SUPPORTING INFORMATION

Self-assembly of flexible β-strands into immobile amyloid-like β-sheets in membranes as revealed by solid-state ^{19}F-NMR

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Materials and methods

Peptide synthesis:

All chemicals required for peptide synthesis were purchased from Novabiochem or Iris biotech and were used without further purification. Peptide synthesis grade solvents (DMF, NMP and DCM) were purchased from Biosolve. Peptides were synthesized on an automated peptide synthesizer using Fmoc-solid phase peptide synthesis protocols with four equivalents excess of amino acids as previously described.[1-4] 3-(trifluoromethyl)-*L*-bicyclopent-[1.1.1]-1-ylglycine (CF_3-*L*-Bpg) or 3-(trifluoromethyl)-*D*-bicyclopent-[1.1.1]-1-ylglycine (CF_3-*D*-Bpg) was coupled manually using 1.2 eq excess of either *L*- or *D*-analogue of Fmoc-CF_3-Bpg. All peptides were produced as C-terminal amides and were cleaved from the resin using TFA: TIS: H_2O (92%:4%:4%, v/v). Peptides were purified using C18 preparative HPLC columns with water : acetonitrile gradients each containing 5 mM HCl especially to avoid TFA contamination of ^{19}F-labeled product as previously described.[5]

Biological test:

MIC determination:

The minimal inhibitory concentration (MIC) was determined against two Gram-positive strains, namely *S. aureus* DSM 1104 and *S. epidermidis* DSM 1798 and also against two Gram-negative strains, namely *P. aeruginosa* DSM 1117 and *E. coli* DSM 1103. Bacteria were grown over night in Müller-Hinton

(MH) broth at 37°C with continuous shaking at 220 rpm. Optical density (OD) of the overnight culture at 550 nm was determined and the culture was subsequently diluted to obtain an OD_{550} of 0.2. This culture was grown for another 2-3 h, during this time the OD_{550} reached a value of about 2.0. This bacterial culture was further diluted with MH broth to obtain a final bacterial concentration of 10^6 CFU/ml. MIC values were determined in a sterile 96-well polystyrene microtiter plate in a total volume of 100 µl, containing 50 µl of bacterial suspension (resulting in a final bacterial concentration of 5 x 10^5 CFU/ml) to which 50 µl of a peptide solution that was added by following serial dilution of peptide stock solution (c=1,024 µg/ml). The mixture was incubated for 20-22 h at 37°C and the bacterial growth was observed via AlamarBlue staining. The MIC value (µg/ml) corresponds to the peptide concentration at which no bacterial growth was observed.

Transmission Electron Microscopy

The ability of $[KIGAKI]_3$ to self-assemble and form fibrils was studied by transmission electron microscopy (TEM) of negatively stained (with 2% uranyl acetate) peptides, that had been previously incubated with a peptide concentration of 2 mM in water for 24 hours.

Circular dichroism

CD sample preparation:

Weighed amounts of the wild type-peptide and/or the CF_3-*L*-Bpg-labelled peptide analogues were dissolved in 10 mM phosphate buffer for preparing stock solutions with concentration between 0.2 and 0.5 mM. Lipids DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol) were used for preparation of multilamellar vesicles (MLVs) and were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Weighed amounts of the powdered lipid were separately dissolved in chloroform/methanol 50%/50% (v/v) to get individual lipid stock solutions of ~7 mM. Aliquots of these stock solutions were mixed to obtain the DMPC/DMPG 4:1 and 1:4 mixtures (in molar ratio). Subsequently, the organic solvents were removed under a gentle stream of nitrogen followed by overnight vacuum pumping to remove the residual solvent traces. The thin lipid film from the respective DMPC/DMPG mixtures was dispersed by addition of 10 mM phosphate buffer

(pH 7.0). The lipid mixture was homogenized and hydrated by vigorously vortexing for 7 × 1 min and by performing seven freeze-thaw cycles. Afterwards, small unilamellar vesicles (SUVs) were formed by sonication of the MLVs for 5 minutes in a strong ultrasonic bath (UTR 200, Hielscher, Germany).

For preparation of the final CD samples, aliquots of KIGAKI-wt and CF_3-*L*-Bpg-labelled peptide analogues from stock solution were added to TFE/10 mM phosphate buffer (50%/50% v/v) solution or liposome dispersion in phosphate buffer. The final peptide concentration in the samples varied between 7.5 and 150 μM, while the lipid concentration in the vesicle dispersions was held at 1.5 mM resulting in peptide-to-lipid ratios between 1:10 and 1:200.

