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Delivering Structural Information on the Polar Face of Membrane-Active Peptides: ^{19}F-NMR Labels with a Cationic Side Chain

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Abstract: *Conformationally constrained non-racemizing trifluoromethyl-substituted lysine isosteres [(E)- and (Z)-TCBLys] with charged side chains are presented as a new type of ^{19}F-NMR labels for peptide studies. Design of the labels, their synthesis, incorporation into peptides and experimental demonstration of their application for solid state NMR studies of membrane-active peptides are described. A series of fluorine-labeled analogues of the helical amphipathic antimicrobial peptide PGLa(Nle) was obtained, in which different lysine residues in the original peptide sequence were replaced, one at a time, by either (E)- or (Z)-TCBLys. Antimicrobial activities of the synthesized analogues were practically the same as those of the parent peptide. The structural and orientational parameters of the helical PGLa(Nle) peptide in model bilayers, as determined using the novel labels confirmed and refined the previously known structure. (E)- and (Z)-TCBLys, as a set of cationic ^{19}F-NMR labels, were shown to deliver structural information about the charged face of amphipathic peptides by solid state ^{19}F-NMR, previously inaccessible by this method.*

Membrane-active peptides (MAPs) realize their functions via interactions with biological membranes and play crucial roles in many fundamental biological processes, including innate immune response,[1] membrane fusion,[2] intracellular transport,[3] amyloidogenesis,[4] among others.[5] The structure, dynamics and alignment of MAPs in lipid bilayers are important parameters for unraveling the molecular mechanisms of their biological actions.

Only very few of the current techniques are suitable for structural studies of MAPs in their membrane-bound state under ambient conditions, because of the inherent complexity of the membrane systems. This complexity and conformational plasticity of MAPs make it nearly impossible to perform X-ray diffraction or electron microscopy studies. The biophysical methods which use oriented membranes (fluorescence-,[6] infrared-,[7] and oriented circular dichroism spectroscopy,[8] colorimetry,[9] interface-sensitive X-ray or neutron scattering[10] and quartz crystal microbalance[11]) provide only low-resolution structural information or suffer from technical problems (e.g. radiation damage or membrane dehydration).

As the most appropriate methodology, solid state NMR spectroscopy (ssNMR) of the peptides labeled by ^{2}H, ^{15}N, ^{13}C, ^{19}F isotopes is used for decades to study MAPs at near-atomic resolution.[12a] In particular, ^{19}F-ssNMR method has been successfully used to obtain structural information for more than a dozen MAPs.[12b–e]

Unlike labeling by ^{2}H, ^{15}N or ^{13}C, introduction of a fluorine-substituted amino acid in place of natural residues, shown to be useful for qualitative structural assessments,[13] might significantly change structure and properties of the labeled peptides.[14] While it might be beneficial for the design of peptides with improved characteristics for practical application, it is completely unacceptable for peptide studies by ^{19}F-ssNMR, where an ideal label should be non-perturbing and conformationally rigid in order to provide correct structural constraints by NMR.[12b–d]

Currently known ^{19}F-labels (**1**–**6**) are shown in Figure 1; they were used to substitute non-polar natural amino acids.[12b–d,15] The only exception is the amino acid **7**[16] which has a polar side chain. To date, no fluorine-substituted amino acids with charged side chains have ever been described as labels for ^{19}F-ssNMR studies of MAPs. One of the reasons for this is the challenging design: a fluorine-containing substituent will perturb the polar charged side chains much more than will modify the hydrophobic non-polar aliphatic residues.

Charged amino acid residues are known to be fundamentally important for the structure and functions of numerous MAPs. For example, interaction of anionic lipid head groups with the positively charged side chains is a prerequisite for the MAPs to bind to natural membranes.[17] Another example is the ladder-like pattern of positively and negatively charged amino acid residues forming multiple salt bridges, which has

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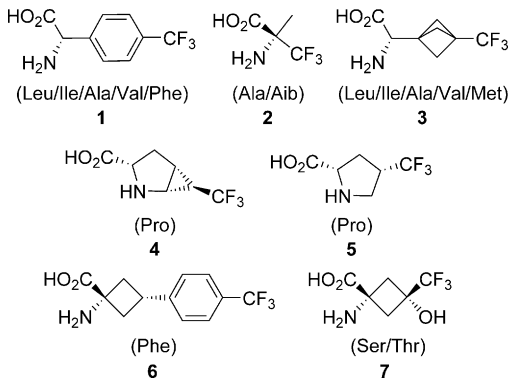
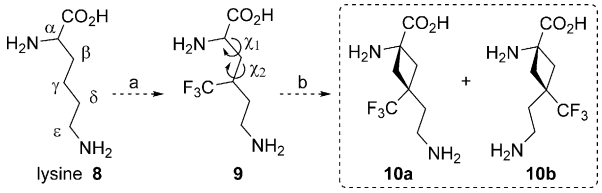


