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Orientation of the antimicrobial peptide PGLa in lipid membranes determined from ^{19}F-NMR dipolar couplings of 4-CF_3-phenylglycine labels

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Abstract

A highly sensitive solid state ^{19}F-NMR strategy is described to determine the orientation and dynamics of membrane-associated peptides from specific fluorine labels. Several analogues of the antimicrobial peptide PGLa were synthesized with the non-natural amino acid 4-trifluoromethyl-phenylglycine (CF_3-Phg) at different positions throughout the α-helical peptide chain. A simple 1-pulse ^{19}F experiment allows the simultaneous measurement of both the anisotropic chemical shift and the homonuclear dipolar coupling within the rotating CF_3-group in a macroscopically oriented membrane sample. The value and sign of the dipolar splitting determines the tilt of the CF_3-rotational axis, which is rigidly attached to the peptide backbone, with respect to the external magnetic field direction. Using four CF_3-labeled peptide analogues (with L-CF_3-Phg at Ile9, Ala10, Ile13, and Ala14) we confirmed that PGLa is aligned at the surface of lipid membranes with its helix axis perpendicular to the bilayer normal at a peptide:lipid ratio of 1:200. We also determined the azimuthal rotation angle of the helix, which agrees well with the orientation expected from its amphiphilic character. Peptide analogues with a D-CF_3-Phg label resulting from racemization of the amino acid during synthesis were separately collected by HPLC. Their spectra provide additional information about the PGLa structure and orientation but allow only to discriminate qualitatively between multiple solutions. The structural and functional characterization of the individual CF_3-labeled peptides by circular dichroism and antimicrobial assays showed only small effects for our four substitutions on the hydrophobic face of the helix, but a significant disturbance was observed in a fifth analogue where Ala8 on the hydrophilic face had been replaced. Even though the hydrophobic CF_3-Phg side chain cannot be utilized in all positions, it allows highly sensitive NMR measurements over a wide range of experimental conditions and dynamic regimes of the peptide.

Keywords: Solid state ^{19}F-NMR; Orientational constraints; Lipid bilayer membrane; Antimicrobial peptide PGLa; 4-Trifluoromethyl-phenylglycine

1. Introduction

Structure analysis of membrane-associated peptides and proteins still presents a challenge to the NMR-spectroscopist, given that isotope labeling is necessary to resolve, assign, and interpret the signals from individual molecular segments. Uniform ^{15}N- or ^{13}C-labeling has the obvious advantage of obtaining all information on a single sample, but the data analysis can be rather demanding [1,2]. On the other hand, there are many instances where a small number of selective labels suffice to reveal the 3D-structure of a peptide together with its alignment and dynamics in the lipid membrane [3–5]. A simple α-helical or rigid β-stranded peptide can be described by three structural parameters only, namely its tilt angle τ, its azimuthal rotation angle ρ, and an order parameter S_{mol} representing its wobble. In such cases it is desirable to obtain the necessary parameters by solid state NMR with the highest possible sensitivity, and with maximum accuracy and experimental ease.

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We have recently proposed a selective labeling scheme based on incorporation of the non-natural amino acid 4-fluoro-phenylglycine (4F-Phg) in the place of any hydrophobic amino acid [6]. Because of the high gyromagnetic ratio of the ^{19}F-nucleus, this has the advantage of requiring very little material and measurement time. The idea behind using 4F-Phg is that the fluorine CSA tensor of the stiff aromatic side chain is located in a well-defined position with respect to the peptide backbone. Its anisotropic chemical shift in a macroscopically aligned membrane sample can thus be directly translated into the individual side chain orientation. From three or more such orientational constraints the overall peptide structure and tilt angle can then be calculated. The only input required for such analysis is the peptide secondary structure (i.e., a postulated model structure may thus be confirmed or rejected), and the side chain torsion angle of 4F-Phg about the C_α–C_β bond needs to be known. In a number of previous membrane-bound peptide structures that were successfully resolved, namely of the antimicrobial gramicidin S and the fusogenic B18 [5,7], the most ambiguous assumption concerned the exact value of this torsion angle. That is because molecular modeling showed the bulky 4F-Phg side chain to prefer different torsion angles depending on both its local environment and the global secondary structure [7,8]. In addition to this source of error it may be difficult to extract the accurate chemical shift values of 4F-Phg: not only is there no easy ^{19}F-NMR referencing procedure available, but bulk magnetic susceptibility effects may furthermore lead to intrinsic lineshifts and broadenings in macroscopically oriented samples. We have recently demonstrated that errors up to 10 ppm may arise if these effects are not taken into account [9,10].

Here, we suggest a more convenient and robust strategy for peptide structure analysis, combining the high sensitivity of ^{19}F-NMR with virtually non-disturbing side chain labeling. Instead of a single fluorine reporter as in 4F-Phg, a trifluoromethyl-group is introduced via the side chain of 4-CF_3-phenylglycine. A simple 1-pulse experiment yields a triplet signal, whose splitting represents the anisotropic intramolecular dipolar coupling within the CF_3-group [11]. As this value is accurately obtained (with no need for chemical shift referencing) it can then be directly translated into the desired side chain alignment. The indispensable sign of the dipolar splitting is available from the anisotropic chemical shift, which moves the triplet signal the same way as the dipolar coupling is being scaled. Another potential advantage of the labeling scheme introduced here, lies in the possibility to extract two orientational constraints about the same peptide plane by analyzing both the L- and D-enantiomer of the 4-CF_3-Phg label.

To illustrate and verify this new dipolar ^{19}F-NMR approach experimentally, we have chosen the antimicrobial peptide PGLa, which has a well-characterized α-helical structure in lipid model membranes and detergent micelles [12,13]. Previous solid state NMR studies on a series of selectively ^{15}N-labeled samples have demonstrated that the helix axis is aligned parallel to the membrane surface ($\tau = 90 \pm 30°$), as expected from its amphiphilic character. Even though the azimuthal rotation angle ρ was not available from this previous analysis, the distribution of cationic and hydrophobic side chains allows a straightforward prediction of how the peptide should be rotationally positioned within the lipid bilayer (Fig. 1). Our aim of this ^{19}F-NMR study is (i) to judge the potentially disturbing effect of the 4-CF_3-Phg side chain on the PGLa secondary structure and its biological function, (ii) to hopefully verify the peripheral alignment of PGLa and determine its azimuthal rotation angle, (iii) to assess the accuracy of the dipolar ^{19}F-NMR analysis and its potential sources of error, and (iv) to discuss the advantages and disadvantages of this new approach in comparison to established labeling methods that can in principle yield the same results.