CD spectropolarimetry

CD spectra of the samples in membrane-mimicking environments such as 50% TFE / 50% phosphate buffer solution, or DMPG/DMPC vesicle dispersions were recorded on a J-815 spectropolarimeter (JASCO, Groß-Umstadt, Germany). Measurements were performed in quartz glass cells (Suprasil, Hellma) of 1 mm path length between 260 and 185 nm at 0.1 nm intervals. Spectra were recorded at 20°C for the buffer solutions and at 30°C for the vesicle suspensions, i.e. above the phase transition temperature of the lipids contained in SUVs, using a water-thermostatted rectangular cell holder. Three repeat scans at a scan-rate of 10 nm min^{-1}, 8 s response time and 1 nm bandwidth were averaged for each sample and the baseline of the respective protein-free sample. After subtracting the baseline spectra from the sample spectra, CD data finally were smoothened by the adaptive smoothing method, which is part of the Jasco Spectra Analysis software. The problem of differential light scattering and absorption flattening in CD spectra of aqueous peptide/liposome dispersions were minimized by taking the following measures:

a) For peptides and proteins measured in liposomes using CD spectroscopy it is recommended in the literature[7-9] to use small unilamellar vesicles (SUVs) rather than LUVs (large unilamellar vesicles). We prepare SUV dispersions as described in the supplementary information, using a very strong ultrasonic bath. In the sonication procedure the sample is cooled with ice water to avoid overheating. In this way we typically end up with an average vesicle size of ~50-60 nm, as confirmed by dynamic light scattering measurements. When applying this procedure to $[KIGAKI]_3$ in DMPC/DMPG

vesicle dispersions, we typically observed only a very slight opaqueness, i.e. scattering effects are largely avoided. We also tried to prepare $[KIGAKI]_3$ in zwitterionic DMPC vesicles, but here the lipid/peptide mixture formed extensive aggregates, making it impossible to measure CD spectra, as these aggregates would precipitate and settle down in the cuvette.

b) Additionally we place the 1 mm cuvette with the liposome sample as close as possible to the PMT detector of the JASCO instrument by flipping the thermostatted sample holder horizontally by 180°. In this way the distance between the sample and PMT is reduced to ~1.5 cm, and the detector´s acceptance angle will improve. Since forward scattering is the predominant process for spherical particles[8], most of the residual scattered light will be collected by the PMT.

Oriented CD (OCD)

Oriented (OCD) samples were prepared with DMPC (P/L 1:100) in the very same way as outlined below for the solid-state 19F-NMR analysis, but using only a single quartz slide as a macroscopic support. Measurements were performed in a home-built OCD cell, which can be rotated about the direction of the incident beam, as explained in detail [6]. Eight spectra at different rotation angles were averaged in order to avoid artifacts due to linear dichroism effects.

Solid state NMR

Sample preparation

Oriented samples for solid-state NMR were prepared as previously described, following a similar procedure as for OCD.[3,10] Usually, 0.1-0.4 mg of CF_3-Bpg labeled peptides were used. The amount of lipid was calculated in each case to obtain the desired peptide-to-lipid molar ratio (P/L). Briefly, lipids were dissolved in chloroform/methanol (1:1, v/v) whereas the peptides were dissolved in methanol /water (2:1, v/v). Dissolved lipids were added to the peptide solution and the resulting clear solution was vortexed, sonicated and uniformly spread over several glass plates. The solvent was allowed to evaporate under vacuum overnight. The glass plates covered with dried lipid-peptide thin film were hydrated in a humidity chamber to obtain well oriented bilayers as previously described.[3]

NMR measurements

All NMR measurements were carried out on a Bruker Avance 500 MHz spectrometer (Bruker Biospin, Karlsruhe, Germany) at 308 K. ^{31}P-NMR was used to check the quality of lipid orientation in the samples, using a Hahn echo sequence with phase cycling.[11] The 90º pulse length was 8 μs, the 180° pulse 16 μs, and the echo time 30 μs. Usually 128 or 256 scans were collected with 2 s relaxation delay time, 100 kHz spectral width, 4096 data points, and proton decoupling with a ttpm20 sequence.[12] Data was processed by zero filling to 16384 data points and a 200 Hz exponential multiplication, followed by Fourier transformation.