Figure 1. Known CF_3-substituted labels for ^{19}F-ssNMR and the natural amino acid residues that they could substitute in MAPs (in brackets, Aib = α-aminoisobutyric acid).

been recently described as a "charge zipper" motif in several MAPs.[18]

Based on ^{19}F-ssNMR analysis, it was recently shown that a lack of ^{19}F-labels on the charged side of an amphipathic peptide can lead to ambiguity in determining its membrane-bound orientation.[19] The polar/charged face of peptides, as of now, has to be considered the side of MAPs largely unexplored by the ^{19}F-ssNMR. Here, we describe the first representatives of charged ^{19}F-labels, i.e., cationic lysine analogues suitable for labeling MAPs.

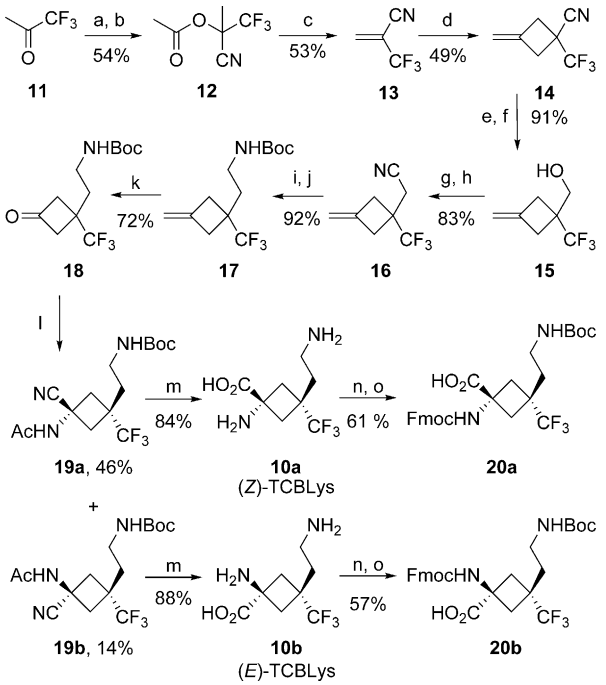
In our design of cationic ^{19}F-labels, lysine (**8**) served as a template; we modified the molecule with the intention of introducing minimal perturbations to the amino acid structure and properties (Scheme 1), as required for the ^{19}F-ssNMR



Scheme 1. Design of lysine analogue TCBLys (both diastereomers are shown) as a label for ^{19}F-ssNMR. a) Introduction of the CF_3-group; b) fixation of χ_1 and χ_2.

studies of MAPs. The γ-position on the lysine side chain appeared to be superior to the other sites for attaching the electron-withdrawing CF_3-substituent (structure **9**). The fluorine-containing group in this position resides the farthest from all the other functional groups and has the least influence on their chemical properties. This is especially important for preserving the high basicity of the ε-amino group, which critically determines the charged character of the amino acid in peptides at ambient pH. A comparison of the pK_a for butan-1-amine (10.7) and 4,4,4-trifluorobutan-1-amine (9.7)[20] justifies this design strategy. The conformational restriction of the fragment connecting the aminocarboxylate moiety to the NMR-reporter CF_3 group can be achieved by incorporating a $-CH_2-$ linker between the α- and γ-positions,[15e,16] leading to the cyclobutane-derived amino acids **10a**,**b** (trifluoromethyl- and cyclobutane-containing lysine analogues, TCBLys) which were selected for the synthesis.

The synthesis (Scheme 2) was based on the construction of 1,3-functionalized cyclobutane derivative **14** using a [2+2] cycloaddition reaction between allene and CF_3-substituted acrylonitrile **13**.[21] In our hands, the transformations leading