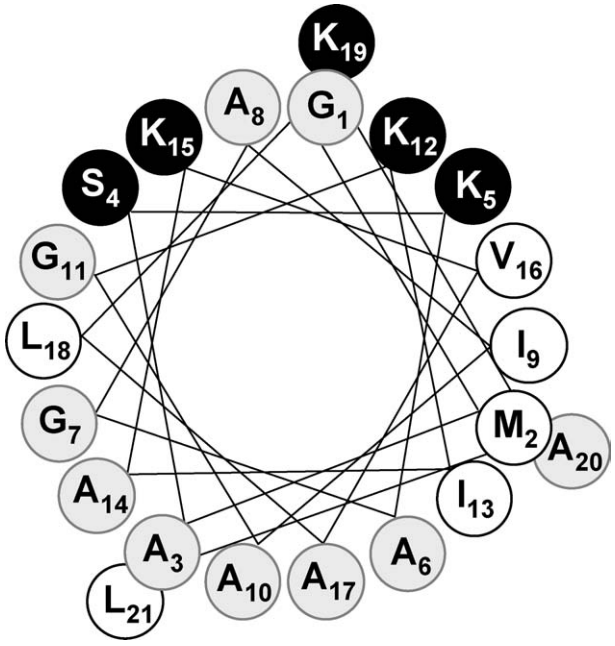

Fig. 1. Helical wheel representation of PGLa with hydrophilic residues in black and hydrophobic residues in white.

2. Materials, methods, and theory

2.1. Peptide synthesis and purification

PGLa (peptidyl-glycylleucine-carboxyamide) and a series of ^{19}F-labeled 4-trifluoromethyl-phenylglycine (CF_3-Phg) analogues were produced the same way as previously reported for 4F-Phg [6]. Within the sequence GMASKAG*AIA*GK*IA*KVALKAL-carboxyamide we

incorporated a single CF_3-Phg at the position of either Ala8, Ile9, Ala10, Ile13, or Ala14. We used a racemic mixture of CF_3-Phg (ABCR, Karlsruhe, Germany). The peptides were synthesized on an Applied Biosystems 433A instrument using standard solid phase Fmoc protocols. The amino acid composition was confirmed by mass spectrometry. The epimers were separated under standard reverse phase HPLC conditions [6]. For each of the five peptide analogues we obtained two clearly separated peaks for the epimers containing L- and D-CF_3-Phg. A second HPLC run with an acetonitrile/water gradient containing 5 mM HCl in the aqueous phase was subsequently performed to remove TFA and avoid any ^{19}F-NMR background, which increased the purity of the peptide to >95%.

2.2. Circular dichroism spectroscopy and antimicrobial activity assays

Circular dichroism spectra were recorded on a Jasco 710 instrument with a 2 mm cuvette in aqueous buffer containing 1 mM NaH_2PO_4/Na_2HPO_4, pH 7.0. To adjust a uniform and accurate peptide concentration for all analogues, UV spectra of both the 1 mg/ml peptide stock solution and the final CD sample were measured. In the stock solution the UV absorption of the CF_3-Phg sidechain at 263 nm was evaluated, and in the CD sample the absorption of the peptide backbone at 190–220 nm was checked. For CD measurements of peptides interacting with model membranes, the lipids (DMPC:DMPG, 60:40) were dissolved and mixed in chloroform/methanol/water, dried under a nitrogen stream, and resuspended in buffer. Small unilamellar vesicles were then produced by ultrasonification. PGLa is virtually fully bound to negatively charged lipid vesicles at low ionic strength when the total negative charge of the lipids exceeds the total positive charges of the peptides [6]. Here we used an 80-fold concentration of negatively charged lipids compared to the concentration of PGLa (25 μM in 2 mm cuvette).

Antimicrobial inhibition assays (both radius of inhibition and MIC determination) were performed as described by Afonin et al. [6].

2.3. NMR sample preparation

For oriented samples a 1:200 peptide:lipid mixture of PGLa and DMPC was prepared and deposited on thin glass slides ($7.5 \times 18 \times 0.08\,mm^3$) as described before [5–7,9]. The peptide (typically 0.25 mg) was dissolved in water, and the lipid in methanol. Both components were mixed to give a 70% methanol solution, and 30 μl drops of the solution were spread onto one side of each glass plate. The membrane films were dried under vacuum, stacked, and hydrated for 2 days at 48 °C in an atmosphere of about 98% relative humidity over a saturated K_2SO_4 solution, such that the membranes spontaneously orient themselves. The stack was immediately wrapped in several layers of parafilm and polyethylene sheet. The quality of orientation of the lipid bilayers was determined with ^{31}P-NMR [14]. Typically 80–90% of the lipid was well aligned between the glass plates.

2.4. Solid state NMR measurements

Measurements were performed on a 500 MHz widebore Unity Inova spectrometer (Varian, Palo Alto, CA) with an external 470 MHz high power amplifier (Creative Electronics, Los Angeles, CA) and a ^{19}F/^{1}H double-tuned flatcoil probe (Doty Scientific, Columbia, SC) [5–7,11,15]. Sample and coil can be manually rotated, but usually the layered sample is aligned horizontally such that the membrane normal is parallel to B_o. Due to technical limitations of the probe ^{1}H-decoupling was limited to about 10–15 kHz, which was sufficient to observe the ^{19}F-labeled peptides in liquid crystalline samples.

2.5. ^{19}F–^{19}F dipolar coupling within the CF_3-group

The general lineshapes of CF_3-labeled groups in powder samples and oriented membranes have been discussed previously [11]. To be able to analyze the dipolar splittings quantitatively, we need to know the static coupling constant of 4-CF_3-Phg. This value was estimated from the distance between the fluorine atoms, given an average C–F bond distance of 1.322 ± 0.015 Å in comparable compounds [16]. Assuming an ideal tetrahedral geometry, the F–F distance is $r_{12} = 2.159 \pm 0.024$ Å. The general dipolar coupling

$$\Delta = \left| 3\hbar\gamma_1\gamma_2 \frac{\mu_0}{4\pi} \frac{1}{r_{12}^3} \frac{3\langle\cos^2\theta\rangle - 1}{2} \right|, \tag{1}$$

between static equivalent spins with $\gamma_F = 2.52 \times 10^8$ rad T^{-1} s^{-1} thus yields a maximum splitting of $\Delta = 31.7 \pm 1.1$ kHz (for $\theta = 0°$). All measurements that we made with CF_3-labeled groups in solid compounds, in lipid membranes, and in solution, are consistent with the assumption that the three fluorines in the CF_3-group give rise to equivalent signals, i.e. they are in fast chemical exchange on the NMR time-scale. This rotational motion around the three-fold symmetry axis of the CF_3-group has to be taken into account by introducing an order parameter $S_{rot} = -1/2$, which makes the maximum observable splitting $\Delta^0_{CF_3} = 15.8$ kHz. Whether the CF_3-group rotates or undergoes 120° jumps or anything in between, does not make a difference. We had previously measured the maximum splitting $\Delta^0_{CF_3}$ within a related aromatic CF_3-group to be about 16 kHz [11], and the corresponding dipolar splitting within a CF_2-segment was reported as 15.5 kHz [17,18].