^{19}F-NMR experiments were performed on a home-built flat-coil ^{19}F/^{1}H probe head, using an anti-ringing sequence to reduce background signals from the probe,[13] with a 2.3 μs 90° pulse, a 1 s relaxation delay time, 500 kHz spectral width, 4096 data points, and proton decoupling with a ttpm20 sequence.[12] Typically between 20000 to 80000 scans were collected. After zero filling to 16384 data points and an exponential multiplication of 200-1000 Hz, a Fourier transformation was performed. Spectra were referenced to a 100 mM NaF solution for which the ^{19}F signal was set to -119.5 ppm. Dipolar splittings were determined from the well resolved ^{19}F-NMR spectra, and were found to be reproducible within 0.5 kHz on repeated measurements.

Orientational analysis

The alignment and dynamics of a rigid peptide in the membrane can conveniently be described by three parameters: the tilt angle τ of the molecular long axis with respect to the bilayer normal, the azimuthal rotation angle ρ around this axis, and the molecular order parameter S_{mol}, which gives as estimate of the dynamics. These angles are defined in **Figure S5**. Given that the effective dipolar tensor is collinear with the C-CF_3 axis of a ^{19}F-labeled peptide, at least three orientational constraints are required to determine τ, ρ and S_{mol}. In practice more than three are needed. In a rapidly rotating CF_3-group the sign of the dipolar splitting is evident from the 1-pulse spectrum, and typically four orientational constraints are sufficient.[14-16]

To calculate orientational constraints from the NMR data, a maximum ^{19}F dipolar splitting of 17.0 kHz was taken for CF_3-Phg, which is an average value for several amino acids with CF_3-groups bound to phenyl rings (as shown in **Table S4**).[17,18]

The peptide $[KIGAKI]_3$ was modeled as an ideal β-sheet, whose alignment was fitted to the orientational constraints. In this molecular frame, the tilt τ defines the angle between the long axis of the sheet (N- to C-terminus) and the bilayer normal. The azimuthal angle ρ is defined as a right-handed rotation around the peptide long axis, with ρ = 0º being defined as the orientation when the vector projecting radially through the C_α atom of Ile-12 is aligned parallel to the membrane plane. The orientation of the C_α-C_β bond in the molecular frame is described by two angles, α and β. β is the angle between the bond vector and the sheet axis (see **Figure S5C**, for an ideal β-sheet this is close to 90º). The angle α is defined by the vector radiating from the β-sheet long axis through the C_α atom and by the projection of the C_α-C_β bond vector onto a plane perpendicular to the sheet (see **Figure S5D**, for an ideal β-sheet this is close to 30º). In the same plane the rotational angle between two consecutive amino acids along the sheet is called ω (see **Figure S5D**, for an ideal β-sheet this is close to 180º). We described the peptide as a β-sheet using β = 89.2º, α = 28.3º, and ω = 178.9º, as deduced from a β-sheet model constructed in SYBYL using φ = –139º and ψ = 135º.[15] While fitting, some other values of ω close to this value were also tried (see below).

To take motional averaging into account a third parameter is introduced in the analysis, a simplified order parameter, S_{mol}, which describes global wobbling motions of the peptide. Its effect in our calculations is to reduce all splittings by a constant factor which can vary between 0 and 1, corresponding to a uniaxial ordering tensor. A value of 1 corresponds to a perfectly rigid peptide without any motion, while a value of 0 corresponds to free isotropic motion, averaging all splittings to zero. In a grid search for the best-fit peptide orientation, the sheet is systematically rotated, and the theoretical quadrupole or dipolar splittings are calculated for different combinations of τ, ρ and S_{mol}. The parameters τ and ρ are changed in steps of 1° from 0 to 180º, and S_{mol} in steps of 0.01 from 0 to 1, to find the parameters giving the lowest root mean squared deviation (rmsd) with respect to the experimental data. The rmsd is defined as

$$\text{Rmsd} = \sqrt{\{\sum_i [\Delta_{exp,i} - \Delta_{calc,i}(\tau,\rho,S_{mol})]^2 / n\}}$$

where $\Delta_{exp,i}$ is the experimental dipolar splitting at label i, $\Delta_{calc,i}(\tau,\rho,S_{mol})$ is the calculated splitting of label i for specific values of τ, ρ and S_{mol}, the sum goes over all labels and n is the number of labels. The values of τ, ρ and S_{mol}, giving the smallest rmsd, are taken as the best-fit values.

