Electronic Supplementary Information

Remarkably high solvatochromism in the circular dichroism spectra of the polyproline-II conformation: limitations or new opportunities?

by V. Kubyshkin, J. Bürck, O. Babii, N. Budisa, and A. S. Ulrich

Index

PCCP, doi: 10.1039/d1cp04551b

Supplementary illustrations

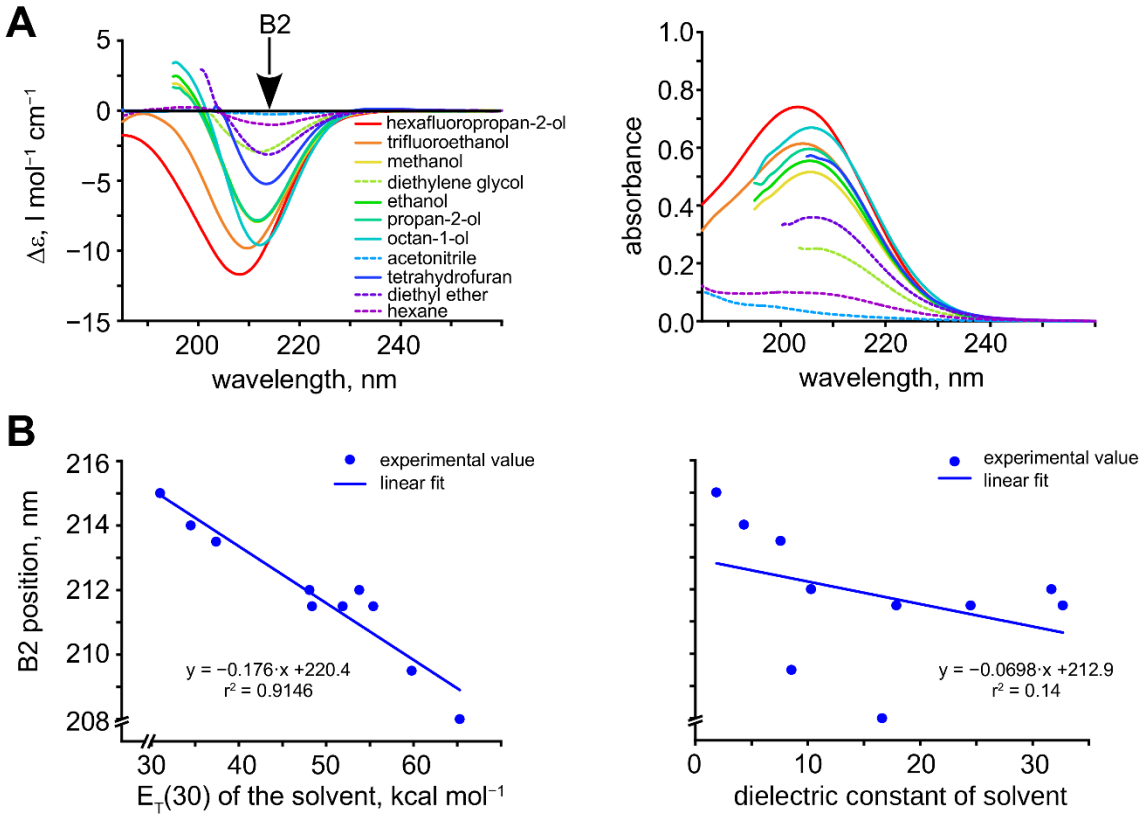

Fig. S1 A Circular dichroism (left) and absorbance spectra (right) of Piv-(Oic)$_{12}$-OCH$_2$CHF$_2$ (**11**) in a series of solvents. Dashed lines indicate spectra in solvents with visually observed reduced solubility. **B** Correlation between the circular dichroism main negative band (B2) position exhibited by Piv-(Oic)$_{12}$-OCH$_2$CHF$_2$ (**11**) with the parameters of the solvents: left – against Reichardt-Dimroth solvent parameter (decent correlation), right – against dielectric constant of the solvent (weak to no correlation).

Table S1 Position of B2 band in spectra of **11** in various solvents.

solvent	B2 position, nm	$E_T(30)$, kcal mol^{-1}	ε
hexafluoropropan-2-ol	208	65.3	16.62
trifluoroethanol	209.5	59.8	8.55
methanol	211.5	55.4	32.7
diethylene glycol	212	53.8	31.69
ethanol	211.5	51.9	24.5
propan-2-ol	211.5	48.4	17.9
octan-1-ol	212	48.1	10.3
acetonitrile	- *	45.6	37.5
tetrahydrofuran	213.5	37.4	7.58
diethyl ether	214	34.5	4.33
hexane	215	31.0	1.88

* very low signal intensity (due to low solubility) did not allow to determine B2 minimum position.

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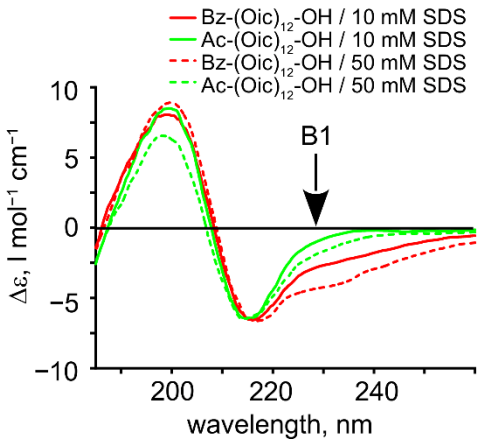

Fig. S2 Spectra of Oic_{12} peptides **8** and **8a** in SDS micelles demonstrate dependence of the B1 feature intensity from the concentration of the detergent and the end capping. Red – N-terminal benzoyl, green – N-terminal acetyl, solid lines – 10 mM SDS, dashed lines – 50 mM SDS.