In an oriented sample (aligned with its normal parallel to B_0) the experimentally observed dipolar splitting Δ_{CF_3} depends directly on the time-averaged value of the angle θ between the CF_3-axis and the membrane normal:

$$\Delta_{CF_3} = \left| \frac{3\langle \cos^2\theta \rangle - 1}{2} \right| \Delta^0_{CF_3}. \quad (2)$$

The time-averaged term $\langle 3\cos^2\theta - 1 \rangle$ results from local oscillations of the side chain and a wobble of the entire peptide within the lipid bilayer. If we approximate these modes as fast isotropic motions, we may then summarize the overall effect of motional averaging by a generalized molecular order parameter $1.0 \geqslant S_{mol} \geqslant 0$, to get

$$\Delta_{CF_3} = \left| \frac{3\cos^2\theta - 1}{2} \right| S_{mol} \times \Delta^0_{CF_3}, \quad (3)$$

where θ is now the effective orientation of the CF_3-axis (which is equivalent to the C_α–C_β bond vector in CF_3-Phg). Note that the dipolar splitting of a horizontally aligned sample cannot tell whether the peptide is undergoing rotation around the membrane normal, as this will only be revealed when the sample is measured at a different tilt angle.

2.6. Determining the sign of the dipolar coupling

When the CF_3-label on the peptide is aligned parallel to the membrane normal ($\theta = 0°$) the maximum dipolar splitting is observed in the triplet signal [11]. As θ approaches the magic angle, the splitting becomes zero, and for $\theta > 54.7°$ it increases again but with a negative sign. Thus any dipolar splitting $|\Delta_{CF_3}| < ((1/2)S_{mol} \times \Delta^0_{CF_3})$ will yield two solutions for the angle θ, one for positive and one for negative dipolar couplings. The sign of a dipolar coupling cannot be measured from the splitting [19], but is readily deduced from the anisotropic chemical shift of the oriented sample. Due to the rotation of the three equivalent fluorine atoms, the ^{19}F CSA tensor is axially symmetric, and the anisotropic chemical shift is also a function of $\cos^2\theta$. Therefore, we could in principle also use the anisotropic chemical shift alone to determine θ, but this is more problematic in view of the need for chemical shift referencing and bulk magnetic susceptibility effects [9,10]. Consequently, we recommend to determine θ accurately from the dipolar splitting, and to use the chemical shift of the triplet signal only to resolve the degeneracy between tilt angles θ above and below the magic angle.

The principal axis values of the CSA tensor of a rotating CF_3-group are not available from the literature but can be estimated from our samples. We found that the most deshielded value lies along the rotational axis. This was concluded from the ^{19}F-NMR spectra of the PGLa-10D-CF_3-Phg analogue in oriented samples, which showed a dipolar splitting of about 11 kHz with a chemical shift of −38 ppm for the central peak of the CF_3-triplet, while the isotropic chemical shift was at −63 ppm. According to Eq. (3), any splitting above 8 kHz can only arise from orientations $0° \leqslant \theta \leqslant 35.3°$. Therefore, anisotropic chemical shifts downfield of the isotropic value correspond to $0° \leqslant \theta \leqslant 54.7°$, and upfield shifts correspond to $54.7° < \theta \leqslant 90°$. We thus define the signed dipolar splitting $\Delta^0_{CF_3}$ to be positive for downfield and negative for upfield chemical shifts, so that from Eq. (3) we get

$$\theta = \arccos \sqrt{\frac{2\Delta_{CF_3}}{3S_{mol}\Delta^0_{CF_3}} + \frac{1}{3}}. \quad (4)$$

The sign of Δ_{CF_3} is the true sign of the dipolar coupling, as the negative sign of the dipolar coupling constant and the negative sign of the rotational order parameter S_{rot} compensate each other [20].

2.7. Determining the orientational constraints

To describe the alignment of PGLa, we defined a molecular frame in which the z-axis is the helix axis of the peptide and the C_α atom of Lys12 lies radially on the y-axis. The helix tilt angle, τ, and the azimuthal rotation of the helix, ρ, define a set of Euler angles $\{0, \tau, \rho\}$ that describes the orientation of the molecule in the laboratory frame, i.e., in the oriented membrane sample. The relative arrangement of the individual labeled CF_3-groups in the molecular frame is given by the assumed molecular model structure. The signed dipolar couplings Δ_{CF_3} of these individual labels are predicted as a function of τ, ρ, and S_{mol}. For all labeled positions, the sum of squared deviations to the experimentally determined couplings, χ^2, is then minimized by a grid search in the region $0 \leqslant \tau < 180°$, $0 \leqslant \rho < 180°$, and $0.5 \leqslant S_{mol} \leqslant 1$. The calculations were done with MATHEMATICA (http://www.wolfram.com), structures were modeled with SYBYL (Tripos, St. Louis, MO) and visualized with MOLMOL [21]. Further details of the procedure are described on our website http://www-ifia.fzk.de/IFIA_Webseiten/Webseiten_Ulrich/index.html, where programs and other scripts that were used for the calculation are publicly available.

3. Results and discussion

3.1. Effect of the CF_3-phenylglycine side chain on peptide properties

It is essential for our investigation that the introduction of a CF_3-label into the PGLa molecule does not change its structural or functional properties. Therefore, we compared the secondary structure of the various labeled PGLa analogues with the wild type peptide by

circular dichroism spectroscopy (CD), and we determined their antimicrobial activities against various strains of bacteria.