In order to get the maximum splittings of 17.0 kHz for all CF_3-Bpg labeled positions, obviously the scaling factor, S_{mol}, should be 1. Also, all C-CF_3 bonds should be oriented exactly along the external magnetic field B_0. This is the case when the peptide forms a completely non-twisted β-sheet (where ω = 180º), which lies flat with the long axis parallel to the membrane surface (meaning at an angle τ = 90º to the membrane normal), and the orientation around the peptide axis is ρ = 61.7º. This can be seen in the following way: Only if the β-sheet is not twisted will the C-CF_3 bond vectors of all positions be parallel. Only if the sheets are parallel to the membrane surface can the C-CF_3 bond vectors get oriented exactly along the membrane normal, which is oriented along B_0 in the macroscopically oriented samples. When ρ = 0º, the vector radially from the peptide long axis through the C_α atom of Ile-12 is aligned parallel to the membrane plane. The angle α gives the orientation of the C_α-C_β bond vector away from this plane, and is 28.3º. The rotation angle ρ is now added to this to give the total angle away from the plane, which should be 90º to get the C_α-C_β bond vector, and the collinear C-CF_3 bond vector, along the membrane normal, which is in turn oriented along B_0 (see **Figure S5D**). This is thus the case when ρ = 61.7º.

The fit was performed with different values of ω close to 180º, and results are shown in **Table S5**. In **Figure S6** are shown dipolar wave curves and rmsd plots of the different fits. For all ω values in the range 176º-184º the shape of the rmsd minimum is very similar, with the best fit values often spread out on an arc, where rmsd values are very similar. The minima are centered close to τ = 90º, ρ = 60º, and S_{mol} is close to 1. Since not all splittings are 17.0 kHz, it is expected that some parameters should deviate from the ideal values mentioned above, but the deviations are small. For ω angles outside the range 176º-184º, rmsd increases very fast, excluding β-sheet structures with a large twist. It can be seen from the curves, that when ω = 180º all labels have the same position around the sheet long axis, which is due to all labeled positions being even so that the angular distance between them is 2n×180º, which is always a multiple of 360º, and the experimental splitting symbols here overlap. When ω gets further away from 360º the angular spread of labels increase, and outside the range 176º-184º this leads to data points which can no longer be well fitted, giving larger rmsd values (see **Figure S6**). Within this ω

range the fits are of similar quality and an exact value of ω can not be determined. It is however clear that it is not far from 180º, meaning that there is almost no twist in the β-sheet.

Additional tables and figures

Table S1. Minimum inhibitory concentration (MIC) values of $[KIGAKI]_3$ and the five ^{19}F-labeled analogues. The MIC values are given in μg/ml.

Peptide name	***S. epidermis*** DSM 1798	***S. aureus*** DSM 1104	***P. aeruginosa*** DSM 1117	***E. coli*** DSM 1103
KIGAKI-wt	16	8	16	16
KIGAKI-6L	8	16	16	16
KIGAKI-8L	16	8	16	8
KIGAKI-10L	32	32	32	32
KIGAKI-12L	16	16	64	16
KIGAKI-14L	8	16	32	16

Table S2. ^{19}F dipolar splittings (in kHz) of IIe-8 replaced with CF_3-*L*-Bpg as a function of peptide concentration. The values were found to be reproducible within 0.5 kHz on repeated measurements.

P/L	**Dipolar splittings (kHz) for KIGAKI-8L**	
	0° tilt	90° tilt
1:800	8.2	-4.2
1:400	14.3	-7.0
1:200	14.4	-7.1
1:100	14.2	-7.0
1:50	13.9	-7.1
1:25	14.6	-7-2

Table S3. ^{19}F dipolar splittings (in kHz) of the all $[KIGAKI]_3$ analogues labeled with CF_3-*L*-Bpg. The values were found to be reproducible within 0.5 kHz on repeated measurements.

Peptide	**P/L = 1:200**		**P/L = 1:800**	
	0° tilt	90° tilt	0° tilt	90° tilt
KIGAKI-6L	15.3	-7.2	8.8	-4.1
KIGAKI-8L	14.4	-7.1	8.2	-4.2
KIGAKI-10L	15.4	-7.1	8.0	-3.9
KIGAKI-12L	16.4	-7.8	8.2	-4.1
KIGAKI-14L	14.1	-7.1	8.4	-4.2

Table S4. Maximum observed ^{19}F dipolar splitting (in kHz) of CF_3-labeled amino acids.[17,18]

Substance	**Dipolar splitting (kHz)**
4-CF_3-Phg	15.4
3-CF_3-Phg	17.5
4-CF_3-Phe	18.5
3-CF_3-Phe	16.5
Average	16.975 ~ 17.0

Table S5. Fitting results of orientational analysis using ^{19}F-NMR dipolar splittings from [KIGAKI]$_3$/DMPC 1:200 samples, with varying values of the structural parameter ω, the angle between two consecutive residues around the peptide axis.