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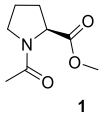
Peptides

Sequences of the peptides analyzed in this study

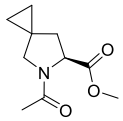
Dipeptide models:

1 Ac-Pro-OCH_3



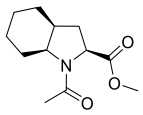
1

2 Ac-Ash-OCH_3



2

3 Ac-Oic-OCH_3



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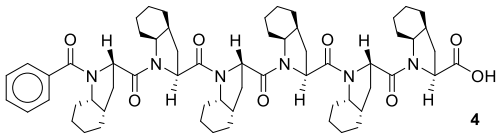
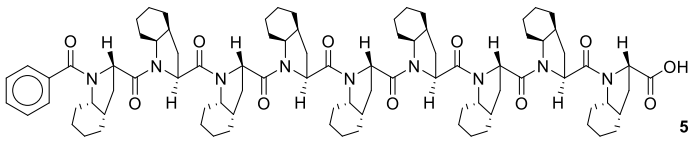
Oligomeric series:

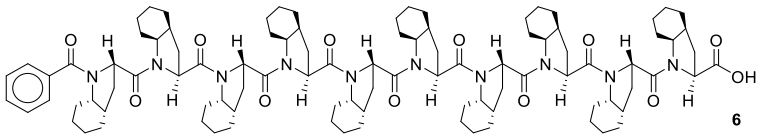
4 Bz-(Oic)$_6$-OH



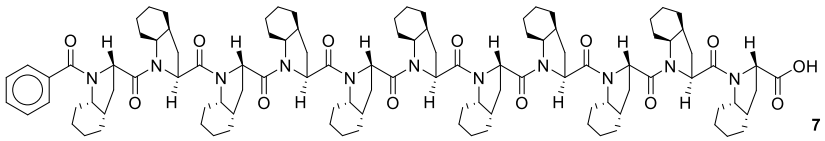
5 Bz-(Oic)$_9$-OH



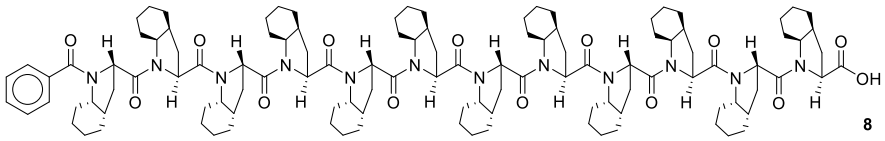
6 Bz-(Oic)$_{10}$-OH



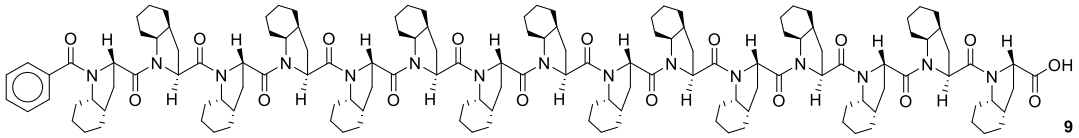
7 Bz-(Oic)$_{11}$-OH



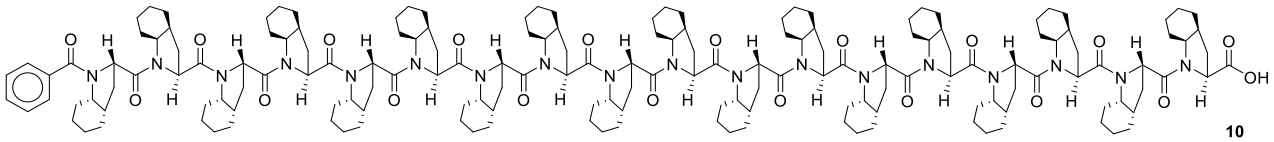
8 Bz-(Oic)$_{12}$-OH



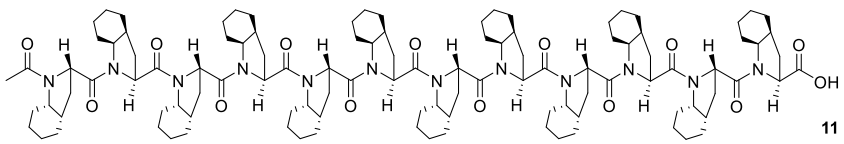
9 Bz-(Oic)$_{15}$-OH



10 Bz-(Oic)$_{18}$-OH



8a Ac-(Oic)$_{12}$-OH



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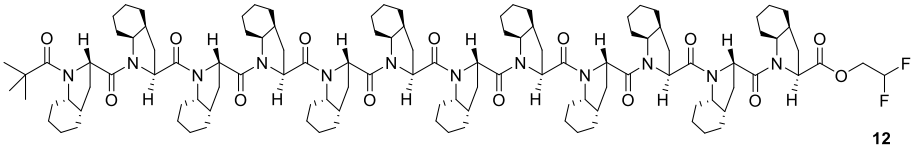
Solvatochromic probe:

