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Chemical Labeling Strategy with (*R*)- and (*S*)-Trifluoromethylalanine for Solid State ^{19}F NMR Analysis of Peptaibols in Membranes

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A chemical ^{19}F-labeling scheme for solid state NMR structure analysis of peptaibols is introduced here and applied to alamethicin (ALM). Like other antimicrobial peptides, this amphiphilic molecule acts by permeabilizing bacterial membranes.[1] It can assemble in lipid bilayers as a putative barrel-stave pore, but its exact backbone conformation, helix alignment, and oligomeric state are still under debate.[2] Given the high proportion of α-aminoisobutyric acid (Aib) in peptaibols,[3] the correspondingly labeled trifluoromethylalanine (CF_3-Ala) should be ideally suited as a reporter group for ^{19}F NMR.[4] Taking advantage of the exceptional sensitivity of fluorine,[5] we determined the conformation, alignment, and dynamics of ALM in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers. By introducing the (*R*)- and (*S*)-stereoisomers of CF_3-Ala as pairs of separate structural reporters, we gained a maximum number of NMR constraints from only a few selectively labeled positions.

From the naturally occurring heterogeneous ALM mixture the sequence of F30/3 (Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phe-ol) was chosen, since an X-ray structure exists for the crystalline form.[6] Liquid state NMR had confirmed the largely helical conformation,[7] which is interrupted by a kink at Pro14. To determine the structure of ALM in a lipid environment by solid state NMR, we replaced a single Aib residue in position 5, 10, or 16 by CF_3-Ala. This amino acid has never before been used for this purpose; hence we aimed to examine it and demonstrate here that it fulfills all criteria of an ideal ^{19}F NMR label, being highly sensitive, containing a spinning CF_3-group that is attached to the peptide backbone, and being structurally virtually unperturbing.[8] Another novel aspect of the Aib-labeling strategy is the fact that twice as many unambiguous (see ref 2b) local NMR parameters can be obtained compared to conventional amino acids. Namely, for each of the three Aib positions to be labeled, two epimeric peptides were synthesized with either the (*R*)- or (*S*)-stereoisomer of CF_3-Ala, resulting in a total of six different ALM analogues.

CF_3-Ala was synthesized as reported.[9] Its incorporation into the peptides was challenging, as the CF_3-group drastically reduces the nucleophilicity of the amine, in addition to its obvious steric hindrance. Since coupling of the subsequent amino acid is slow in solid phase synthesis,[10] three tripeptides were prepared first in solution [see Supporting Information (SI)] as N^{α}-protected building blocks: Ala-(CF_3-Ala)-Ala, Val-(CF_3-Ala)-Gly, and Val-(CF_3-Ala)-Aib.

The use of racemic *N*-benzyloxycarbonyl-CF_3-Ala yielded epimeric mixtures for each tripeptide, and the isomers containing (*R*)- and (*S*)-CF_3-Ala could be separated by flash chromatography. Given that they are separatable, this approach is preferable over stereoselective synthesis, since the subsequent NMR structure analysis relies on the use of both epimers. The purity of the peptide fragments was confirmed by HPLC, ^{1}H and ^{19}F NMR, and mass spectrometry (see SI). To assign the (*R*)- and (*S*)-stereocenters, each of the six compounds was hydrolyzed and compared by analytical HPLC with the retention profile of pure H-(*R*)-CF_3-Ala-OH and H-(*S*)-CF_3-Ala-NH_2 on a C18 column with a chiral mobile phase.[11] The full-length ALM sequences were then completed by manual solid phase peptide synthesis and purified by semipreparative HPLC (see SI).

To examine the structural and functional compatibility of the CF_3-Ala substitutions, circular dichroism (CD) spectra were measured for ALM F30/3 and the six ^{19}F-labeled analogues under membrane-mimicking conditions (50% TFE in phosphate buffer, Figure 1A,B). All line shapes are very similar with a high helical content of 40−50% according to quantitative deconvolution by standard algorithms.[12] This confirms that a substitution of Aib by CF_3-Ala does not perturb the backbone in either configuration. Functional tests of all ALM analogues by bacterial growth inhibition assays gave the same conclusion (see SI).

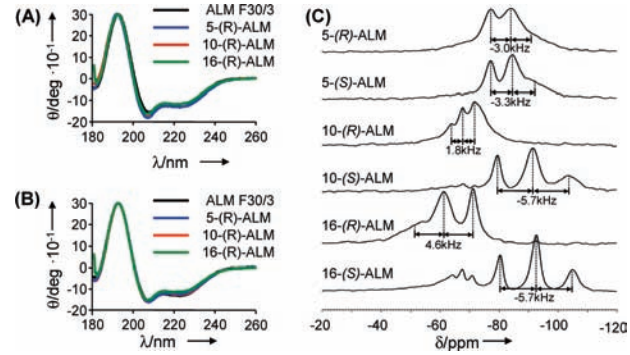

Figure 1. (A, B) CD spectra of the six ^{19}F-labeled ALM analogues (with (*R*)- and (*S*)-CF_3-Aib) and of the wild type F30/3, recorded in 1:1 (v/v) TFE/buffer. (C) Solid state ^{19}F NMR spectra of the same analogues, reconstituted in macroscopically aligned DMPC bilayer and measured at 308 K with the membrane normal parallel (0°) to the static magnetic field.