Circular dichroism spectra of the peptides were acquired in the presence of negatively charged DMPC/DMPG vesicles (Fig. 2). Its interaction with the lipid membrane transforms the peptide from a random coil conformation to a helical structure. For wild type PGLa and most of the analogues we found a helix content of about 50–60%, in agreement with earlier studies [12]. It did not differ significantly for the analogues with either L- or D-CF_3-Phg labels (Table 1). However, an accurate determination of the helical content depends critically on the peptide concentration in the solution. While the concentration of the labeled peptides could be determined rather exactly from UV absorption of the aromatic ring at 263 nm, this was not possible for the wild type peptide. The apparent difference between wild type PGLa and the labeled peptides is therefore not really significant.

Interestingly, a considerable reduction in helical content was observed for the L- and D-epimers labeled at position Ala8. These peptides also exhibited a visibly reduced solubility. In contrast to all other labeled positions, Ala8 is situated on the hydrophilic face of the helix, as illustrated in Fig. 1. The large hydrophobic CF_3-Phg sidechain in this position thus seems to disturb the amphiphilic character of the peptide that is necessary for helix formation. The fact that Ala8 is close to the less structured N-terminal part of PGLa may also contribute to this structural deviation. We had previously observed that the somewhat less hydrophobic fluoro-phenylglycine label in position Ala8 did not disturb the structure to such extent [6].

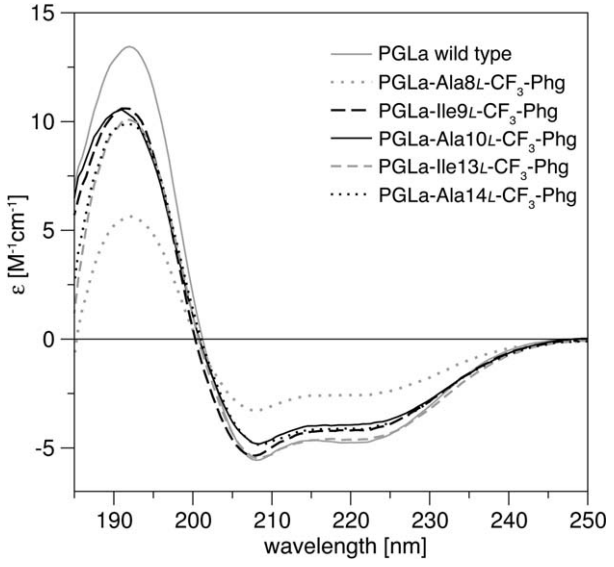

Fig. 2. Circular dichroism spectra of wild type PGLa and its analogues labeled with a single L-CF_3-Phg in position 8,9,10,13, or 14 in the presence of small unilamellar vesicles at a molar ratio of peptide:DMPC:DMPG = 1:120:80.

Table 1
Helix content of PGLa and its CF_3-Phg-labeled analogues (%)

Position/replaced with	L-CF_3-Phg	D-CF_3-Phg
Ala 8	38	19
Ile 9	53	53
Ala 10	48	51
Ile 13	54	48
Ala 14	48	50
Wild type	60	

Antimicrobial activity against 7 Gram-positive and 5 Gram-negative strains of bacteria was tested with agar diffusion assays. Again, PGLa labeled in position Ala8 showed a considerably reduced activity for both L- and D-forms. All other analogues showed an antimicrobial activity similar to wild type PGLa (Fig. 3). Some of the labeled peptides even showed a higher activity than the wild type—in particular the activity of PGLa-10-CF_3-Phg and PGLa-14-CF_3-Phg against two strains of *Pseudomonas aeruginosa* was increased. A similar but less pronounced increase had been previously observed for PGLa-10-fluoro-phenylglycine [6], suggesting that the increased activity is due to the higher overall hydrophobicity of the aromatic side chain in place of Ala. Generally, the D-CF_3-Phg epimers had a lower activity compared to the respective L-CF_3-Phg epimers. Although the differences were small, this was highly significant ($p < 10^{-7}$ in paired t test) for all measurements taken together.

In addition, the minimal inhibitory concentrations of PGLa wild type and the CF_3-Phg analogues were measured for *Escherichia coli* ATCC 25922 and for *Staphylococcus aureus* SG 511 with a standard microbroth dilution method. Between the eight L- and D-analogues

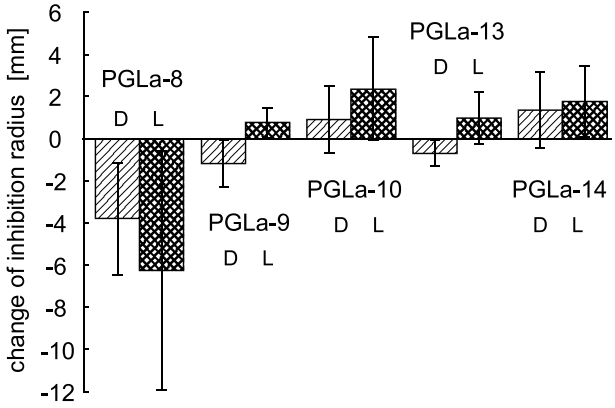

Fig. 3. Effect of the L- and D-CF_3-Phg label on antibacterial activity. Wild type PGLa and its analogues with L- and D-CF_3-Phg in position 8,9,10,13, or 14 were studied by agar diffusion assays. Of the 12 strains of bacteria tested, two were resistant and one gave ambiguous results. For the remaining 9 bacterial strains the average change in the radius of inhibition compared to wild type PGLa is shown together with the standard deviation.

labeled in positions 9,10,13, and 14 and the wild type PGLa the differences in minimal inhibitory concentration were no more than one dilution step of factor 2.

These results justify the use of PGLa analogues with CF_3-Phg labels in positions 9,10,13, or 14 to study the orientation and dynamics of PGLa in the membrane. We did not, however, employ any peptide with a CF_3-Phg label in position 8 for any further experiments.

3.2. Different relaxational behavior of dipolar peaks

The signal of the three equivalent fluorines of the CF_3-group is split into a triplet under the influence of the homonuclear dipolar coupling, which is scaled by fast rotation around the tilted C_ϵ–C_ζ axis and any further molecular wobble. The expected three narrow signals with peak intensities of 1:2:1 are not as well resolved in these samples as they had been previously described for small and highly mobile organic molecules in oriented membranes [11]. In this case of a less mobile peptide, the triplet is always asymmetric with a narrow intense peak near the isotropic chemical shift, and with the broadest peak at the furthest distance away from the isotropic value (Fig. 4). We attribute this asymmetry to a differential transverse relaxation rate of the three signal components, which is modulated by fluctuations of the angle θ between the CF_3-axis and the static magnetic field on the time-scale of the experiment. The anisotropic chemical shift and the homonuclear dipolar coupling in the rotating CF_3-group are both proportional to $3\cos^2\theta - 1$, and at a magnetic field of 11.7 T they have approximately the same magnitude. In one of the three spectral components these two orientation-dependent effects have opposite signs and partially compensate one another. The resonance frequency of this component is therefore close to the isotropic chemical shift value. The central component of the triplet appears at the pure anisotropic chemical shift, as the dipolar contribution is zero. In the third component the two orientation-dependent effects add up, hence the chemical shift of this component depends most strongly on the instantaneous orientation of the CF_3-axis. Consequently, any slow fluctuations in the local tilt angle of the CF_3-group lead to a very different T_2-broadening of the three spectral components. Thus, the observed peak broadening is attributed to slow dynamics rather than insufficient proton decoupling. As we are interested in the orientation-dependence of the signal we cannot make constructive use of this effect. For future analysis of the interaction of a CF_3-group with other nuclei, however, it may be worthwhile considering the development of TROSY experiments [22].