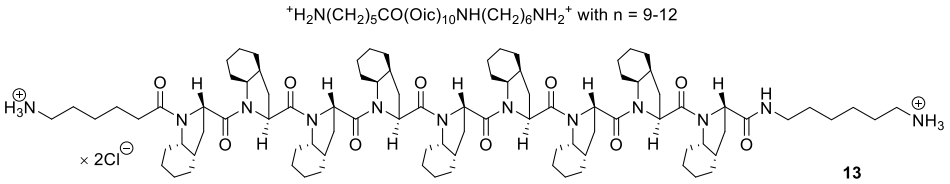
11 Piv-$(Oic)_{12}$-OCH_2CHF_2



12

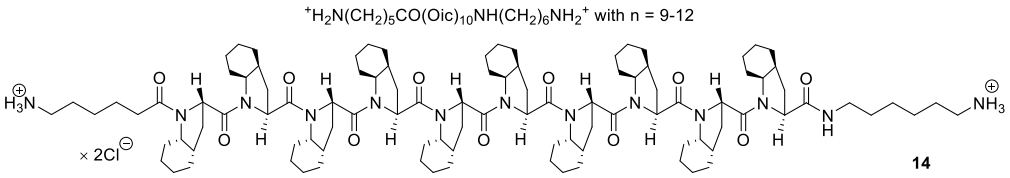
Transmembrane state testing peptides:

12 H_2N-$(CH_2)_5$-CO-$(Oic)_9$-NH-$(CH_2)_6$-NH_2 × 2HCl



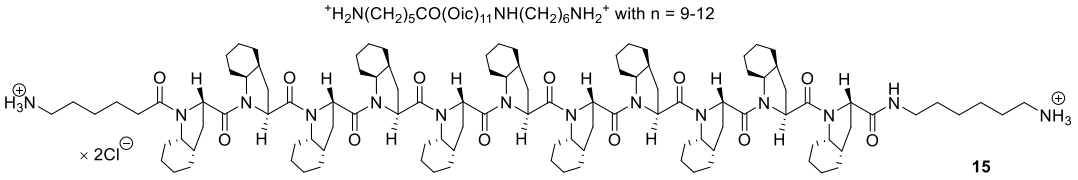
13

13 H_2N-$(CH_2)_5$-CO-$(Oic)_{10}$-NH-$(CH_2)_6$-NH_2 × 2HCl



14

14 H_2N-$(CH_2)_5$-CO-$(Oic)_{11}$-NH-$(CH_2)_6$-NH_2 × 2HCl



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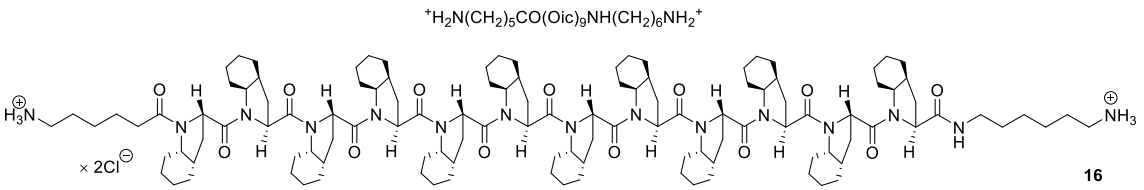
15 H_2N-$(CH_2)_5$-CO-$(Oic)_{12}$-NH-$(CH_2)_6$-NH_2 × 2HCl



16

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Synthesis

Dipeptide models **1**-**3** were prepared and characterized previously.[S1,S2] All other peptides were prepared by manual Fmoc-based solid phase peptide synthesis using Fmoc-Oic-OH and Fmoc-Oic-preloaded 2-chlorotrotyl resin as described.[S3,S4] A brief description of the synthesis is given below.

To overcome the severe hydrophobicity issues, which lead to a reduction in resin swelling and hinder the coupling reaction, low resin loading at around 0.1 mmol g^{-1} and dichloromethane – dimethylformamide (1:1) mixture as the solvent for the coupling steps were used. For peptides **4**-**15** couplings were performed by 2 equiv of Fmoc-Oic-OH, 2 equiv TCTU (*N*,*N*,*N*′,*N*′-tetramethyl-*O*-(6-chloro-1*H*-benzotriazol-1-yl)uronium tetrafluoroborate) and 4 equiv DIPEA (*N,N*-diisopropylethylamine). Fmoc deprotection was done by 20% piperidine in dimethylformamide. Linear peptides were capped by treatment with 20 equivalents of benzoyl chloride / acetic anhydride / pivaloyl chloride and excessive DIPEA. Peptides were cleaved off the resin by treatment with hexafluoropropan-2-ol – dichloromethane (1:3 v/v) mixture. The peptides **5**-**11** were additionally purified using a previously described precipitation from methanol.[S3] Peptide **11** was esterified by difluorodiazoethane as described.[S5]

Peptides **12**-**15** were synthesized using the same coupling conditions as above, with the modification that HATU ((1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate)) was used instead of TCTU. After coupling of the last building block 6-(Boc-amino)hexanoic acid the linear sequences were cleaved off the resin by treatment with hexafluoropropan-2-ol – dichloromethane (1:3 v/v) mixture and freeze-dried. Then, the C-terminus of the peptides was modified by coupling in solution the *N*-benzyloxycarbonyl-1,6-diaminohexane moiety: 2 equiv of *N*-carbobenzyloxy-1,6-diaminohexane hydrochloride, 2 equiv HATU, 5 equiv DIPEA in acetonitrile – dichloromethane (1:1 v/v) mixture, coupling time 2 h. The reaction mixture was poured into water and taken up by dichloromethane followed by removing of the solvent on rotary evaporator. The Cbz group was removed by hydrogenolysis: 1 bar hydrogen, 5% palladium on charcoal in methanol: water (1:1 v/v) mixture.[S4] The peptides were additionally purified by reverse phase HPLC using acetonitrile-water gradient containing hydrochloric acid.

