leatherhead, UK) was used for CD measurements. The solutions were measured in a quartz cell with a pathlength of 1.0 mm (Hellma 110-QS). For automated peptide synthesis, a Syro I Peptide Synthesizer (MultiSynTech GmbH, Witten, Germany) was employed.

Synthesis of the Ac-(4S)Xaa-OMe model compounds 7S-10S

Synthesis of methyl ester functionalized triazolyl proline 7S



Ac-(4*S*)Azp-OMe (0.15 mmol, 1.0 eq) was dissolved in 100 µl ^{t}BuOH and 200 µL H_2O were added. After the addition of propargyl methylester (3.0 eq) a freshly prepared aqueous solution of 0.4 M $CuSO_4 \cdot 5H_2O$ (0.1 eq) and a 1 M solution of sodium ascorbate (0.2 eq) was added. The suspension was stirred at room temperature for 20 h. The reaction mixture was diluted with 5 ml water and extracted with EtOAc (4x 1 ml). The combined organic layers were concentrated under vacuum and the residue was purified by flash chromatography.

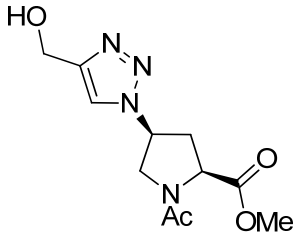
The NMR-spectrum showed two conformers (*cis* and *trans* conformation around the tertiary amide bond) in a ratio of 1:2.7 in D_2O.

^{1}H-NMR (500 MHz, D_2O, 25°C): δ/ppm (major conformer) = 8.68 (s, 1H; triazole), 5.43 (m, 1H; Hγ), 4.72 (dd, *J* = 9.3 Hz, 4.1 Hz, 1H; Hα) 4.36 (dd, *J* = 12.1 Hz, 4.2 Hz, 1H; Hδ), 4.32 (dd, *J* = 12.1 Hz, 6.2 Hz, 1H; Hδ), 3.92 (s, 3H; OCH_3 (triazole)), 3.63 (s, 3H; OCH_3 (Pro)), 3.00 (ddd, *J* = 14.3 Hz, 9.4 Hz, 6.4 Hz, 1 Hz, 1H; Hβ), 2.89 (dt, *J* = 14.3 Hz, 4.3 Hz, 1H; Hβ), 2.17 (s, 3H; Ac). (minor conformer) = 8.64 (s, 1H; triazole), 5.38 (m, 1H; Hγ), 4.98 (dd, *J* = 8.1 Hz, 3.1 Hz, 1H; Hα), 4.20 (dd, *J* = 13.7 Hz, 2.9 Hz, 1H; δ), 4.15 (dd, *J* = 13.9 Hz, 5.8 Hz, 1H; Hδ), 3.92 (s, 3H; OCH_3 (triazole)), 3.62 (s, 3H; OCH_3 (Pro)), 3.06 (m, 1H; Hβ), 2.07 (s, 3H; Ac). **^{13}C-NMR** (126 MHz, D_2O, 25°C): δ/ppm (major conformer) = 173.2, 172.9, 162.1, 139.2, 128.7, 59.4, 57.6, 53.0, 52.6, 52.3, 34.1, 21.3. (minor conformer) = 174.0, 172.9, 162.0, 139.2, 128.7, 59.2, 58.5, 53.2, 52.6, 51.4, 35.8, 21.2.

The conformers were assigned based on a NOESY-spectrum.

MS (ESI): *m/z* = 319.2 $[M+Na]^+$ (10%), 615.0 $[2M+Na]^+$ (100%), M = 296.11 g mol^{-1} calcd for $C_{12}H_{16}N_4O_5$.

Synthesis of hydroxy functionalized triazolyl proline 8S



Ac-(4*S*)Azp-OMe (0.15 mmol, 1.0 eq) was dissolved in 100 µl ^{t}BuOH and 200 µL H_2O were added. After the addition of propargyl methylester (3.0 eq) a freshly prepared aqueous solution of 0.4 M $CuSO_4 \cdot 5H_2O$ (0.1 eq) and a 1 M solution of sodium ascorbate (0.2 eq) was added. The suspension was stirred at room temperature for 20 h. The reaction mixture was diluted with 5 ml water and extracted with CH_2Cl_2 (4x 3 ml). The combined organic layers were concentrated under vacuum and the residue was purified by flash chromatography.

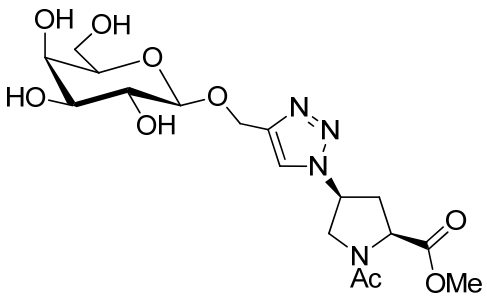
The NMR-spectrum showed two conformers (*cis* and *trans* conformation around the tertiary amide bond) in a ratio of 1:2.7 in D_2O.

^{1}H-NMR (400 MHz, D_2O, 25°C): δ/ppm (major conformer) = 7.96 (s, 1H; triazole), 5.26-5.20 (m, 1H, Hγ), 4.61 (dd, *J* = 9.3 Hz, 4.3 Hz, 1H; Hα), 4.58 (s, 2H; C*H*$_2$OH), 4.21 (dd, *J* = 12.0 Hz, 4.1 Hz, 1H; Hδ), 4.16 (dd, *J* = 12.0 Hz, 6.3 Hz, 1H; Hδ), 3.50 (d, 3H; OMe), 2.91-2.80 (m, 1H; Hβ), 2.05 (s, 3H; Ac). (minor conformer) = minor: 7.92 (s, 1H; triazole), 5.20-5.16 (m, 1H; Hγ), 4.83 (d, *J* = 8.8 Hz, 1H; Hα), 4.56 (s, 2H; C*H*$_2$OH), 4.09 (d, *J* = 13.5 Hz, 1H; Hδ), 3.99 (dd, *J* = 13.6 Hz, 6.3 Hz, 1H; Hδ), 3.49 (s, 3H, OMe), 2.95 (d, *J* = 14.5 Hz, 1H; Hβ), 2.74 (dt, *J* = 14.2 Hz, 4.4 Hz, 1H; Hβ), 1.95 (s, 3H; Ac).

^{13}C-NMR (126 MHz, $CDCl_3$, 25°C): δ/ppm (major conformer) = 171.5 (amide), 169.4 (ester), 148.4 (triazole), 120.6 (triazole), 58.1 (Cγ), 57.2 (Cα), 56.5 (CH_2OH), 52.7 (OMe), 52.4 (Cδ), 34.8 (Cβ), 22.3 (Ac).

MS (ESI): *m/z* = 291.1 $[M+Na]^+$ (100%), 559.2 $[2M+Na]^+$ (51%), M = 268.12 g mol^{-1} calcd for $C_{11}H_{16}N_4O_4$.