ω / °	**S_{mol}**	**τ / °**	**ρ / °**	**rmsd / kHz**
170	1.00	89	42	5.36
172	1.00	89	46	3.21
175	0.98	86	52	1.06
176	0.95	86	54	0.77
177	0.93	85	56	0.68
178	0.98	102	59	0.71
179	0.97	102	61	0.76
180	0.95	92	73	0.79
181	1.00	74	62	0.77
182	0.98	102	65	0.71
183	0.94	83	67	0.68
184	0.95	86	69	0.77
185	0.98	86	71	1.06
188	1.00	89	77	3.21
190	1.00	89	81	5.36

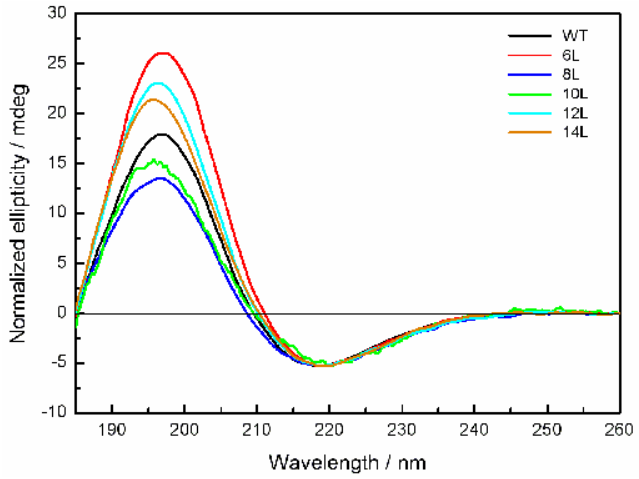

Figure S1. CD spectra of $[KIGAKI]_3$ and ^{19}F-labeled analogues in DMPC/DMPG (3:1) vesicles at a P/L = 1:50. The positions labeled with CF_3-*L*-Bpg are marked in the insert. The spectra are normalized to the minimum intensity of the negative band of KIGAKI-wt at 218.5 nm to show the same intensity at their corresponding minimum, in order to illustrate the relative similarities/differences in the line shapes.

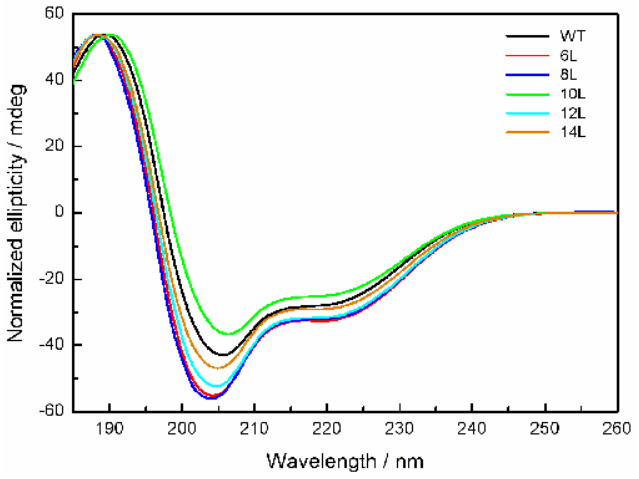

Figure S2. CD spectra of $[KIGAKI]_3$ and ^{19}F-labeled analogues in 10 mM PB /TFE (1:1 v/v). The positions labeled with CF_3-*L*-Bpg are marked in the insert. The spectra are normalized to the maximum intensity of the positive band of KIGAKI-wt at 189.3 nm to show the same intensity at their particular maximum, in order to illustrate the relative similarities/differences in the line shapes