The identity and purity of peptides were confirmed by mass-spectrometry (Tables S2-S3) and NMR analysis as shown on Fig. S3-S4. The spectra of peptides **4-10** demonstrate rising intensity of key peptide resonances against the terminal benzoyl group with rising peptide length.

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Analytical data

Table S2 Electrospray – time of flight mass-spectra analysis for peptides **4**-**11**

ID	peptide	Expected mass	Observed mass
4	Bz-$(Oic)_6$-OH	$[M+H]^+$ 1029.6	1029.6
5	Bz-$(Oic)_9$-OH	$[M+H]^+$ 1482.9	1483.9
		$[M+2H]^{2+}$ 742.0	742.0
6	Bz-$(Oic)_{10}$-OH	$[M+H]^+$ 1634.0	1635.0
		$[M+2H]^{2+}$ 817.5	818.0
7	Bz-$(Oic)_{11}$-OH	$[M+H]^+$ 1786.1	1786.1
		$[M+2H]^{2+}$ 893.6	893.6
8	Bz-$(Oic)_{12}$-OH	$[M+H]^+$ 1937.2	1937.2
		$[M+2H]^{2+}$ 969.1	969.1
9	Bz-$(Oic)_{15}$-OH	$[M+H]^+$ 2390.5	2390.5
		$[M+2H]^{2+}$ 1195.8	1195.8
10	Bz-$(Oic)_{18}$-OH	$[M+2H]^{2+}$ 1422.4	1422.9
		$[M+3H]^{3+}$ 948.6	948.3
8a	Ac-$(Oic)_{12}$-OH	$[M+H]^+$ 1875.2	1875.2
		$[M+2H]^{2+}$ 938.1	938.1
11	Piv-$(Oic)_{12}$-OCH_2CHF_2	$[M+H]^+$ 1981.55	1980.63

Table S3 Matrix-assisted laser desorption/ionization – time of flight mass-spectra analysis for peptides **12**-**15**

ID	peptide	Expected mass	Observed mass
12	H_2N-$(CH_2)_5$-CO-$(Oic)_9$-NH-$(CH_2)_6$-NH_2	$[M+Na]^+$ 1613.2	1611.8
13	H_2N-$(CH_2)_5$-CO-$(Oic)_{10}$-NH-$(CH_2)_6$-NH_2	$[M+Na]^+$ 1764.4	1762.8
14	H_2N-$(CH_2)_5$-CO-$(Oic)_{11}$-NH-$(CH_2)_6$-NH_2	$[M+Na]^+$ 1915.6	1913.8
15	H_2N-$(CH_2)_5$-CO-$(Oic)_{12}$-NH-$(CH_2)_6$-NH_2	$[M+Na]^+$ 2066.8	2065.0