Synthesis of the galactose containing triazolyl proline 9S



The synthesis was carried out as previously described with the propargyl methyl ester. As the alkyne propargyl β–D-galactopyranoside[1] was used and the reaction was carried out under microwave irradiation at 80 °C during 2 h instead of 20 h stirring at room temperature. After concentration under reduced pressure the residue was subjected to reversed phase flash chromatography.

The NMR-spectrum showed two conformers (*cis* and *trans* conformation around the tertiary amide bond) in a ratio of 1:2.7 in D_2O.

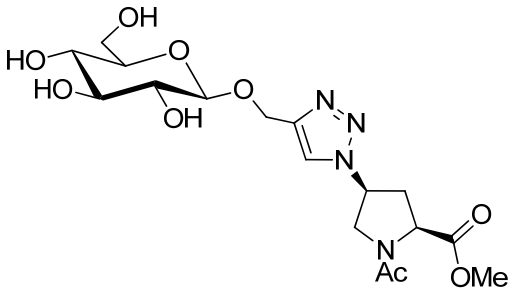
^{1}H-NMR (500 MHz, D_2O, 25°C): δ/ppm (major conformer) = 8.19 (s, 1H; triazole), 5.41-5.34 (m, 1H; Hγ), 4.99 (d, *J* = 12.6 Hz, 1H; H1'), 4.86 (d, *J* = 12.6 Hz, 1H; H1'), 4.73 (dd, *J* = 4.0 Hz, 9.2 Hz, 1H; Hα), 4.46 (d, *J* = 8.0 Hz, 1H; H1), 4.35-4.27 (m, 2H; Hδ), 3.91 (d, *J* = 2.3 Hz, 1H; H4), 3.80-3.66 (m, 2H; H6), 3.78-3.68 (m, 1H; H5), 3. 68-3.59 (m, 1H; H3), 3.64 (s, 3H; OMe), 3.51 (t, *J* = 8.8 Hz, 1H; H2), 3.04-2.93 (m, 1H; Hβ), 2.87 (dt, *J* = 3.9 Hz, 14.1 Hz, 1H; Hβ), 2.17 (s, 3H; OAc). (minor conformer) = 8.15 (s, 1H; triazole), 5.34-5.29 (m, 1H; Hγ), 4.96 (d, *J* = 12.6 Hz, 1H; H1'), 4.84 (d, *J* = 12.6 Hz, 1H; H1'), 4.19, (d, *J* = 13.7 Hz, 1H; Hδ), 4.12 (dd, *J* = 6.3 Hz, 13.7 Hz, 1H; Hδ), 3.09 (*d*, *J* = 14.5 Hz, 1H, Hβ), 2.07 (s, 3H, OAc).

^{13}C-NMR (126 MHz, D_2O, 25°C): δ/ppm (major conformer) = 173.3 (amide), 173.0 (ester, 144.2 (triazole quart.), 124.6 (triazole tert.), 101.9 (C1), 75.2 (C5), 72.7 (C3), 70.6 (C2), 68.6 (C4), 61.7 (C1'), 61.0 (C6), 58.9 (Cγ), 58.1 (Cα), 53.1 (OMe), 52.4 (Cδ), 35.8 (Cβ), 21.3 (OAc).

The signals were assigned based on COSY-, HMBC- und HMQC-spectra. And the conformers were assigned based on a NOESY-spectrum.

MS (ESI): *m/z* = 453.4 $[M+Na]^+$, M = 430.17 g mol^{-1}calcd for $C_{17}H_{26}N_4O_9$.

Synthesis of the glucose containing triazolyl proline 10S



The synthesis was carried out as previously described with the propargyl methyl ester. As the alkyne propargyl β–D-glucopyranoside[2] was used and the reaction was carried out under microwave irradiation at 80 °C during 2 h instead of 20 h stirring at room temperature. After concentration under reduced pressure the residue was subjected to reversed phase flash chromatography.

The NMR-spectrum showed two conformers (*cis* and *trans* conformation around the tertiary amide bond) in a ratio of 1:2.7 in D_2O.

^{1}H-NMR (500 MHz, D_2O, 25°C): δ/ppm (major conformer) = 8.19 (s, 1H; triazole), 5.41-5.35 (m, 1H; Hγ), 4.98 (d, *J* = 12.5 Hz, 1H; H1'), 4.86 (d, *J* = 12.6 Hz; H1'), 4.73 (dd, *J* = 4.2 Hz, 9.3 Hz, 1H; Hα), 4.53 (d, *J* = 8.1 Hz, 1H; H1), 4.35-4.27 (m, 2H; Hδ), 3.91 (d, *J* = 12.4 Hz, 1H; H6), 3.71 (dd, *J* = 6.0 Hz, 12.4 Hz, 1H; H6), 3.63 (s, 3H; OMe), 3.50-3.42 (m, 2H; H3&5), 3.38 (d, *J* = 9.4 Hz, 1H; H4), 3.29 (d, *J* = 8.1 Hz; H2), 3.02-2.94 (m, 1H; Hβ), 2.87 (dt, *J* = 4.3 Hz, 14.2 Hz, Hβ) 2.17 (s, 3H, Ac). (minor conformer) = 8.15 (s, 1H; triazole), 5.35-5.30 (m, 1H; Hγ), 4.96 (d, *J* = 12.7 Hz, 1H; H1'), 4.85 (d, *J* = 12.6 Hz, 1H; H1'), 4.99-4.94 (m, 1H; Hα), 4.52 (d, *J* = 8.4 Hz, 1H; H1), 4.19 (d, *J* = 13.7 Hz, 1H; Hδ), 4.12 (dd, *J* = 6.4 Hz, 13.7 Hz, 1H; Hδ), 3.63 (s, 3H; OMe), 3.36 (d, *J* = 9.5 Hz, 1H; H4), 3.27 (d, *J* = 8.4 Hz, 1H; H2), 3.09 (d, *J* = 14.4 Hz, 1H; Hβ), 3.05-3.97 (m, 1H; Hβ) 2.07 (s, 3H; Ac).

^{13}C-NMR (126 MHz, D_2O, 25°C): δ/ppm (major conformer) = 173.3 (ester), 173.0 (amide), 124.7 (triazole) 101.3 (C1), 76.0, 75.7 (C3&5), 73.0 (C2), 69.6 (C4), 61.8 (C1'), 60.7 (C6), 59.0 (Cγ), 57.7 (Cα), 53.1 (OMe), 52.4 (Cδ), 34.2 (Cβ), 21.3 (Ac).

The signals were assigned based on COSY-, HMBC- und HMQC-spectra. And the conformers were assigned based on a NOESY-spectrum.

MS (ESI): *m/z* = 453.4 $[M+Na]^+$, M = 430.17 g mol^{-1}calcd for $C_{17}H_{26}N_4O_9$.