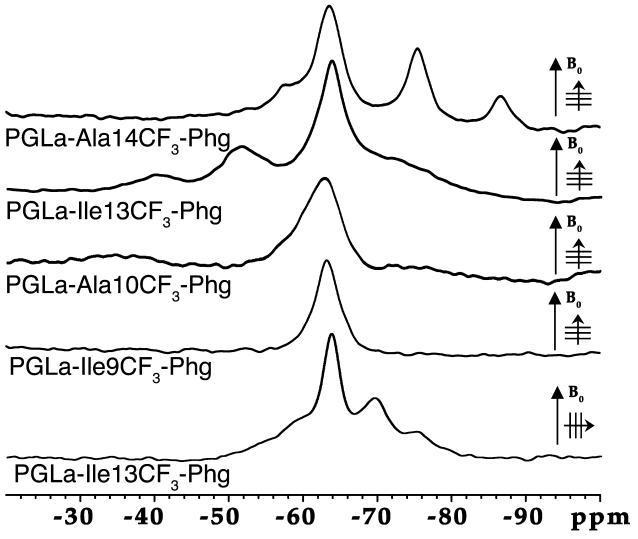

Fig. 4. ^{19}F-NMR spectra of oriented samples of PGLa analogues labeled with L-CF_3-Phg in position 9,10,13, or 14 with a molar ratio of peptide:DMPC = 1:200. 1-pulse spectra were recorded for 1–3 h at 35 °C, with ^{1}H decoupling of 10 kHz. All samples are oriented with the membrane normal parallel to the field, and the PGL-13-CF_3-Phg sample is also shown in the perpendicular orientation. Despite the fast relaxation of some signal components it is possible to read the splitting due to the homonuclear dipolar coupling within the CF_3-group directly from these triplets:

Position replaced with L-CF_3-Phg	Ile 9	Ala 10	Ile 13	Ala 14
Dipolar coupling (kHz)	0	0	5.6	−5.4

In this work, we determined the signed dipolar coupling from a simple NMR experiment with a 90° ^{19}F-pulse followed by continuous wave ^{1}H-decoupling during acquisition. The gap between the ^{19}F-pulse and the start of acquisition was compensated by first order phase correction or backward linear prediction. This experiment was slightly more sensitive than any echo experiment, which gave nearly identical spectra. We also performed CPMG experiments on the CF_3-groups with the oriented peptide samples [11,23,24]. However, these data did not provide any real advantage, as the accuracy of the dipolar coupling was not critical to determine the orientation of the molecule (see below). On the other hand, the CPMG experiments required very accurate optimization of the pulse length, had a lower sensitivity, and for some samples (with broad peaks in the 1-pulse experiment) they failed to show a recognizable signal after several hours of measurement time.

3.3. Orientation of PGLa in the lipid membrane

To determine the alignment of the PGLa molecule with respect to the lipid bilayer in the liquid crystalline state, we had to make certain assumptions concerning its structure and dynamics. In a first approach we assumed that the molecule has a regular α-helical structure. We used a pitch angle $\omega = 99.8°$ between successive residues in the helical wheel and a side chain orientation of $\alpha = 53.2°$ and $\beta = 121.1°$ (see http://www-ifia.fzk.de/IFIA_Webseiten/Webseiten_Ulrich/index.html),

which corresponds to a poly-Ala helix with $\varphi = -58°$ and $\psi = -47°$ as constructed by SYBYL. The effect of all motions namely of the molecule in the membrane and of the side chain within the molecule are approximated by a single isotropic order parameter S_{mol}, which is supposed to scale all dipolar couplings by the same factor. With these assumptions we searched for the molecular orientation $\{\tau, \rho\}$ and order parameter (S_{mol}) that provide the best fit with the experimentally determined dipolar couplings of the four PGLa analogues labeled with L-CF_3-Phg (Fig. 5). We found a global minimum for the sum of squared deviations between experimental and simulated data with $\chi^2 = 0.4\,kHz^2$, which was a very acceptable fit. The corresponding peptide tilt angle of $\tau = 89°$ is in good agreement with previous ^{15}N-NMR data on PGLa [13], and the azimuthal rotation angle $\rho = 106°$ agrees well with the expected alignment of the amphiphilic helix (Fig. 6).

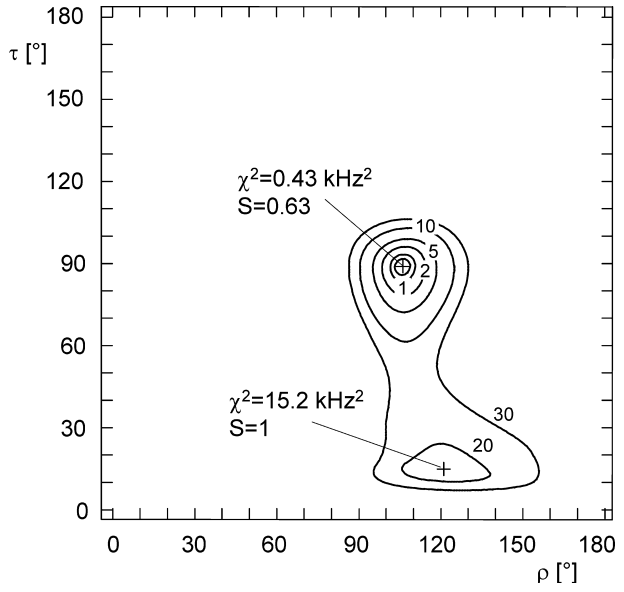

Fig. 5. Fit between the four experimentally measured dipolar splittings (see Fig. 4) and the simulated values that are obtained by systematically rotating the molecule through all possible orientations τ (helix tilt angle) and ρ (azimuthal rotation angle). The molecule is modelled as a regular α-helix with $\omega = 100°$, $\alpha = 53°$, and $\beta = 121°$. For every combination of angles $\{\tau, \rho\}$ the minimum of χ^2 in the complete range of feasible order parameters $0.5 < S_{mol} < 1.0$ is determined and displayed as a contour plot.