PCCP, doi: 10.1039/d1cp04551b

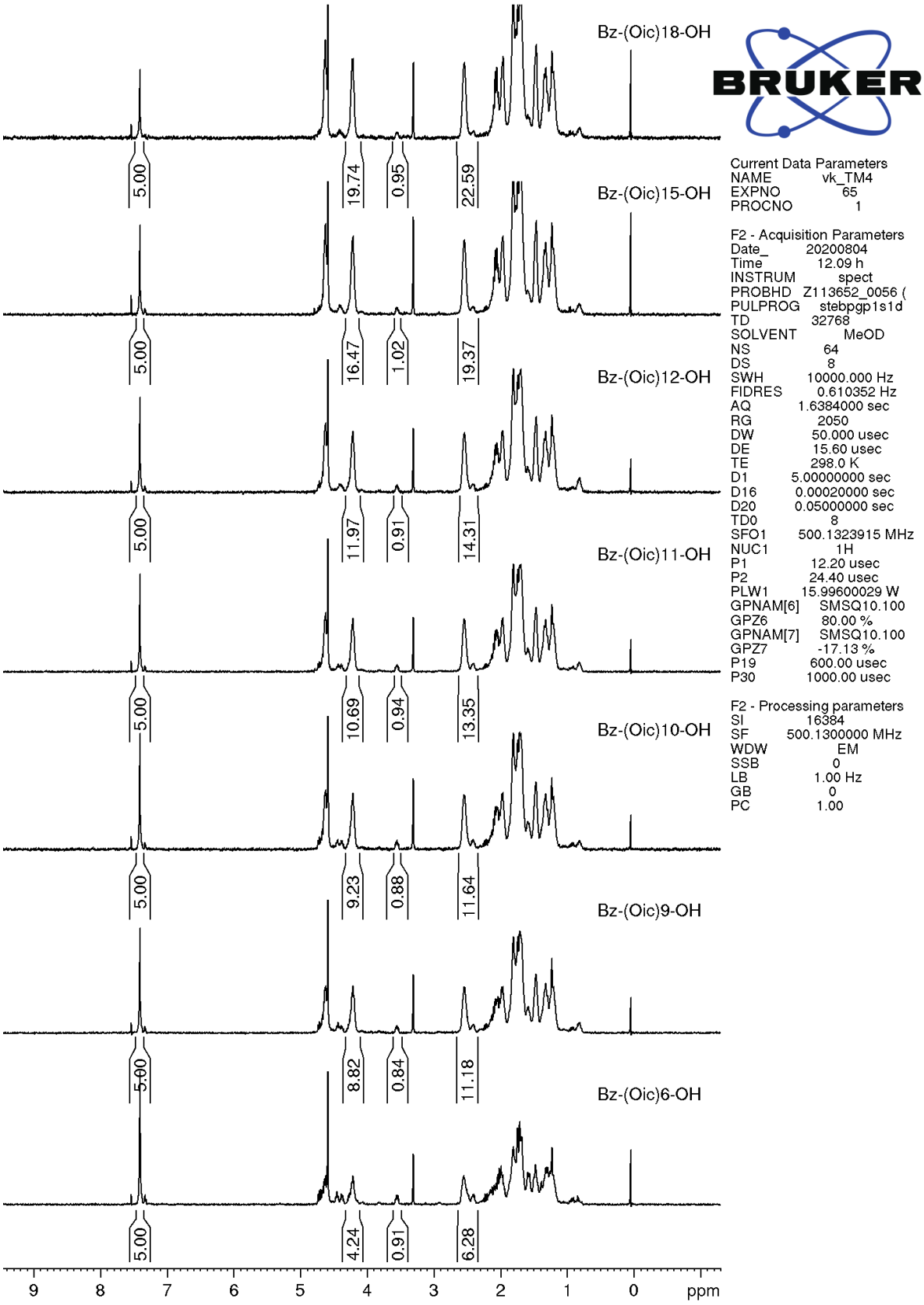

Fig. S3 ^{1}H NMR spectra of peptides **4**-**10** in deuteromethanol – deuterochloroform (1:1) mixture.

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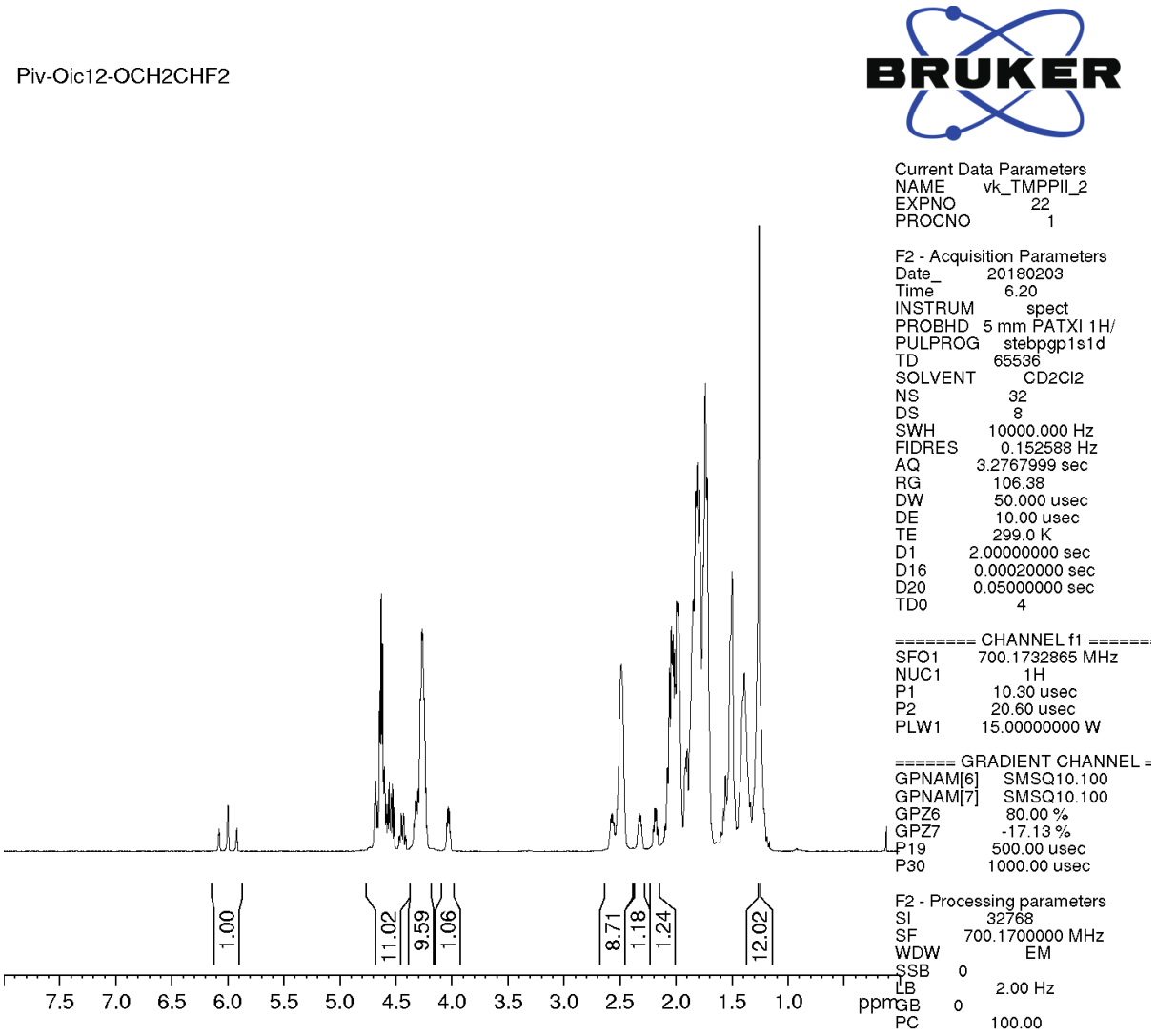

Fig. S4 ^{1}H NMR spectrum of Piv-Oic$_{12}$-OCH$_2$CHF$_2$ (**11**) in dichloromethane-d$_2$.