Fmoc-building blocks

The synthesis of the tripeptidic building blocks Fmoc-Pro-(4*S*)Azp-Gly-OH[2] and Fmoc-Pro-Hyp(TBDPS)-Gly-OH[3] was described before.

Synthesis and analytical data of the CMPs 1S-5S

General protocols

protocol A – peptide coupling

Fmoc-Pro-Yaa-Gly-OH (3 eq) and HCTU (3 eq) were each dissolved in DMF (0.5 M). These solutions and $^{i}Pr_2NEt$ (9 eq, dissolved in NMP, 3M) were added to the suspension of the amino functionalized resin in DMF. The mixture was shaken for 60 min and washed with DMF (5x).

protocol B – Fmoc-deprotection

40% piperidine in DMF was added to the resin and followed by shaking for 3 min. The solution was filtrated and the procedure was repeated for 10 mins with 20 % piperidine in DMF. The resin was washed with DMF (7x).

protocol C – Acetylation

$^{i}Pr_2NEt$ (30 eq) and Ac_2O (30 eq) were added to the resin suspended in CH_2Cl_2. This mixtrue was shaken for 1 h and washed with CH_2Cl_2 (5x).

protocol D – click chemistry

The solid phase bound peptide was suspended in DMF and $[Cu(MeCN)_4]PF_6$ (0.5 eq per azide) was added. After addition of TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine) (0.5 eq per azide) the mixture was sonicated. The alkyne (3 eq) was added and the mixture was sonicated again. The mixture was heated for 2 h at 60 °C in a Biotage Initiator micro wave synthesizer. The resin was washed with a solution of sodium diethyldithiocarbamate (125 mg in 25 ml DMF with 125 µl $^{i}PrNEt_2$) (5x), DMF (3x) and CH_2Cl_2 (5x).

protocol E – removal of the silyl protecting group

The solid phase bound peptide was suspended in a 1M solution of TBAF (13 ml/ g resin) and shaken over night and the resin was washed with THF (5x), DMF (5x) and CH_2Cl_2 (5x).

protocol F – cleavage off the resin

The resin was shaken 1h in a mixture of TFA/H_2O/TIS (95:2.5:2.5) and the liquid was filtrated. This procedure was repeated twice and the collected liquids were concentrated under

reduced pressure. After addition of cold Et_2O a white solid precipitated which was centrifuged and the supernatant was decanted. The solid was suspended in Et_2O, sonicated, centrifugated and the supernatant was decanted. The residual white solid was dried under reduced pressure, dissolved in water and lyophilized to obtain a white foam.

protocol G – HPLC purification

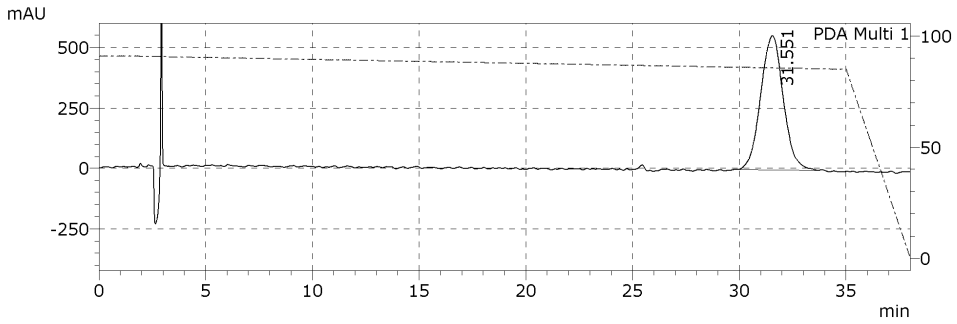
MeCN (A) and water containing 1% of MeCN and 0.1% TFA (B) were used as eluents. The flow used for semipreparitive HPLC was 5 ml/min and 1 ml/min for analytical HPLC. The oven was heated to 65 °C to prevent triple helix formation. After semipreparative purification all collected fractions were analyzed by analytical HPLC again and only pure fractions were combined.

CMP 1S

Solid phase bound **5S** was functionalized using propargyl methyl ester according to protocol D. The silyl protecting groups were removed according to protocol E. The cleavage off the resin and the purification was in analogy to the procedure for **5S**.

HPLC: t_R=31.6 min; gradient: 91 % to 85% B over 35 min at 65°C.

MS (ESI): *m/z* = 2040.2 $[M+H]^+$, M = 2038.9 calcd for $C_{90}H_{127}N_{25}O_{30}$.



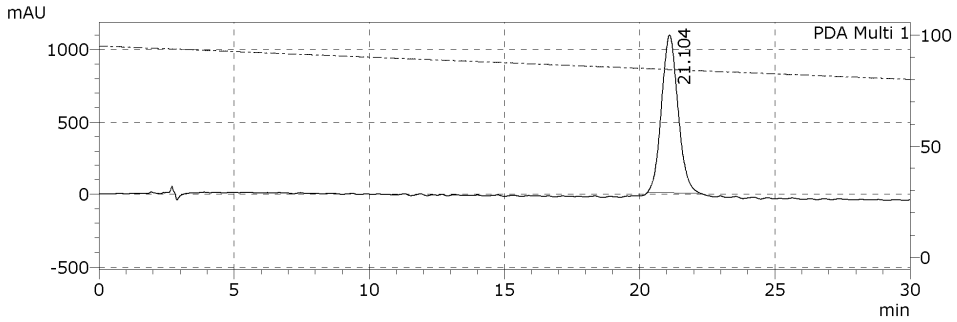
PDA Multi 1 @ 210 nm

CMP 2S

Solid phase bound **5S** was functionalized using propargyl alcohol according to protocol D. The silyl protecting groups were removed according to protocol E. The cleavage off the resin and the purification was in analogy to the procedure for **5S**.

HPLC: t_R=21.1 min; gradient: 95 % to 80% B over 30 min at 65°C.

MS (ESI): *m/z* = 2010.6 $[M+2H]^{2+}$ (100%), 2010.6 $[M+H]^{+}$ (13%), M = 2010.9 calcd for $C_{89}H_{127}N_{25}O_{29}$.



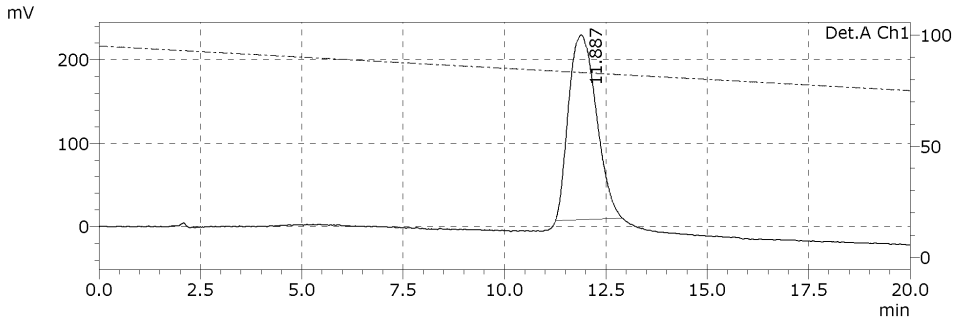
PDA Multi 1 @ 210 nm

CMP 3S

Solid phase bound **5S** was functionalized using propargyl β-D-galactopyranoside according to protocol D. The silyl protecting groups were removed according to protocol E. The cleavage off the resin and the purification was in analogy to the procedure for **5S**.