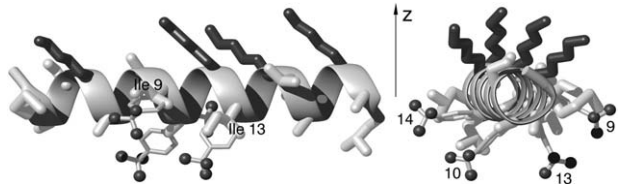

Fig. 6. Regular α-helical model of PGLa in the best fit orientation with $\tau = 89°$, $\rho = 106°$. The charged lysine side chains are shown in dark grey. The z-axis marks the direction of the membrane normal.

The order parameter of $S_{mol} = 0.6$ indicates that the peptide is reasonable mobile in the liquid crystalline lipid bilayer at the low peptide:lipid ratio of 1:200, as seen before for other comparable systems [5,7]. There is only one further local minimum observed ($\tau = 14°$, $\rho = 121°$, $S_{mol} = 1$), but it has a much higher χ^2 deviation of $15.7\,kHz^2$ and can be safely ignored.

In a second approach we constructed the four PGLa analogues with individual L-CF_3-Phg labels as α-helices ($\varphi = -58°$; $\psi = -47°$) and performed a simple energy-minimization to relax the respective local structure and accommodate the bulky L-CF_3-Phg side chains. The orientations of the four C_ϵ–C_ζ axes were again used to determine the overall peptide alignment. With these molecular models we obtained unexpectedly a very good fit for a tilted transmembrane orientation of the helix. This search revealed another minimum for a slightly rotated peripheral orientation although its fit with $\chi^2 = 1.9\,kHz^2$ was not as good (Table 2).

We are able to exclude the unexpected and artefactual solution corresponding to a tilted transmembrane orientation of PGLa, because it is not compatible with the coupling data measured for the corresponding D-CF_3-Phg analogues as discussed below. Nevertheless, the comparison between the different structural models (ideal α-helix versus relaxed conformations) shows that the calculations are sensitive to small changes in the molecular structure. Therefore, we have to consider the effect of any subtle structural changes on the calculation of the molecular orientation very carefully.

3.4. Variation in the model structure

We will now discuss the assumptions made to determine the orientation of the PGLa molecule, and how this result is affected by any deviations from these assumptions.

(a) For the orientational analysis we assume a known molecular structure. It is evident from CD measurements that PGLa assumes a helical structure upon interaction with lipid membranes. High resolution ^{1}H-NMR studies in micelles have shown that the helix is quite rigid in the central and C-terminal part, while the N-terminus is more flexible [13]. These studies did not provide a sufficiently well-resolved structure that would have allowed us to reliably derive the side chain orientations in the molecular coordinate system. In addition, even though our CD measurements have shown very little changes in helicity, the introduction of a CF_3-Phg label may slightly modify the local structure.

(b) We assume that the hydrophobic CF_3-Phg label has no effect on the orientation of the molecule in the lipid bilayer, when substituted for another hydrophobic side chain. The microbial inhibition assays have indeed shown that the labels do not lead to any principal changes in the interaction of the molecules with the

Table 2
Best fit orientations for various model structures of PGLa

Structure				Orientation			
	ω	α (°)	β (°)	χ^2 (kHz^2)[d]	τ (°)	ρ (°)	S_{mol}
α-Helix, standard[a]	99.8	53.2	121.1	**0.39**	**89**	**106**	**0.63**
α-Helix, energy-minimized[b]	91.7– 98.4	43.6–51.9	109.8–111.6	0.10	29	117	0.85
				1.94	**90**	**119**	**0.57**
α-Helix, adapted[c]	100	47	110	0.51	29	113	0.99
				0.97	**98**	**112**	**0.60**
α-Helix, adapted[c]	96	47	110	**0.08**	**99**	**103**	**0.80**
				5.00	29	108	1.00
3_{10}-Helix[a]	119	53.5	111.2	13.90	43	148	1.00
π-Helix[a]	85.3	52.5	131.1	10.40	82	164	0.84
β-Sheet[a]	178.9	28.3	89.2	53.40	142	40	1.00

The peptide orientation is calculated for structural models with the following sidechain arrangements: [a]Secondary structure of poly-Ala chains as constructed in SYBYL with $\varphi = -58°/\psi = -47°$ (α-helix); $\varphi = -50°/\psi = -28°$ (3_{10}-helix); $\varphi = -57°/\psi = -71°$ (π-helix); and $\varphi = -139°/\psi = 135°$ (β-sheet). [b]α-Helix after 500 steps of energy-minimization. [c]Regular α-helices with slightly modified structure (see text). [d]The global minimum of χ^2 and all local minima with $\chi^2 < 10\,\text{kHz}^2$ are shown.

membrane. A variation in the azimuthal rotation angle ρ by several degrees is rather likely, however, as the different retention times of wild type PGLa and the CF_3-Phg analogues in reverse phase HPLC demonstrate that there exist detectable differences in the hydrophobic surfaces of these molecules.

(c) We assume that the fast molecular motions of the peptide (i.e., its deviations from the effective orientation) are not only isotropic, but also that they are identical at all labeled sites. This is most probably not the case, neither for the motion of the whole molecule in the membrane, nor for the internal motion of the respective side chain within the molecule. Consequently, the dipolar couplings of the individual labels will be averaged to a different extent and cannot all be described by exactly the same order parameter S_{mol}. We have to ignore the effects of such anisotropic and non-uniform dynamic modes, because they cannot be described by any simple model.

These limitations of our model assumptions are the major source of uncertainty, compared to which the experimental errors in the dipolar couplings are less critical for an accurate determination of the molecular orientation. Therefore, we have to validate the data evaluation, by (i) systematically examining the deviations resulting from different assumed conformations, (ii) by introducing an error function that takes additional sources of errors into account, and (iii) by including the D-CF_3-Phg peptide analogues in the analysis.