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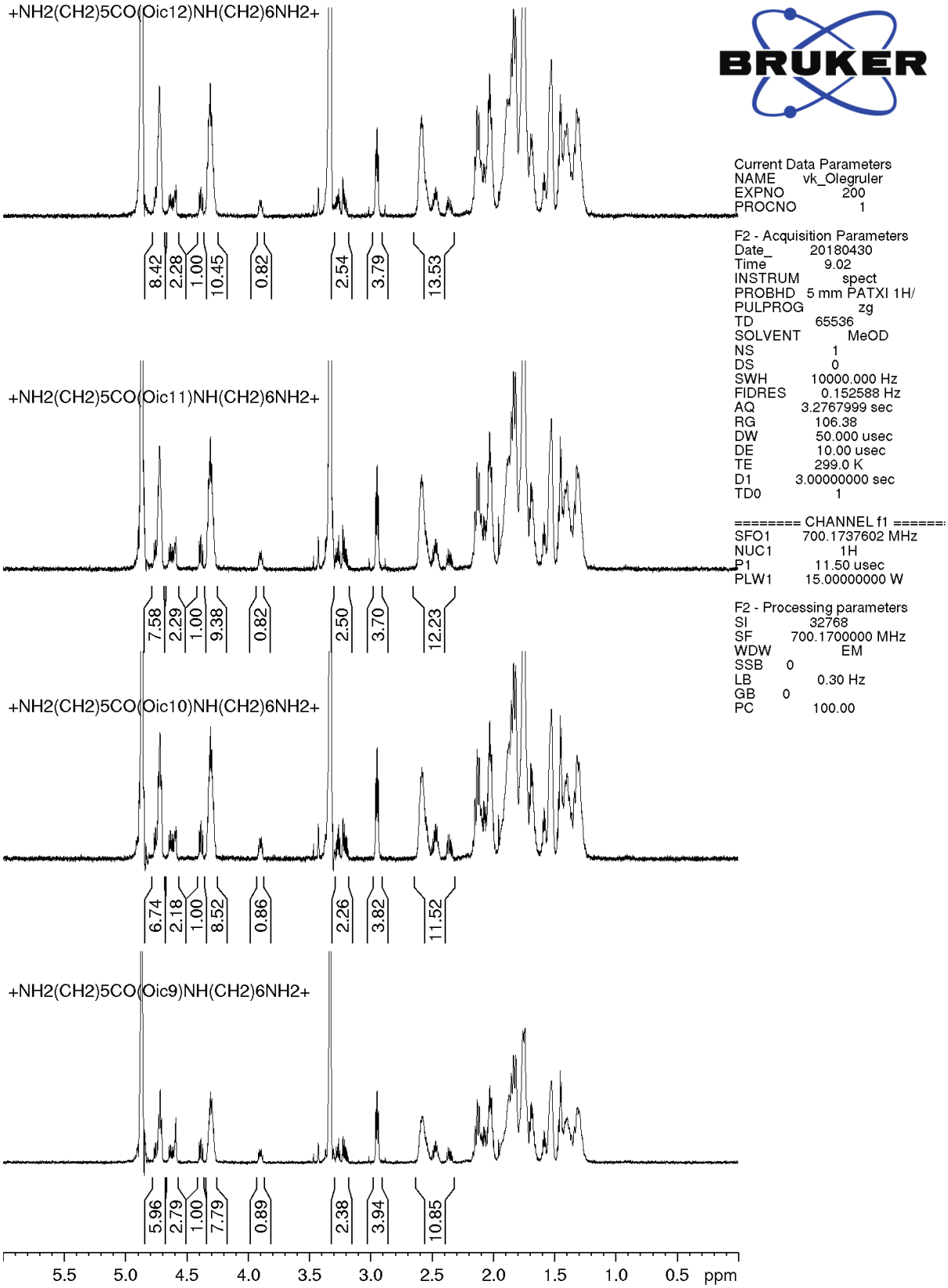

Fig. S5 ^{1}H NMR spectra of the peptides **12**-**15** in deuteromethanol – chloroform mixture.

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Conventional circular dichroism measurements

Sample preparation

For dipeptide measurements, the compounds **1**-**3** were taken in 1.00-2.00 mg amount into vials, where they were dissolved in 1.00 ml dichloromethane each. An aliquote corresponding to 1 μmol was taken to another vial, and the solvent was let to dry out on air. The remaining compound was dissolved in 1.00 ml water or octan-1-ol prior to measurements.

For measurements of oligomeric peptides **4**-**10**, the peptides were taken in 1.00-2.00 mg amount into vials. They were dissolved in 1.00 ml of dichloromethane – methanol (1:1) mixture. Aliquotes corresponding to 1 μmol amide were taken to other vials, and the solvent was let to dry out on air. The remaining peptide was dissolved in octan-1-ol or a detergent solution in deionized water prior to measurements. The residual peptides in the vials before aliquoting were dissolved in deuteromethanol – deuterochloroform (1:1 v/v) mixture for NMR measurements. The latter showed that a comparison between the concentration expected from gravimetry and the one found in NMR spectra were consistent within ±10% accuracy.