Scheme 2. Synthesis of (*Z*)-TCBLys (**10a**), (*E*)-TCBLys (**10b**) and their orthogonally *N*-protected derivatives (**20a**,**b**) for Fmoc-SPPS. a) KCN, H_2SO_4, <10 °C, 15 min; b) AcCl, reflux, 5 h; c) pyrolysis, 550 °C, 4 h; d) allene, PhH, hydroquinone, 200 °C, 24 h; e) KOH, EtOH–H_2O, reflux, 12 h; f) $LiAlH_4$, Et_2O, RT, 12 h; g) CH_3SO_2Cl, Et_3N, CH_2Cl_2, −10 °C, 30 min; h) KCN, DMF, 100 °C, 12 h; i) $LiAlH_4$, Et_2O, RT, 12 h; j) Boc_2O, RT, 2 h; k) $NaIO_4$, $RuCl_3$, CH_3CN–H_2O, RT, 30 min; l) NH_3, Ti(O-*i*-Pr)$_4$, TMSCN, *i*-PrOH, RT, 1 h, then Ac_2O, Et_3N, THF, RT, 12 h, chromatographic separation; m) 6 N HCl, reflux, 12 h, ion-exchange chromatography; n) Boc_2O, TMSCl, DIPEA, CH_2Cl_2, −15 °C, 18 h; o) FmocCl, RT, 5 h.

to the cyclobutane ring formation were scalable, despite the high temperature required for the pyrolysis of **12**. The subsequent hydrolysis of the nitrile group in **14** followed by $LiAlH_4$ reduction produced the alcohol **15**. It was transformed to nitrile **16** via the corresponding mesylate. Reduction of the nitrile group in **16** with $LiAlH_4$ furnished a volatile primary amine, which, in its crude form, reacted with di-*tert*-butyl pyrocarbonate (Boc_2O) to give the Boc-protected derivative **17**. Oxidative cleavage of the double bond in **17** by $NaIO_4$–$RuCl_3$ afforded the Boc-*N*-protected amino ketone **18**. A modified Strecker reaction using Ti(O*i*-Pr)$_4$ as water scavenger and mild Lewis acid[22] was employed to obtain a mixture of diastereomeric acylated amino nitriles (**19a:19b** = 3:1) after treating the intermediate amino nitrile with acetic anhydride. The diastereomers were separated by column chromatography, and the major isomer was identified as (*Z*)- by NMR and X-ray analysis (see the Supporting

Information). Refluxing each aminonitrile (**19a**,**b**) in 6 N HCl achieved full hydrolysis and provided the amino acids **10a**,**10b**.

To be suitable for Fmoc (Fmoc = 9-fluorenylmethoxycarbonyl) solid state peptide synthesis (Fmoc-SPPS), the amino groups in the synthesized lysine analogues **10a**,**b** should be orthogonally protected. Selective N^{ε} protection could be accomplished using 1.3 equiv. Boc_2O at low temperature (−15 °C); only a trace amount of the N^{α}-Boc-derivative was formed. A subsequent one-pot reaction with FmocCl led to the smooth formation of compounds **20a** and **20b**. This approach yielded regioselective orthogonal protection without the need to isolate the intermediate compounds.

Next, we tested compatibility of **20a**,**b** with Fmoc-SPPS by synthesizing Lys/TCBLys-substituted analogues of PGLa(Nle), a Met/Nle (Nle = norleucine) mutant of the antimicrobial peptide PGLa. This peptide is a typical representative of α-helical amphipathic peptides whose thorough ^{19}F-ssNMR analysis has been recently performed using non-polar ^{19}F-label **3**.[23] The lysine residues at positions 12, 15, and 19 were substituted, one at a time, with either (*E*)- or (*Z*)-TCBLys to yield a total of 6 labeled analogues (Figure 2). Despite being