First, we compared the best fitting orientations for different molecular conformations. Energy-minimization had shown that the side chain angle β of L-CF_3-Phg tends to be reduced compared to a poly-Ala helix, and that the angle between two adjacent residues in the helical wheel is rather variable. Hence, we calculated the orientational fits for a large number of regular α-helical structures with ω between 95 and 102°, and β between 105 and 120°. Steps were small enough (1–5°) to see a smooth shift in the results of the fit. Only two of these datasets are shown in Table 2. The side chain angle α was not varied in this series, but a change of α lead to a change of $-\rho$ by the same amount without affecting the quality of the fit. In addition we evaluated four of these structures after energy-minimization. All α-helical structures, both regular and energy-minimized ones, had a local minimum in the range of $\rho = 100$–120°, and $\tau = 88$–99°. This indicated an uncertainty in the molecular orientation of about 10–20° resulting from small variations in molecular structure. The uncertainty due to experimental inaccuracy (about $\pm 0.5\,\text{kHz}$) was considerably smaller.

We also examined structures other than α-helix. Even though it is clear that a 3_{10}- or π-helical structure is incompatible with an amphiphilic distribution of side chains in PGLa, we tested whether they would fit the experimental data. For these regular helical structures we obtained minima with rather high χ^2 (Table 2). Nonetheless, after locally adjusting certain side chain orientations by less than 10° accurate fits could be obtained (not shown). A putative β-sheet structure was altogether incompatible with the experimental data. Generally, we found that small changes in the model structure have a large influence on the quality of the best fit. Nevertheless, it is impossible to determine the exact structure of the molecule from these data, as there are too many parameters for fine-adjustment. We can rather hope to find a possible range of orientations for certain groups of structures, thereby, excluding some other groups of structures that are not compatible with the

data. It should be noted that each individual side chain can generally fit the measured dipolar coupling in four different azimuthal orientations ρ for any given set of τ and S_{mol} values. Therefore a "false" structure in a "false" orientation may also happen to fit the experimental data very well.

This first strategy could not account for deviations like variations in peptide alignment as discussed under (b) above. Following a second strategy, therefore, we assigned an estimated error both to the experimentally determined dipolar couplings and to the orientation of each individual side chain, i.e., to the angle between each CF_3-axis and the membrane normal. The deviation χ^2 is normalized on the basis of these error estimates (http://www-ifia.fzk.de/IFIA_Webseiten/Webseiten_Ulrich/index.html). χ^2 is expected to increase with the number of experimental constraints and to decrease with the number of fitted parameters. An objective criterion as to which χ^2 values represent acceptable solutions cannot be stated, because the error estimates are subjective and neither the couplings nor the parameters form sets of independent data. We may consider sets ρ, τ, S_{mol} with $\chi^2 < 10$ as possible solutions and sets with $\chi^2 < 1$ as a good agreement with the experiment. This strategy has several advantages. It allows to assign different error margins to individual labels, and conformational errors are correctly translated into couplings with the $\cos^2$ function. Basically, this second strategy led to the same conclusions as the first one, as shown in Fig. 7A.

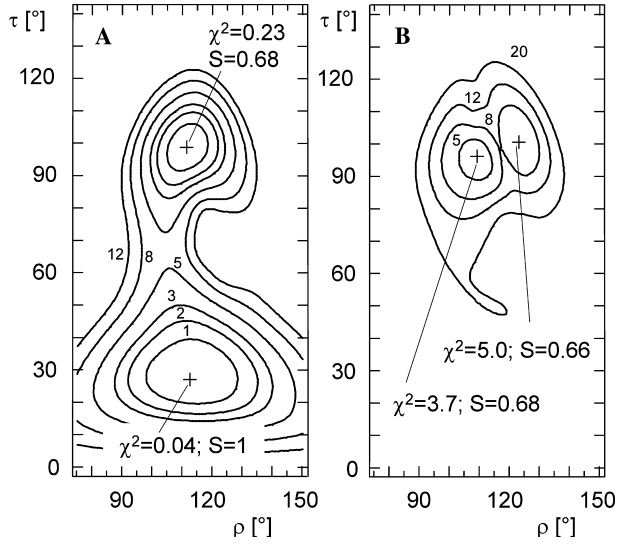

Fig. 7. Fit between the measured and simulated dipolar splittings as a function of τ and ρ for order parameters $0.5 < S_{mol} < 1.0$. In (A) the dipolar couplings of the L-CF_3-Phg labels in position 9,10,13, and 14 are used, while in (B) the couplings of both L- and D-CF_3-Phg labels in these positions are used. In contrast to Fig. 4, $\omega = 99.9°$, $\alpha = 47.3°$ (L) and $-40.1°$ (D), $\beta = 110.7°$ (L) and $70.1°$ (D). χ^2 is normalized to the following scaling factors: all couplings to 0.5 kHz, the angle to the membrane normal to 10° for L- and 20° for D-CF_3-Phg analogues, except for 14L, 9D, and 13D with an additional 5° or 10° uncertainty due to changes of hydrophobic moment.

3.5. Peptide analogues with D-CF_3-phenylglycine

As a by-product of the purification of the labeled all-L-PGLa analogues we had also obtained peptides with a single D-CF_3-Phg side chain. We analyzed their ^{19}F-NMR spectra in DMPC membranes (Table 3) to evaluate their potential for structure analysis. In case they turn out to be reliable sources of information, this would double the number of independent parameters for calculating the overall peptide alignment. To interpret the corresponding data, the local structure of the D-CF_3-Phg label in the molecule has to be known. Major distortions of the α-helical structure are not expected for the following reasons: first, circular dichroism detected no significant reduction of the overall helicity (Fig. 2). Second, by high resolution ^{1}H-NMR we have previously shown that a ^{19}F-labeled amino acid with very similar steric restrictions, D-4-fluoro-phenylglycine, substituted at position Ala10 of PGLa does not cause a local break in the helix [6]. Third, computer modeling shows that in none of the labeled positions in PGLa the D-CF_3-Phg side chain would require major structural rearrangements to relax the surrounding steric contacts. That is, energy-minimization of the D-analogues changed the orientation of the CF_3-axis in the molecule by less than 6° starting from a regular α-helix with $\varphi = -58°$; $\psi = -47°$. On the other hand, a statistical analysis had reported an undisturbed α-helical structure to be quite rare for any D-amino acid situated between two L-amino acids [25]. We also observed a slightly reduced antimicrobial activity for the D-CF_3-Phg analogues compared to the respective L-CF_3-Phg epimers. Taken together, we expect the D-CF_3-Phg side chains to assume a local conformation that does not differ much from an α-helix, but we cannot predict the exact conformational deviation.