The peptide **11** was used for studying the solvatochromic effects of various organic solvents. The solvents: hexafluoropropan-2-ol, trifluoroethanol, methanol, diethylene glycol, ethanol, propan-2-ol, octan-1-ol, acetonitrile, tetrahydrofuran, diethylether and hexane were all spectroscopy grade. A weighed-in amount of 66-116 μg peptide was dissolved in the corresponding volume of solvent (400 to 700 μl) to adjust a constant amide concentration (mean residue concentration) of 1 mM. At this concentration the peptide was completely soluble in most of the solvents resulting in clear solutions except for acetonitrile, hexane, diethylether and diethylene glycol, where a slight turbidity due to undissolved particles could be seen. The latter samples were centrifuged at 13000 rpm for 10 min and the clear supernatant was finally used for the CD measurement.

PCCP, doi: 10.1039/d1cp04551b

Measurements

Circular dichroism spectra for dipeptide models **1**-**3** and oligomeric peptides **4**-**10** were recorded on Jasco J-810 spectropolarimeter in 0.2 nm intervals between 260 and 180 nm for water, between 300 and 195 nm for octan-1-ol, and between 300 and 180 nm for detergent micelles. The samples were measured at 298 K in 1 nm quartz cell, all samples were prepared to have a final 1 mM amide concentration. Spectra were recorded at 50 nm min^{-1} scan rate, a response time 2 s and a spectral bandwidth 0.5 nm. After subtraction of the solvent reference spectrum, the spectra were smoothened using adaptive smoothening, which is a part of Jasco *Spectra Analysis* software. Absorbance for peptides **4**-**10** is shown on Fig. S6.

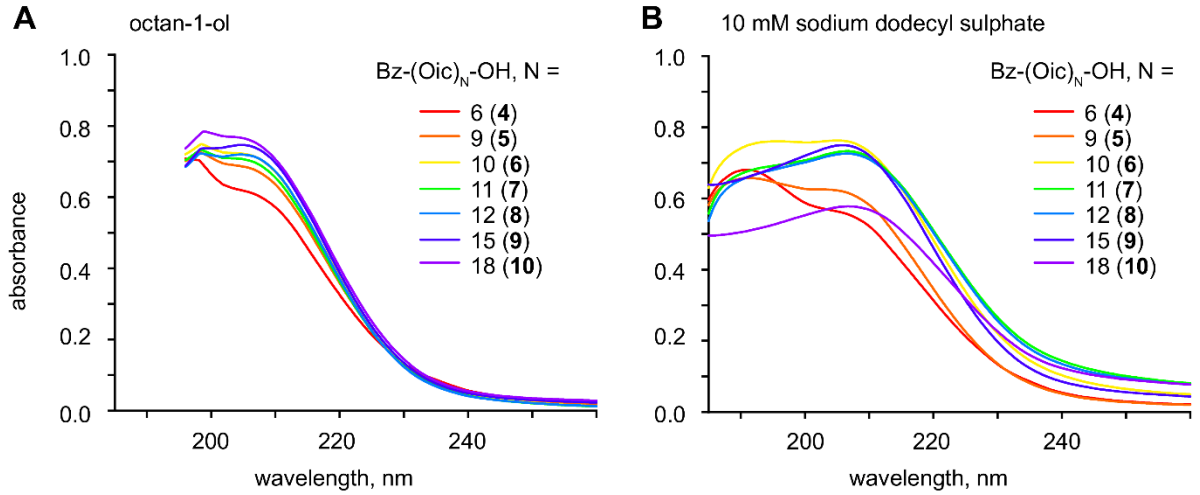


Fig. S6 Absorbance spectra for the peptides **4**-**10** **A** in octan-1-ol and **B** in sodium dodecyl sulphate micelles.

Circular dichroism spectra of peptide **11** were recorded on a Jasco J-815 spectropolarimeter between 260 and 185 nm at 0.1 nm intervals, using a rectangular 1 mm quartz glass cuvette, as previously reported.[S6] For each temperature and for the baseline (peptide-free solvent reference), three repeat scans at a scan rate of 10 nm min^{-1}, a response time of 8 s, and a spectral bandwidth of 1 nm were averaged. After subtraction of the pure solvent reference, CD data were baseline-corrected and processed using Savitzky-Golay smoothening which is part of the Jasco *Spectra Analysis* software.