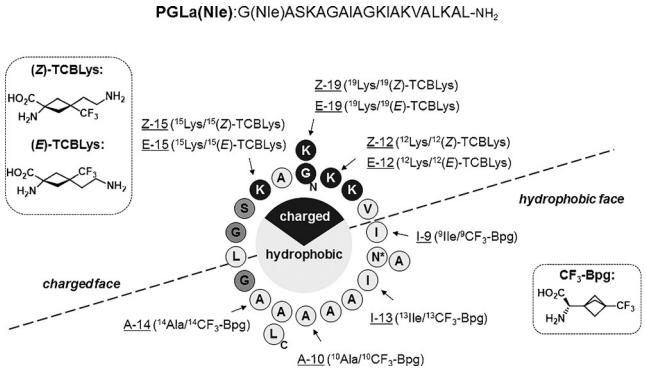
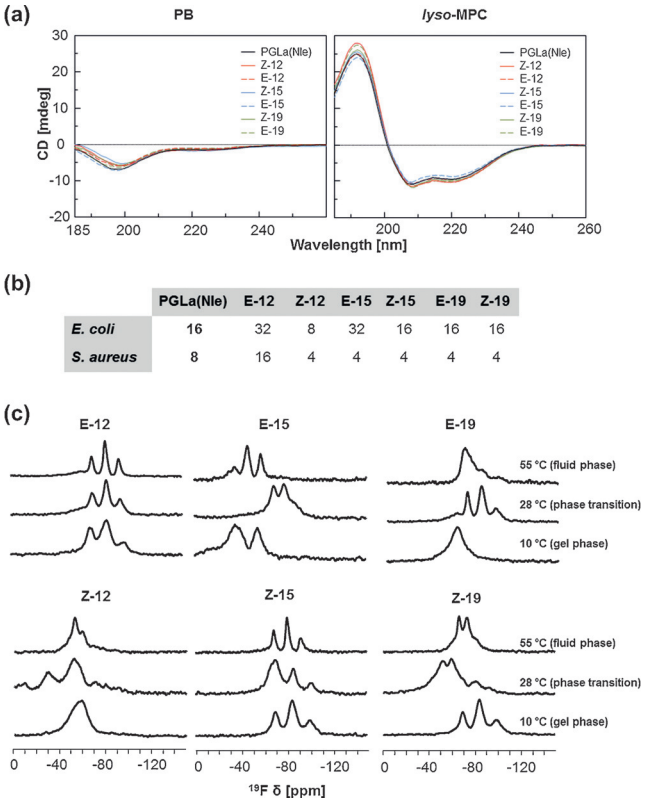


Figure 2. Amino acid sequence, helical wheel and amphipathicity of the α-helical PGLa(Nle); shown by arrows are the positions labeled with the new ^{19}F-label **10a** and **10b** (TCBLys) and previously addressed with **3** (CF_3-Bpg); mutations are indicated in brackets (N* = Nle).

α,α-disubstituted, **20a** and **20b** showed sufficient reactivity, similar to that of Fmoc-Lys(Boc)-OH, and were thus found to be fully compatible with Fmoc-SPPS.

One of the main goals of developing TCBLys was to obtain a ^{19}F-label with structure and properties similar to lysine. As a first criterion to assess the similarity, we evaluated the basicity of the ε-amino group of the ^{19}F-label (see the Supporting Information). The pK_a values in the (*E*)- and (*Z*)-TCBLys residues (as determined using *E*-19 and *Z*-19 peptides) were 9.3 ± 0.1 and 9.7 ± 0.1, respectively. These values are close to the corresponding pK_a value of the lysine side chain (10.3). Hence, the impact of the CF_3-group on the basicity of the (*E*)- and (*Z*)- residues is small.

The structural impact of the Lys/TCBLys-substitution was further evaluated by circular dichroism (CD) spectroscopy (Figure 3a). All six tested peptides adopted random coil conformations in aqueous phosphate buffer (PB) while being structured as α-helices in the presence of *lyso*-lipid (*lyso*-MPC) micelles. Therefore, we concluded that residues of the TCBLys diastereomers resemble lysine residues regarding their structural impact on the polypeptide backbone.



	PGLa(Nle)	E-12	Z-12	E-15	Z-15	E-19	Z-19
E. coli	16	32	8	32	16	16	16
S. aureus	8	16	4	4	4	4	4

Figure 3. Properties of the Lys/TCBLys mutants of PGLa(Nle). a) CD spectra in aqueous (left: PB) and membrane-mimicking (right: micelles of *lyso*-MPC in PB, peptide/detergent = 1/100 mol mol^{-1}) environments. All peptides are at 0.05 mg mL^{-1} concentration. b) MIC (μg mL^{-1}) against *E. coli* and *S. aureus*; c) Representative ^{19}F-ssNMR spectra from labeled peptides in oriented DMPC multibilayers as a function of temperature; peptide/lipid = 1/50 mol mol^{-1}; the bilayer normal was aligned at 0° to the direction of the static magnetic field.

Determination of the minimum inhibitory concentrations (MICs) of the Lys/TCBLys mutants served as a sensitive assay to probe for functional perturbations imposed by the labeling. Gratifyingly, we found that all mutant peptides were antimicrobially active. As the MIC values of the labeled peptides were close to that of the parent peptide (Figure 3b), it is safe to conclude that the labeling does not affect significantly the key function of PGLa(Nle).

PGLa(Nle) was previously shown to adopt an amphipathic α-helical structure in membranes, and re-orient with respect to the bilayer normal when the lipids change their phase state.[20] Using identical conditions, we measured the ^{19}F-ssNMR spectra of all TCBLys-substituted peptides in oriented 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) bilayers. We observed three distinct orientational states of the labeled peptides at different temperatures (Figure 3c), in full agreement with the previous study.