HPLC: t_R=11.9 min; gradient: 95 % to 75% B over 20 min at 65°C using a Jupiter column.

MS (ESI): *m/z* = 1087.8 $[M+2H]^{2+}$ (100%), 2174.2 $[M+H]^{+}$ (40%), M = 2173 calcd for $C_{95}H_{137}N_{25}O_{34}$.



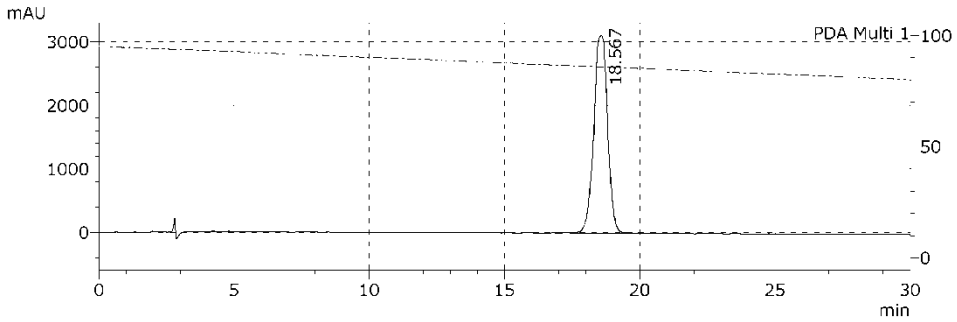
PDA Multi 1 @ 210 nm.

CMP 4S

Solid phase bound **5S** was functionalized using propargyl β-D-glucopyranoside according to protocol D. The silyl protecting groups were removed according to protocol E. The cleavage off the resin and the purification was in analogy to the procedure for **5S**.

HPLC: t_R=18.6 min; gradient: 95 % to 80% B over 30 min at 65°C using a Jupiter column.

MS (ESI): *m/z* = 1087.0 $[M+2H]^{2+}$ (100%), 2172.7 $[M+H]^{+}$ (17%), M = 2173 calcd for $C_{95}H_{137}N_{25}O_{34}$.



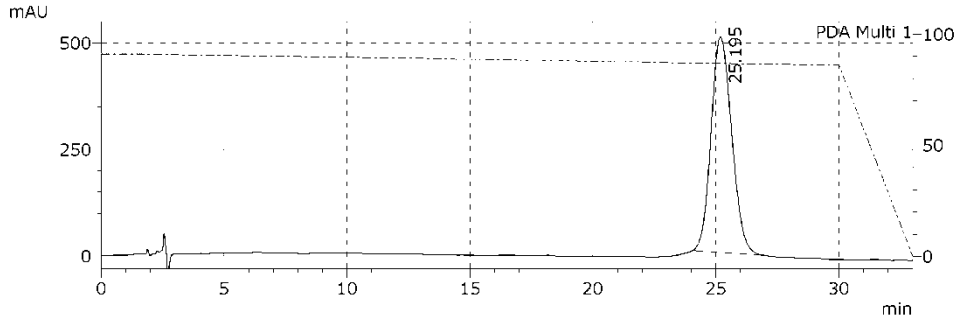
PDA Multi 1 @ 210 nm.

CMP 5S

The peptide was synthesized on a Rink amide ChemMatrix resin (0.47 mmol/g) using a peptide synthesizer. The Fmoc-Pro-Hyp(TBDTPS)-Gly-OH building block was coupled according to protocol A, followed by a Fmoc deprotection according to protocol B. Those steps were repeated twice followed by a coupling of Fmoc-Pro-(4*S*)Azp-Gly-OH[2] according to protocol A. After Fmoc deprotection (protocol B) Fmoc-Pro-Hyp(TBDPS)-Gly-OH was coupled three times (protocol A) followed by an Fmoc deprotection (protocol B) after every coupling. The peptide was then acetylated according to protocol C. The silyl protecting groups were removed according to protocol E and the peptide was cleaved off from the resin according to protocol F. The crude product was purified by HPLC (protocol G).

HPLC: t_R=25.2 min; gradient: 91 % to 85.9 % B over 30 min at 65°C.

MS (ESI): *m/z* = 1978.6 $[M+Na]^+$ (100%), M = 1955 calcd for $C_{86}H_{123}N_{25}O_{28}$.



PDA Multi 1 @ 210 nm

General procedure for CD-spectroscopic analysis

Stock solutions of the lyophilized peptides with a concentration of ≈ 0.8 mM (by weight) in a solution of 50 mM AcOH were prepared. Those were diluted with the buffer to a concentration of 0.20 mM. The solutions were equilibrated at 5°C for >24 h before the measurements. CD spectra were recorded using a spectral bandwidth of 1 nm at 10 °C with a time constant of 5 s and a step resolution of 1 nm. The unfolding processes were followed at the maxima of the previously recorded spectra with a spectral bandwidth of 1 nm and a time constant of 12 s. The temperature was increased in 1 °C steps and the data point was recorded after an equilibration time of 100 s.

General procedure for the determination of T_m

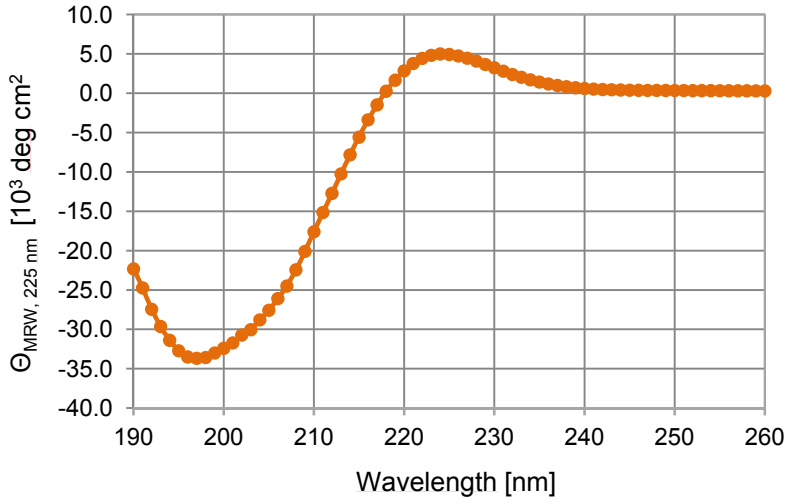
The recorded data was fitted to an all or none transition in which three single strands combine to a triple helix as previously reported by Engel *et al.*[4, 5]. The initial values of the parameters ΔH and T_m were set to -500 kJ/mol and 40 °C. The initial values for A, B, θ_n, θ_d were estimated with the simplex fit function of Micromath Scientist 3.0 (ΔH and T_m were fixed during this estimation). The final fitting was performed using the least-square fit function of Scientist 3.0. The model used is shown below.