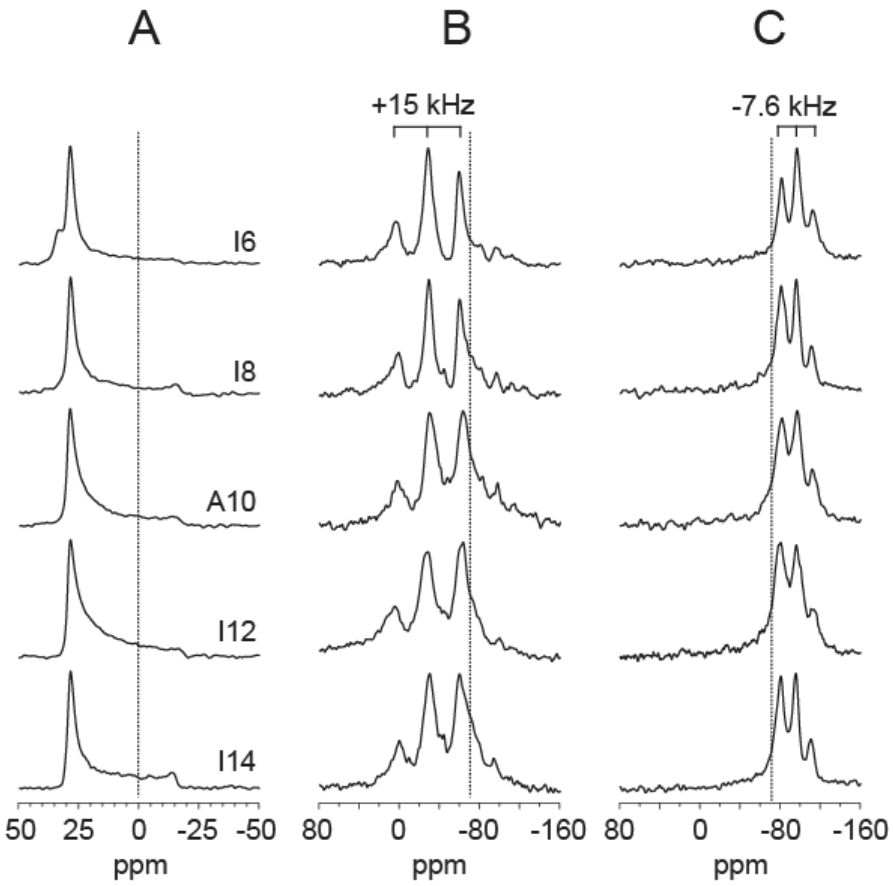

Figure S3. Solid- state ^{19}F-NMR of $[KIGAKI]_3$ at P/L=1:200 for all labeled positions measured in DMPC at 35ºC. The positions labeled with CF_3-*L*-Bpg are indicated close to the ^{31}P spectra (A). (B) ^{19}F-NMR at 0° tilt and (C) ^{19}F-NMR at 90° tilt. The isotropic frequency is marked as a dashed line, and the splittings of the dipolar triplets are indicated. The exact values of splittings are listed in Table S3.

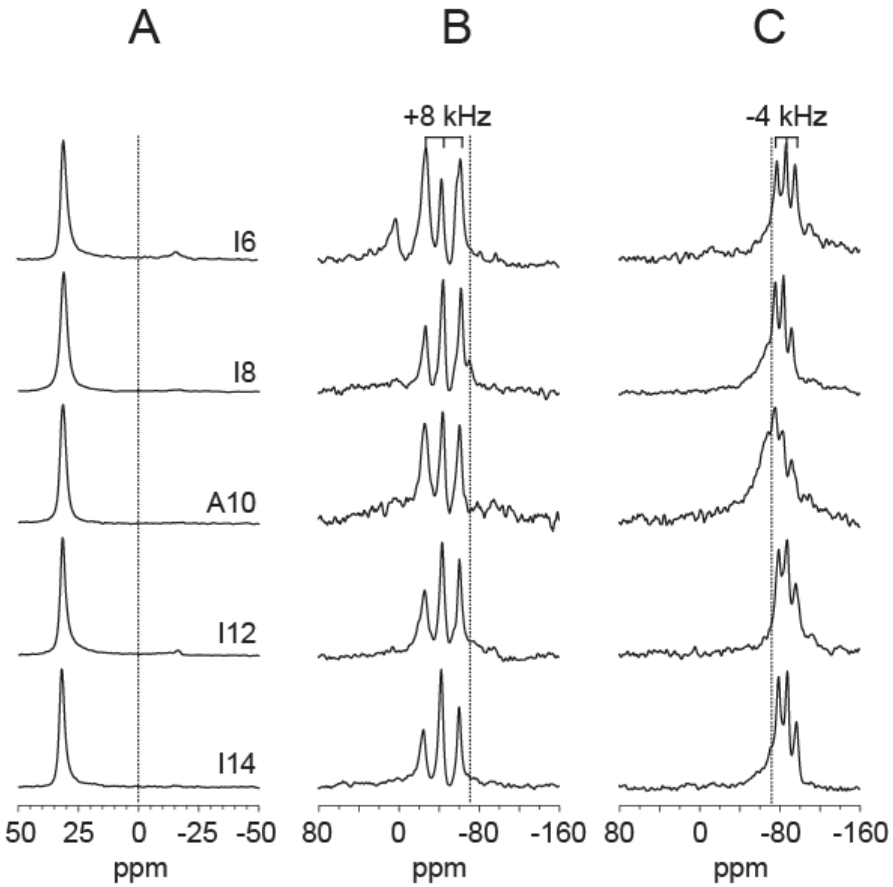

Figure S4. Solid- state ^{19}F-NMR of $[KIGAKI]_3$ at P/L=1:800 for all labeled positions measured in DMPC at 35ºC. The positions labeled with CF_3-*L*-Bpg are indicated close to the ^{31}P spectra (A). (B) ^{19}F-NMR at 0° tilt and (C) ^{19}F-NMR at 90° tilt. The isotropic frequency is marked as a dashed line, and the splittings of the dipolar triplets are indicated. The exact values of splittings are listed in Table S3.