The earlier ^{19}F-ssNMR structure analysis of PGLa(Nle)[23] (Figure 4a) had been performed with only four structural constrains, which is the minimal number required to find an unambiguous solution for the three orientational parameters

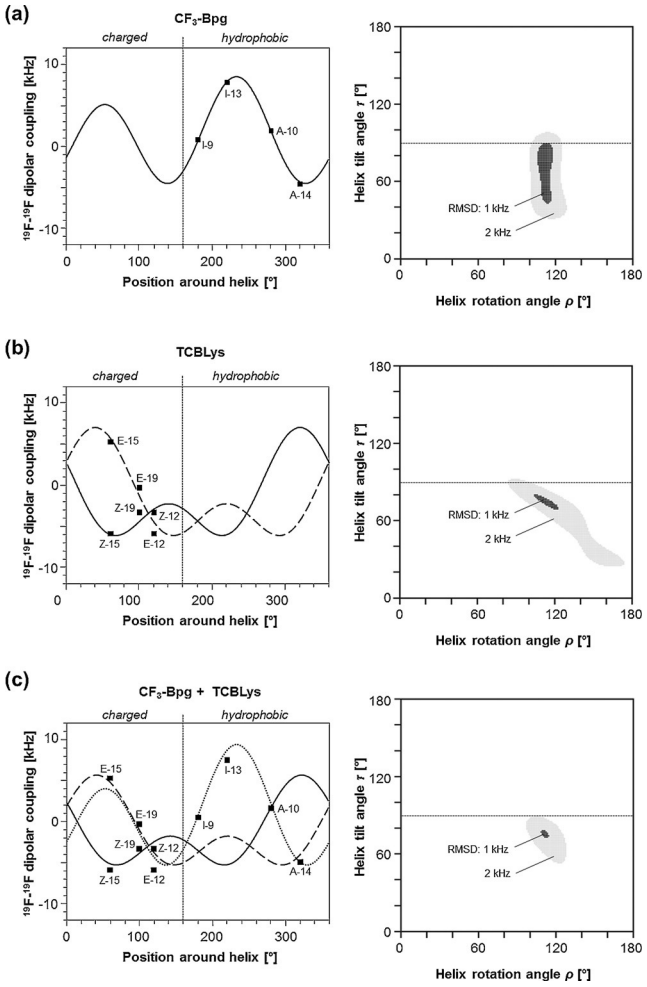


Figure 4. ^{19}F-ssNMR structure analysis of PGLa(Nle) in fluid DMPC bilayers. The best-fit dipolar wave plots (left panels) and corresponding error plots (right panels: RMSD τ–ρ) are calculated from experimental ^{19}F-ssNMR constraints based on a) four CF_3-Bpg-labels, b) six TCBLys-labels, and c) four CF_3-Bpg plus six TCBLys-labels. RMSD = root mean square deviation.

(τ, ρ, S_{mol}) that define the peptide alignment in the membrane. In this work, we have now sampled new orientational constraints from the three lysine positions in PGLa(Nle) on the previously inaccessible charged face of the helix. Two diastereomers of the ^{19}F-label doubled the number of constraints from one position. The use of two stereoisomers had already been demonstrated to be effective in determining the membrane orientation of an alamethicin with another ^{19}F-label **2**.[15d] In the case of PGLa(Nle), six independent TCBLys constraints yielded a structural solution (Figure 4b) that is fully consistent with the previous analysis. Moreover, using the entire set of ^{19}F-ssNMR data for PGLa(Nle) labeled with **3**, **10a**, and **10b**, we could further refine the orientation of this peptide in lipid bilayers. As seen from Figure 4c, when four CF_3-Bpg and six TCBLys labels were used together, the best-fit solution is much more precise, in terms of both the tilt angle τ and the azimuthal rotation angle ρ.

In conclusion, we have designed and synthesized conformationally restricted (*E*)- and (*Z*)-1-amino-3-(2-aminoethyl)-3(trifluoromethyl)cyclobutane carboxylic acids for ^{19}F-ssNMR structural studies of MAPs. These ^{19}F-labels are the first of their kind with a charged (cationic) side chain, thus suitable to probe the polar face of amphiphilic peptides. Both diastereomers of the novel TCBLys-label were demonstrated to have close structural and functional similarity to lysine. The two diastereomers could be used as independent labels in a single position to obtain two independent orientational constraints. The simultaneous use of hydrophobic as well as charged ^{19}F-labels was demonstrated to considerably increase the precision of the peptide orientational analysis, and will thus extend the applicability of ^{19}F-ssNMR to previously inaccessible systems.

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