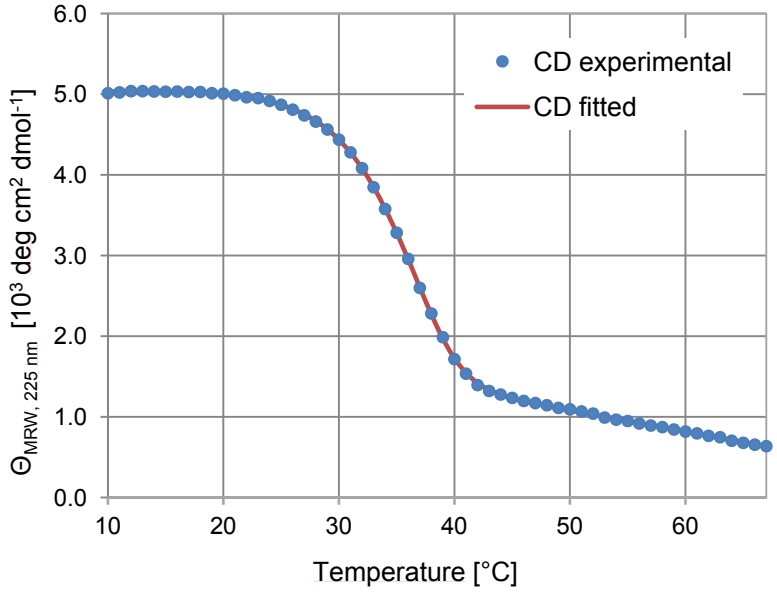
```
// Model two state
IndVars: TEMP
DepVars: F, CD, K
Params: H, DEU, REFU, DEN, REFN, Tm


R=8.31

K=EXP(H/(R*(TEMP+273.15))*((TEMP+273.15)/(Tm+273.15)-1)-ln(0.75*0.0002^2))

P=1/(3*K*(0.0002^2))

U=(-P/2+(P^2/4+P^3/27)^(1/2))^(1/3)
V=-(P/2+(P^2/4+P^3/27)^(1/2))^(1/3)
F= U+V+1


CDU=REFU+DEU*(TEMP+273.15)
CDN=REFN+DEN*(TEMP+273.15)
CD=F*(CDN-CDU)+CDU
```

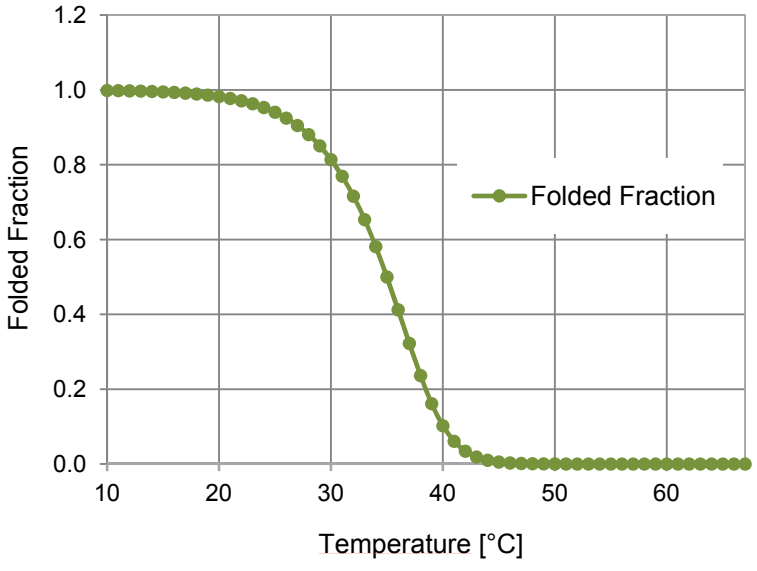
Repeated experiments showed that the Tm values are reproducible within an error of determination that can be estimated to be less then ±0.4 °C. The T_m values were rounded to integers.