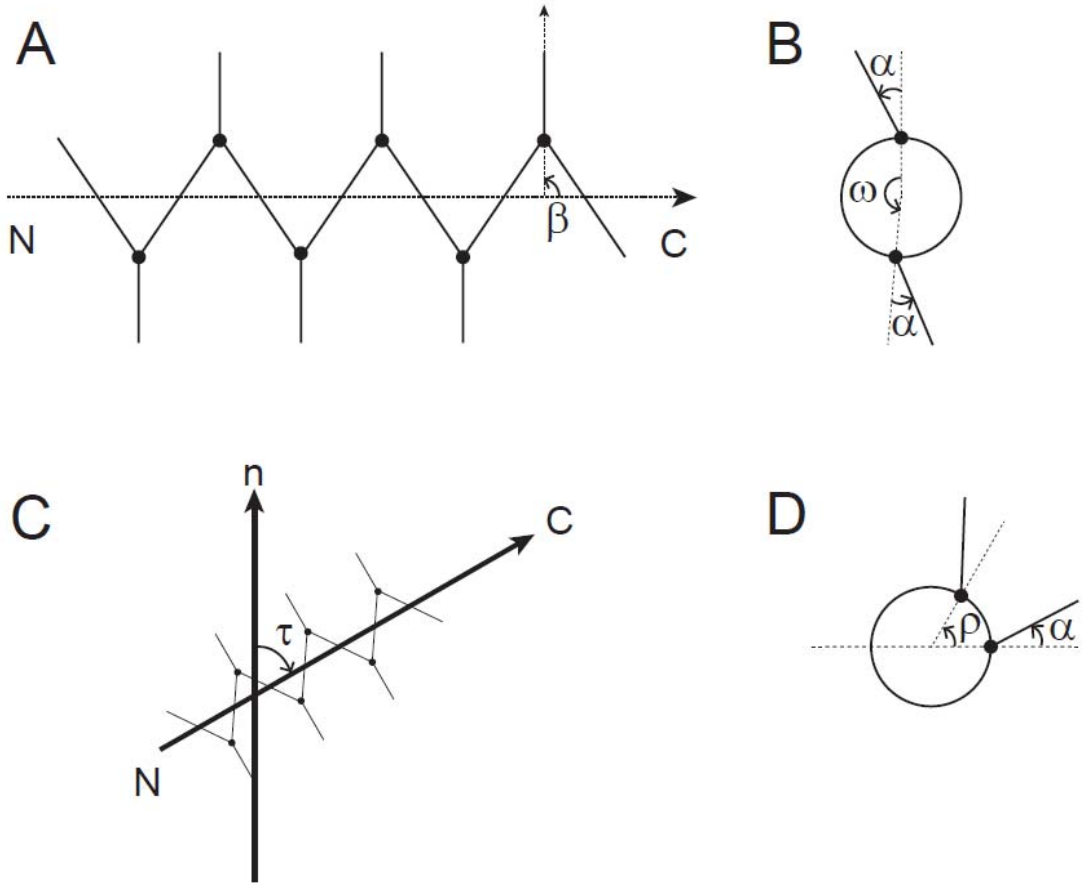

Figure S5. Definition of angles. A schematic β-sheet is shown where C_α atoms are marked by small black circles, the backbone is shown as lines connecting these atoms, and side chains are marked by C_α-C_β bond vectors. (A) β is the angle between the C_α-C_β bond and the long axis of the sheet, which is close to 90º in an ideal β-sheet. (B) The sheet seen from the side, the long axis points out of the picture in the center of the circle. α is the angle between the vector from the center of the axis to the C_α atom, and the C_α-C_β bond. ω is the angle between the C_α-C_β bonds of consecutive residues in the sheet, and is close to 180º. (C) The peptide tilt angle τ is defined as the angle between the long axis of the sheet (from N to C) and the membrane normal (n). (D) The rotation angle ρ defines a rotation around the long axis. For ρ = 0º, the angle between the vector from the center of the axis to the C_α atom of Ile-12 is in the membrane plane. This vector defines the y axis, around which the peptide tilt angle is also defined.