For solid state ^{19}F NMR analysis, all peptides were reconstituted into macroscopically oriented DMPC bilayers at a molar peptide-to-lipid (P/L) ratio of 1/10, as previously described for other peptides.[13] The narrow ^{31}P NMR lineshapes of all samples demonstrated a high quality of lipid alignment (e.g., SI).

The ^{19}F NMR spectra show that all six ALM analogues were well oriented, since no "powder patterns" were present (Figure 1C). From the triplet splittings of the CF_3-labels the ^{19}F−^{19}F dipolar

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couplings could be extracted. Measurements at different sample inclinations (0° and 90° with respect to magnetic field) show that the peptides undergo unrestricted rotational diffusion in the liquid crystalline bilayer on the millisecond time scale, since the splittings decrease at 90° by a factor of −1/2 due to motional averaging (cf. previous reports).[5b,13,14] The dipolar coupling of each CF_3-group was converted into a local orientational constraint for the corresponding labeled position.[5b,15] The resulting six parameters were used in a grid search to determine the orientation of ALM in terms of its tilt angle (τ), azimuthal rotational angle (ρ), and dynamic parameter S_{mol}, as described in the SI. For an initial assessment, several different helical conformations were examined as simplified input models, namely a straight α-helix, a straight 3_{10}-helix, and the crystal structure (PDB-code: 1AMT).

None of these initial model structures gave a self-consistent solution with an acceptable χ^2 minimum. Given the pronounced and possibly flexible kink at Pro14, we then excluded the two constraints of position 16. The remaining four constraints on the N-terminal helical segment (positions 5 and 10) gave an excellent fit with a low χ^2 (Figure 2A). This best-fit solution suggests that the N-terminal α-helix is oriented with a tilt angle τ of 8° ± 4°, corresponding to an almost upright transmembrane alignment. The azimuthal rotation ρ of nominally 82° ± 30° is intrinsically poorly defined due to this upright alignment. Other conformations such as a 3_{10}-helix and the crystal structure were also tested, but these χ^2 minima were not satisfactory (see SI). The observed conformation and alignment of ALM in DMPC (Figure 2B) are thus fully consistent with previous reports on the orientation of the N-terminal helix.[2a,6,16] The high order parameter (S_{mol} = 0.99) indicates that the peptide does not undergo any long-axial wobble in the lipid bilayer, which is plausible when several monomers are assembled as an oligomer.[17] Indeed, this observation supports the formation of a barrel-stave pore under the present sample conditions (P/L = 1:10) as has been suggested before.[18]

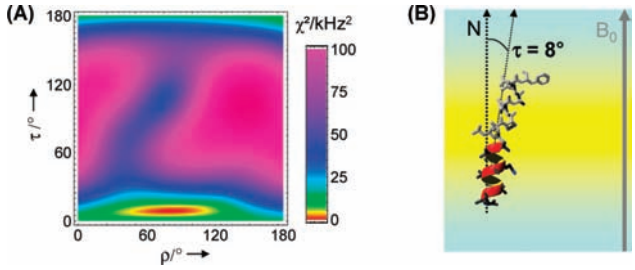

Figure 2. (A) ^{19}F NMR data yield a ρ/τ plot with a unique χ^2-minimum for the N-terminal segment (based on the four labels from positions 5 and 10), revealing an α-helical conformation with best-fit values of $\tau \approx 8°$, $\rho \approx 82°$, and $S_{mol} \approx 1$. The two C-terminal constraints from position 16 deviate from this alignment (when modeled as a straight α-helix or 3_{10}-helix), confirming that the structure is interrupted by a kink. (B) Visualized alignment of the N-terminal α-helix (in red) of ALM with a tilt angle of $\tau \approx 8°$ relative to the membrane normal in a DMPC bilayer (the kinked C-terminus is shown gray; drawing created with MOLMOL).[19]

In summary, three different Aib residues of the peptaibol alamethicin were substituted with (*R*)- and (*S*)-CF_3-Ala to obtain six ^{19}F-labeled analogues by a combination of solution phase and solid phase peptide synthesis protocols. Having proven that the secondary structure and antimicrobial function of these ALM analogues remained unperturbed, the local orientations of the CF_3-groups were measured by solid state ^{19}F NMR. Structure calculations yielded a unique and accurate picture of the N-terminal segment of ALM in DMPC membranes. These results show *per se* that (i) its conformation is α-helical (but not 3_{10}) with a kink presumably at Pro14; (ii) the N-terminal helix has a transmembrane alignment with an 8° tilt angle; and (iii) its mobility suggests an oligomeric assembly that does not wobble but is nevertheless able to diffuse laterally in the bilayer. Given the high sensitivity of ^{19}F NMR and having demonstrated here the particular utility of this new Aib-labeling scheme for peptaibols, the inclusion of an additional pair of CF_3-Ala labels in the C-terminus could be used in the future to complete the picture of the full-length peptide. Thus, it will be possible to examine the influence of peptide concentration and lipid composition on the behavior of ALM, specifically concerning its postulated switch between a monomeric state and an oligomeric assembly, and to answer the question of whether the kink at Pro14 participates in a conformational change upon activation and pore formation.

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Supporting Information Available: Full experimental details regarding materials, methods, synthesis of ^{19}F-labeled Aib, tripeptides and full ALM analogues, and their characterizations. This material is available free of charge via the Internet at http://pubs.acs.org.

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