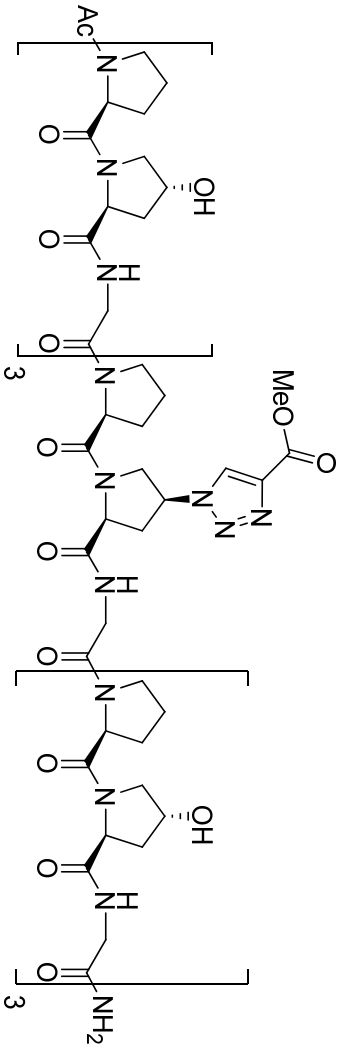
CD Spectra and Tm analysis

CMP 1S

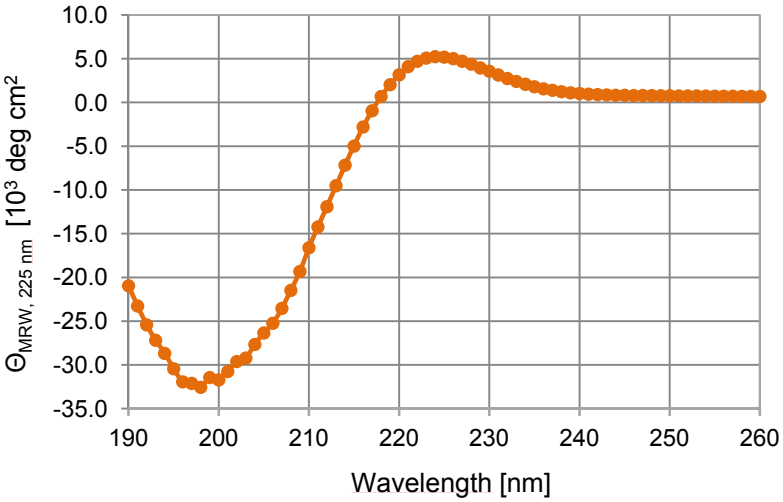


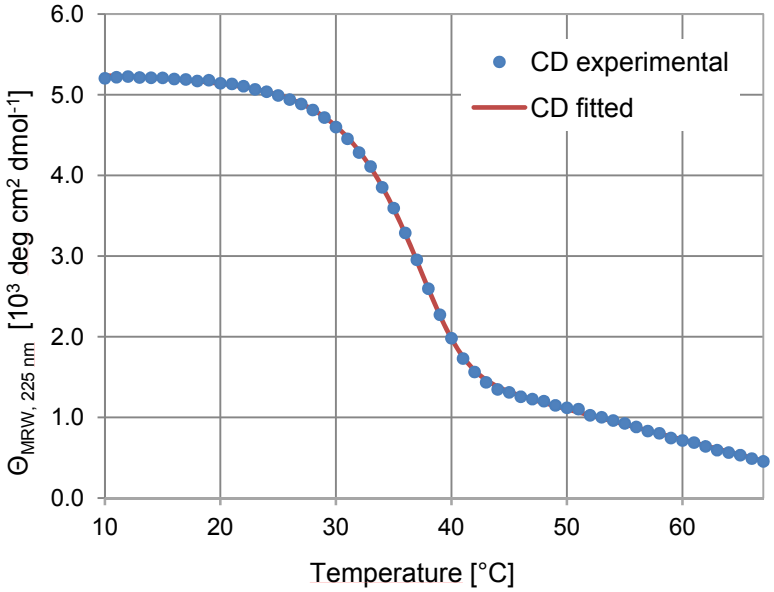


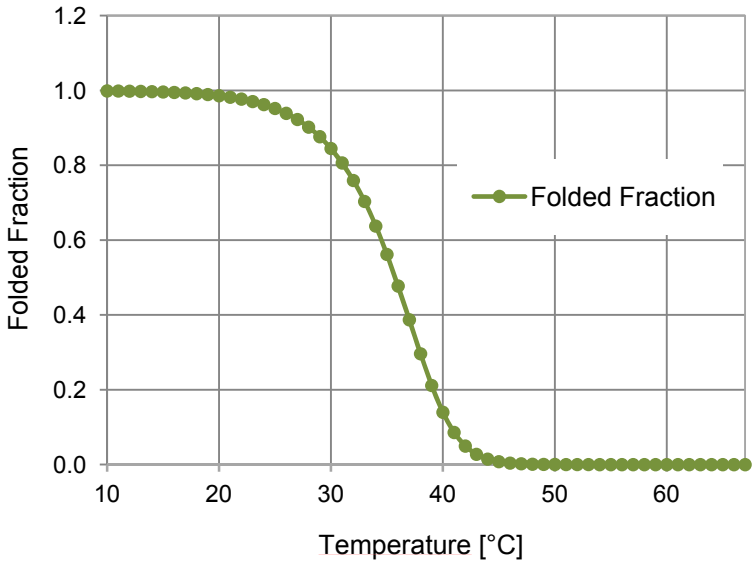


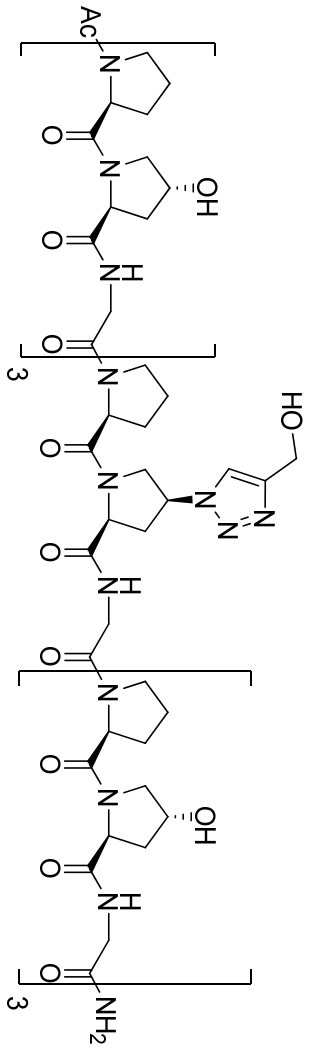


CMP 2S

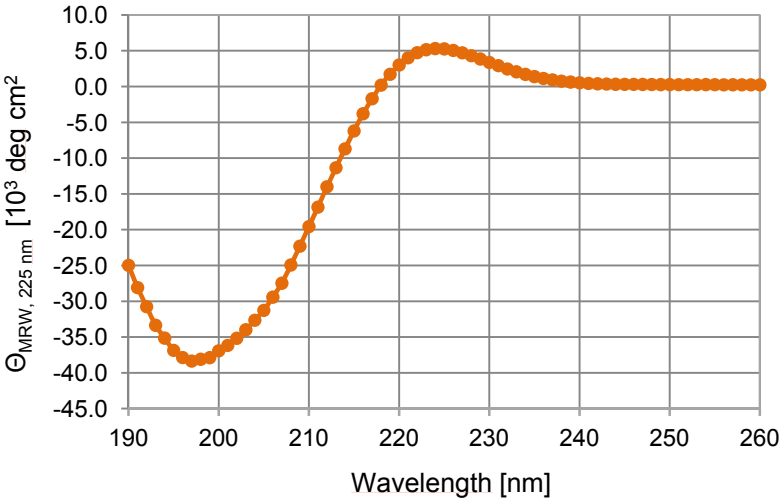

10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
-30.0
-35.0
190
200
210
220
230
240
250
260
Wavelength [nm]
Θ MRW, 225 nm [10³ deg cm²]

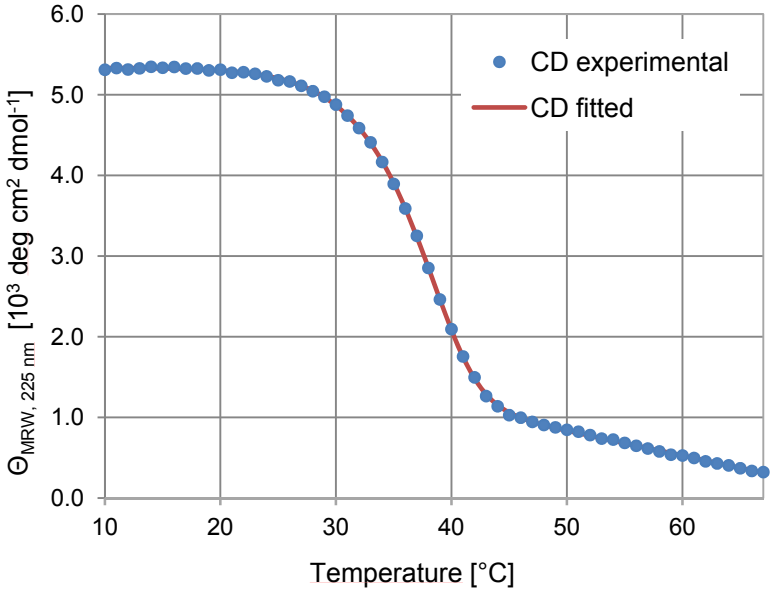

6.0
5.0
4.0
3.0
2.0
1.0
0.0
10
20
30
40
50
60
Temperature [°C]
CD experimental
CD fitted
Θ MRW, 225 nm [10³ deg cm² dmol⁻¹]