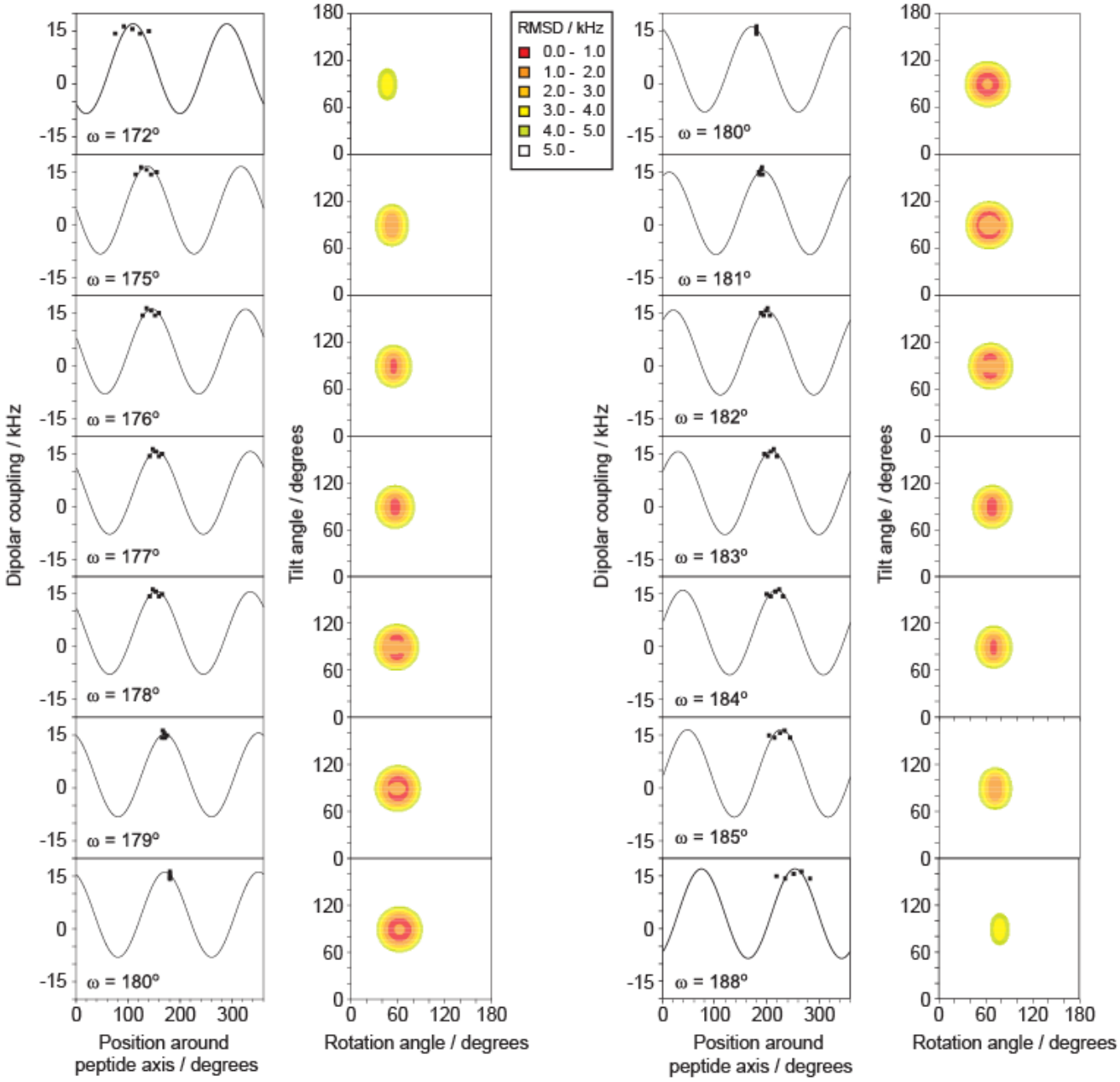

Figure S6. Orientational analysis of $[KIGAKI]_3$ in DMPC at P/L=1:200. For each value of ω, a dipolar wave and an rmsd plot is shown. The symbols show experimental splitting for each label, together with the position of the label around the peptide axis, and the best-fit calculated curve for the orientational parameters. The rmsd plot gives the color coded rmsd values as a function of peptide orientation, as defined by τ and ρ angles.

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