As a first observation we note that the dipolar coupling data from the D-CF_3-Phg analogues are not fully compatible with a regular α-helical structure. The best fit has a χ^2 of 26 kHz^2 and gives $\tau = 102°$, $\rho = 139°$, and $S_{mol} = 0.8$. Starting from an energy-minimized structure, we found a minimum of $\chi^2 = 23$ kHz^2 for $\tau = 97°$, $\rho = 138°$ and $S_{mol} = 0.8$. By slightly varying the angles of a regular α-helical structure we did not achieve any acceptable reduction of χ^2 either. These data thus suggest that some considerable local deviations from the α-helical model are induced by D-CF3-Phg, after all.

Table 3
Dipolar coupling of PGLa analogues with D-CF_3-Phg label

Position replaced with D-CF_3-Phg	Ile 9	Ala 10	Ile 13	Ala 14
Dipolar coupling (kHz)[a]	0	12.1	−3.5	8.2

[a] Couplings were measured in oriented samples with a 1:200 molar peptide:DMPC ratio at 35 °C.

Although no reliable stand-alone solution is found, nevertheless, the tilt angle τ of the best fit solutions is close to the expected alignment of PGLa. We also believe that the apparent increase in ρ compared to the azimuthal orientation of the L-CF_3-Phg analogues may be rationalized by two arguments. First, the D-CF_3-Phg side chains are in close contact with the i + 3rd residues. The entropy of such a conformation is very low, and in view of the high flexibility of the peptide the average distance between these side chains will probably be larger than that of the single energy-minimized structure. For some more realistic structural models with larger α we indeed found fits with lower ρ values (not shown). Second, the extended hydrophobic CF_3-Phg side chain in the D-enantiomer is expected to modify the hydrophobic moment of the peptide compared to the wild type or L-CF_3-Phg analogues. If this effect is visualized in a lipid environment, the helix would indeed become rotated towards the direction of larger ρ values.

Despite the uncertainty concerning the exact conformation of the D-CF_3-Phg analogues, their experimental data are useful to exclude certain putative solutions that have emerged from the analysis of the L-CF_3-Phg labels alone. Namely, for several α-helical structures and the 3_{10}-helix a second local minimum have suggested a solution with a low τ value, corresponding to a tilted transmembrane orientation rather than a peripheral peptide alignment (Table 2). In any model with such small tilt angle, it is clear that the set of individual side chains must show similar dipolar couplings. The couplings of the D-CF_3-Phg analogues, however, gave values between −3.5 and +12.1 kHz (Table 3), which clearly excluded such transmembrane orientation.

A more quantitative approach was used to include the data from the D-CF_3-Phg analogues into the orientational fit. We followed the second strategy discussed above and simply assigned large error estimates to the side chain angle α of the D-CF_3-Phg analogues. For the α-helical structures the peripheral orientation now emerges as a unique solution (Fig. 7B). The 3_{10}-helix model can also be excluded by this strategy, as it does not yield any acceptable solution (not shown).

3.6. Interaction of PGLa with gel phase membranes

The robustness of the ^{19}F 1-pulse experiment allowed us to measure the dipolar couplings under a wide range of experimental conditions and to monitor the behavior of the peptide throughout the lipid phase transition of the DMPC membranes. In the lipid gel phase at 15 °C, we found a slight increase in all dipolar couplings, when the sample was measured in the standard orientation with the membrane normal parallel to the magnetic field. The orientational fit for the regular α-helix has a minimum at virtually the same orientation as in the liquid crystalline phase, but with an order parameter that is increased by about 12% (to $S_{mol} = 0.71$ for the regular standard helix shown in Table 2). When the sample was measured with the membrane normal perpendicular to the field, the signals of the peptide in the fluid and gel phases of the lipid differed qualitatively. In the fluid phase membrane the anisotropic chemical shift and dipolar coupling were scaled with respect to the horizontal membrane alignment by an order parameter of −1/2, as expected for fast rotation of the peptide around the membrane normal (Fig. 4). Experiments with vertically tilted samples in the gel phase showed only a broad component around the isotropic position. The rotation of the peptide obviously stops or becomes so slow that the different orientations of the label are not averaged, and the other components of the signal are broadened and disappeared in the background. Thus, we conclude that the interaction of PGLa with DMPC does not depend on the phase state of the lipid. The observed increase in the order parameter very likely reflects a reduced overall mobility of the peptide in the more rigid gel phase membrane. The fast rotation around the membrane normal is stopped as expected for an inserted peptide.

4. Conclusions

The high intrinsic sensitivity of the ^{19}F nucleus and the simple 1-pulse NMR sequence make the proposed dipolar approach very robust under a wide range of experimental conditions. Analogous experiments with membrane-active peptides based on ^{15}N labeling suffer from several limitations. Fast motion makes the ^{1}H–^{15}N cross polarization inefficient. Slow motion leads to fast transverse relaxation and reduces the sensitivity, which is anyway not very high for ^{15}N. With the CF_3-label, on the other hand, we measured dipolar couplings from 0.25 μmol of peptide in 2 h. Only some small dipolar couplings are virtually obscured by the linewidth itself and have to be approximated. Our general conclusions are summarized as follows:

- ^{19}F homonuclear dipolar couplings within the CF_3-group of 4-trifluoromethyl-phenylglycine provide a very accurate measure of the orientation of the amino acid side chain, which is rigidly attached to the peptide backbone.
- A simple 1-pulse ^{19}F-NMR experiment allows the simultaneous determination of the anisotropic chemical shift and the homonuclear dipolar coupling, thus, uniquely defining the effective tilt angle of the CF_3-axis with respect to the external field.
- Using four CF_3-labeled peptide analogues we could confirm that PGLa is aligned at the surface of DMPC bilayers at a peptide:lipid ratio of 1:200, with the helix axis perpendicular to the membrane normal.

- We have measured the azimuthal rotation angle of the PGLa helix, which agrees well with the orientation expected from its amphiphilic character.
- Peptide analogues with a D-CF_3-Phg label provide only qualitative information about the peptide structure and orientation, which can be used to supplement the more reliable data from the L-CF_3-Phg labels.
- The CF_3-Phg side chain is not compatible with all hydrophobic positions along a peptide sequence, hence the labeled analogues have to be critically tested for an intact conformation and biological function.
- The simple 1-pulse ^{19}F-NMR experiment allows highly sensitive measurements over a wide range of conditions (such as very low peptide concentration [9]) and dynamic regimes. This makes it both an attractive alternative and a complement to conventional NMR experiments based on ^{15}N- or ^{2}H- labeling to monitor the orientation and dynamics of peptides and proteins in the membrane.

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