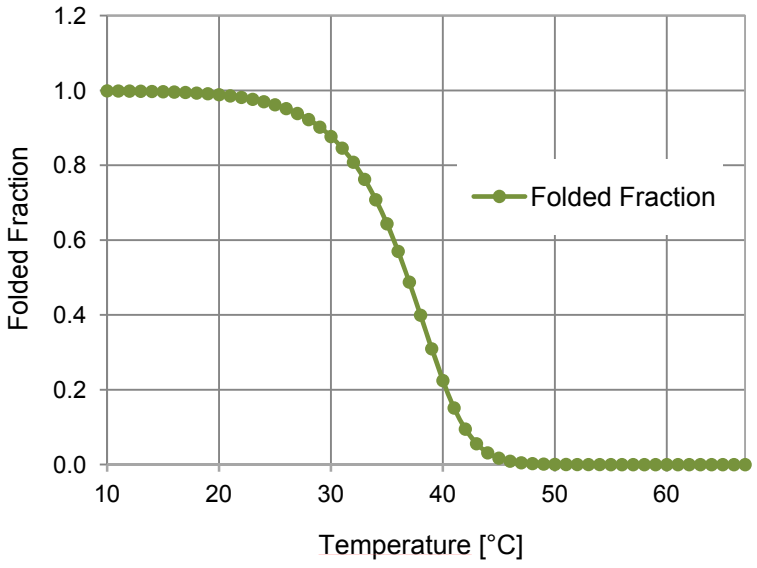

1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
20
30
40
50
60
Temperature [°C]
Folded Fraction