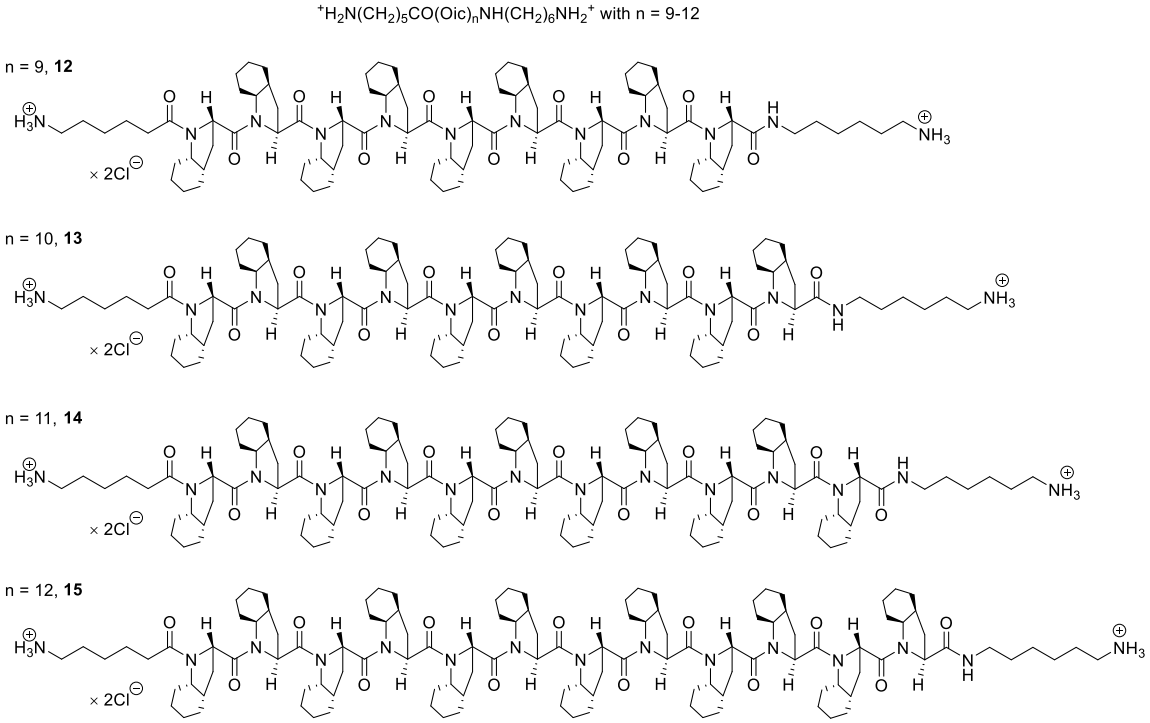
Finally, all spectra were converted to Δε units based on the weighed-in peptide amount and the volume of the sample for concentration determination, the mean residue weight of the peptide and the optical path length of the cuvette.

PCCP, doi: 10.1039/d1cp04551b

Synchrotron circular dichroism measurements

Sample preparation

For measurements in liposomes the following synthetic lipids were taken: 1,2-dilauroyl-*sn*-glycero-3-phosphatidylcholine (DLPC, 12:0/12:0 PC),1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC, 14:0/14:0 PC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC, 18:1/18:1 PC) and 1,2-dieicosenoyl-*sn*-glycero-3-phosphatidylcholine (DEiPC, 20:1/20:1 PC). Four oligo peptides **12**-**15** were examined:



To reconstitute the oligo-Oic peptides in liposomes, the lyophilized peptide powders and the lipids each were dissolved in chloroform – methanol 1:1 mixture (v/v). An aliquot of the lipid stock solution was mixed with an aliquot of an oligo-Oic peptide stock solution to reach the desired concentrations for a peptide-to-lipid molar ratio of 1:40. The organic solvent was removed with a flow of nitrogen for 30 min and afterwards the samples were dried for 3 h under vacuum to remove residual solvent. The lipid/peptide film formed in the vial was dispersed in phosphate buffer (PB, 10 mM, pH 7) and homogenized by being vortexed for 10 min, followed by 10 freeze (liquid nitrogen, 77 K) − thaw (water bath, 313 K) − vortex cycles. Small unilamellar vesicles (SUVs) were generated by sonication for 16 min in a high-power ultrasonic bath with a beaker-shaped sonotrode (UTR 200, Hielscher, Germany). The water of the ultrasonic bath was thermostated at 308 K to avoid overheating of the samples. Liposome samples were always kept above the lipid phase transition temperature before measurement. The oligo-Oic peptide concentration in the final liposome samples was between 0.48 and 0.80 mM as determined from the weighed-in peptide amount in the stock solution, the dilution factor and the volume of the solution.

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Measurements

For achieving a better signal-to-noise ratio CD spectra of the vesicle dispersions were recorded at the UV-CD12 beamline of the Karlsruhe Research Accelerator (KARA) synchrotron facility in Karlsruhe, Germany (Karlsruhe Institute of Technology). The beamline components and its experimental end-station have been previously described in detail.[S7] A cylindrical quartz glass cuvette (121.000-QS, Hellma, Müllheim, Germany) the exact optical path length of which had been determined by UV-Vis interferometry as 45.2 µm was used for the SRCD spectral scans of all liposome samples. Liposome spectra were recorded at 30 °C using a cell holder thermostated by Peltier elements.

Ten scans were collected of each sample at a scan rate of 18 nm min^{-1}, a 0.3 s lock-in time, a 1.5 s dwell time and a spectral bandwidth of 1 nm at 0.5 nm intervals between 260 and 185 nm. The averaged spectrum of the corresponding liposome background spectrum in 10 mM PB was subtracted from the averaged sample spectrum to get the corrected lineshape. Raw CD spectral data were processed by using CDToolX software[S8] to get the averaged, baseline-corrected and Savitzky-Golay smoothened spectra. Finally, all spectra were converted to Δε units based on the weighed-in peptide amount and the volume of the sample for concentration determ ination, the mean residue weight of each peptide and the optical path length of the used cuvette.

PCCP, doi: 10.1039/d1cp04551b

Supplementary References

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[S3] V. Kubyshkin and N. Budisa, *J. Pept. Sci.*, 2018, **24**, e3076. Doi: 10.1002/psc.3076
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