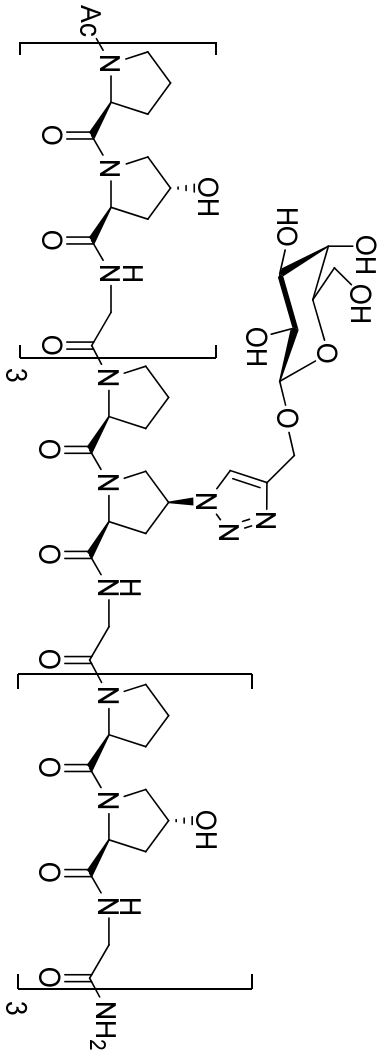

Ac
N
O
OH
NH
3
HO
N
N
N
NH2

CMP 3S

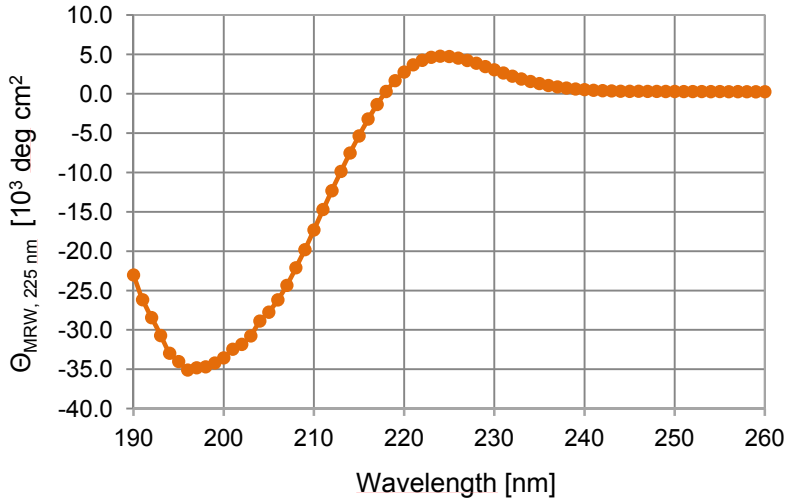


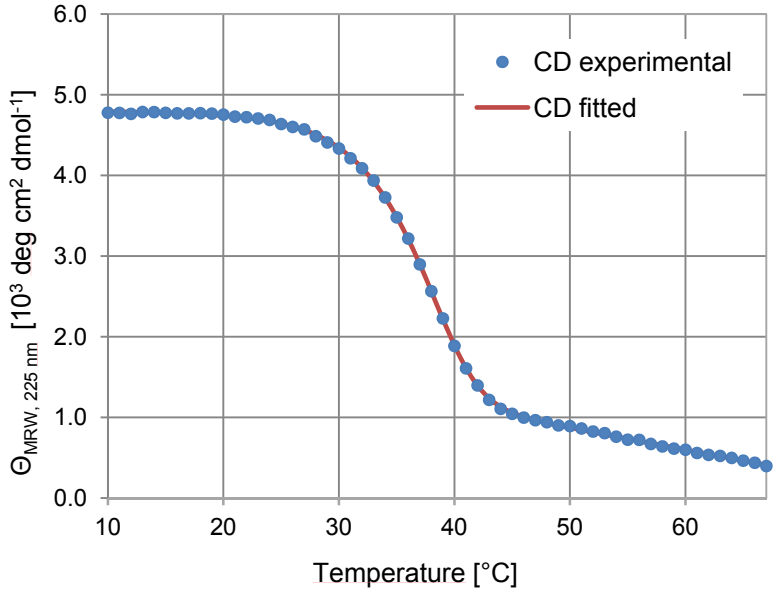


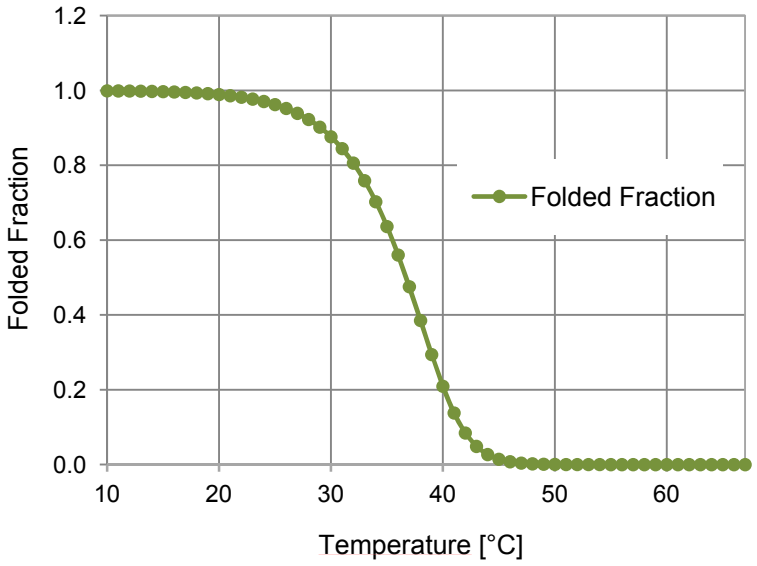


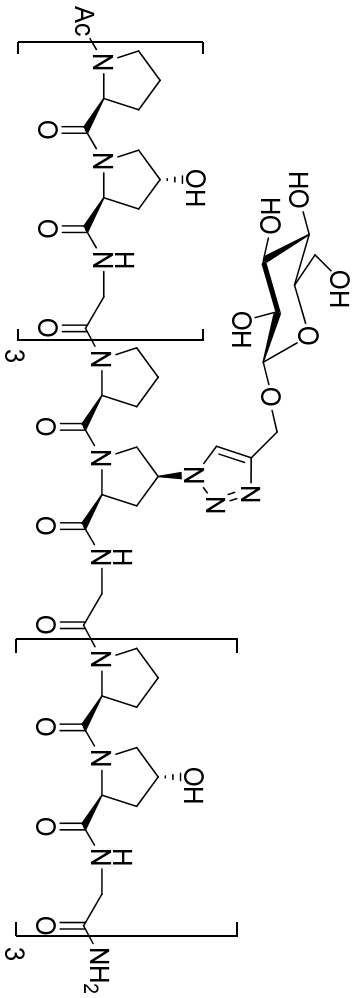


CMP 4S

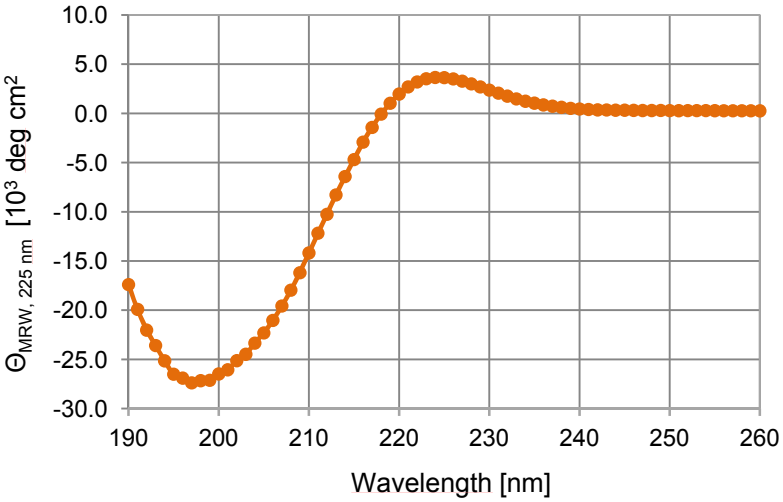


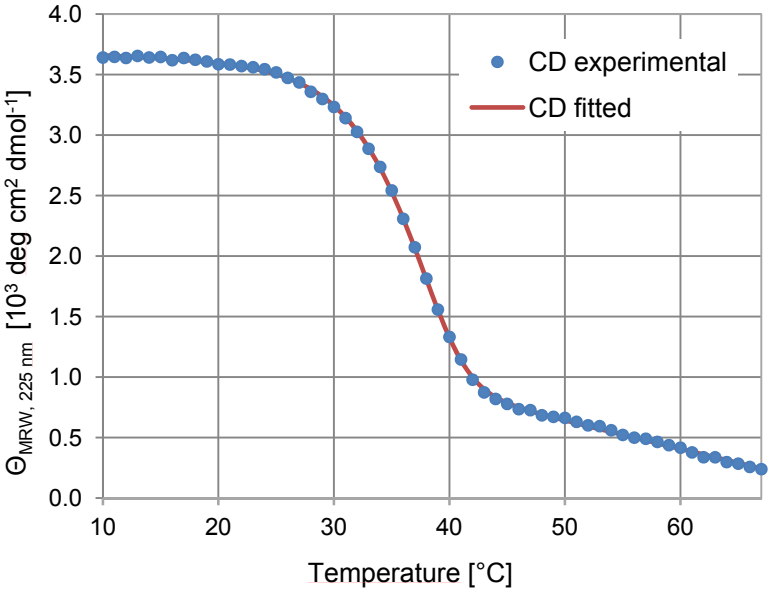


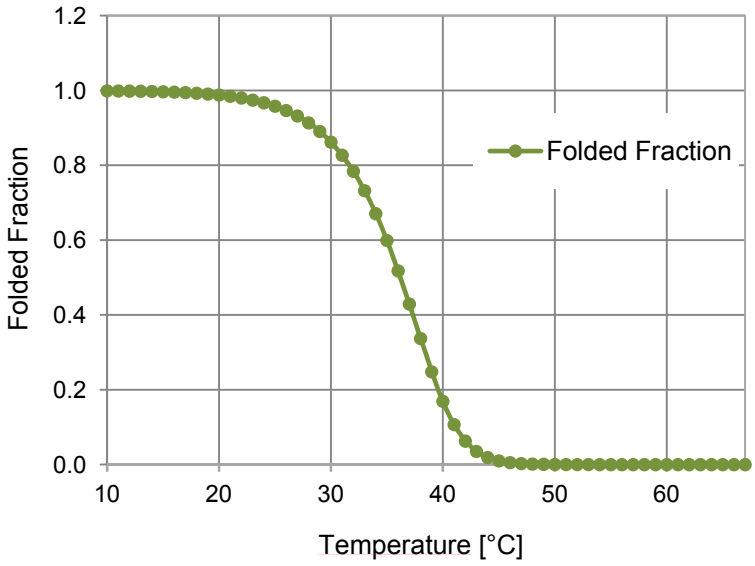


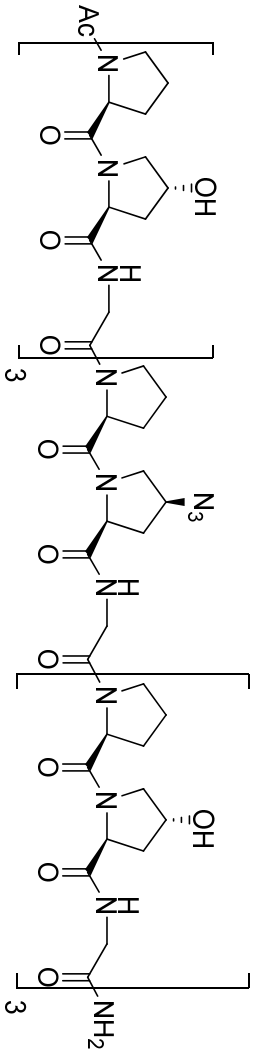


CMP 5S









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