Amino Acids

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Design, Synthesis, and Application of a Trifluoromethylated Phenylalanine Analogue as a Label to Study Peptides by Solid-State ^{19}F NMR Spectroscopy**

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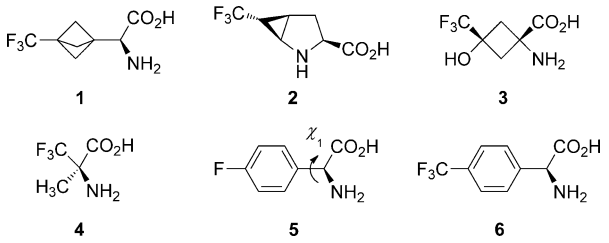
^{19}F NMR spectroscopy is a powerful technique to resolve the structure and dynamics of peptides and proteins. The large gyromagnetic ratio, the absence of a biological background, the high sensitivity of the chemical shift to the local environment, and the spin of $^1/_2$ make fluorine an attractive nucleus for NMR spectroscopy.[1] Solid-state ^{19}F NMR spectroscopy of macroscopically oriented samples, in particular, is used to determine the structure, alignment, and dynamics of membrane-bound peptides under quasi-native conditions.[2] To this aim, suitable fluorine-labeled amino acids have to be incorporated selectively into the polypeptide backbone. However, they have to meet several strict criteria: 1) they must be conformationally constrained to place the ^{19}F reporter group in a well-defined position relative to the peptide backbone; 2) they must be compatible with standard procedures for solid-phase peptide synthesis (SPPS); 3) they must not perturb the native structure or function of the peptide. These criteria preclude the use of virtually all readily available fluorinated analogues of proteinogenic amino acids,[3] as because of their conformational flexibility they do not fulfill criterion 1.

Recently, several trifluoromethyl-substituted compounds **1**–**4** have been designed and applied as labels for solid-state ^{19}F NMR spectroscpy, specifically to replace either bulky aliphatic amino acids (**1**),[4] or proline (**2**),[5] or serine/threonine (**3**),[6] or α-aminoisobutyric acid (**4**)[7] in membrane-active peptides. However, the aromatic phenylalanine analogues **5** and **6**, which have also been explored, showed significant disadvantages, as they racemized under standard Fmoc SPPS conditions (Fmoc = 9-fluorenylmethyloxycarbonyl) and thus did not meet criterion 3.[2a,c,8] Purification of the target peptides was problematic,[8] and in some cases even impossible.[9] Furthermore, analysis of the chemical shift anisotropy of the ^{19}F atom in **5** had to take into account the side chain χ_1 angle and required complicated referencing.[10] We thus aimed to expand the scope of available labels and report herein the rational design, synthesis, and validation of a novel aromatic label for solid-state ^{19}F NMR spectroscopy to replace phenylalanine.



In peptides and proteins, the aromatic ring of phenylalanine can participate in noncovalent inter- and intramolecular interactions with other aromatic residues (π–π interaction),[11] with the positively charged side chains of arginine, lysine, and histidine (cation–π interaction),[12] with negatively charged glutamic and aspartic acid (anion–π interaction),[13] with the charged peptide termini, and with nucleic acids,[14] lipids,[15] and carbohydrates.[16] Phenylalanine-rich sequences are reported to assist other molecules crossing the blood-brain barrier,[17] to specifically bind the export proteins of *M. tuberculosis*,[18] and certain proline-rich peptide motifs,[19] to mediate homopeptide self-assembly in solution,[20] and to guide the assembly of transmembrane helices.[21] In membrane proteins phenylalanine is often found to be preferentially located near the hydrophobic–hydrophilic interface of the lipid bilayer.[22] Therefore, a specific label that can

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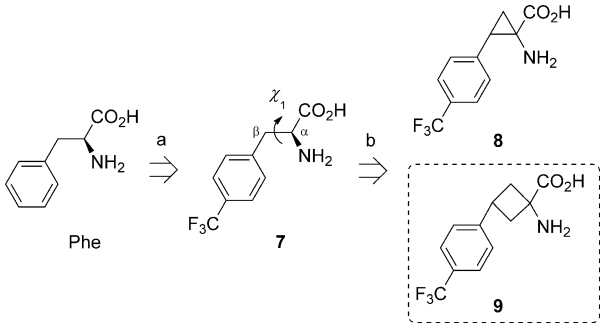
appropriately replace phenylalanine residues in peptides will open new possibilities to study a broad range of important targets under biologically relevant conditions by using solid-state ^{19}F NMR spectroscopy.

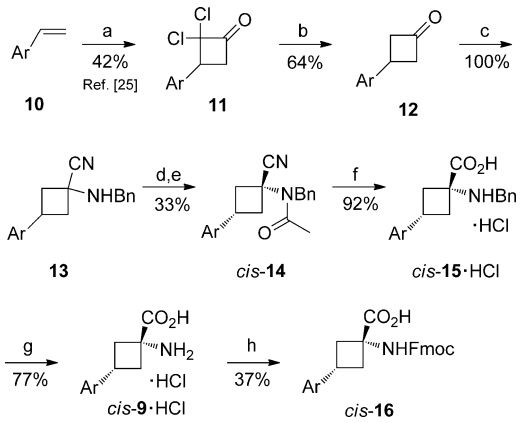
The principles of designing the target structure are summarized on Scheme 1. We chose a CF_3 moiety as a reporter group in view of the simple spectral analysis that requires no referencing, and the advantageous relaxation



Scheme 1. Design of a novel ^{19}F NMR label (**9**) to specifically replace phenylalanine in membrane-bound peptides: a) attachment of the CF_3 reporter; b) restriction of the molecular mobility.

times of this rapidly rotating group.[8a,b] The CF_3 substituent was placed in the *para* position of the phenyl group (**7**) (Scheme 1 a) to maintain the symmetry of the rotationally unrestricted phenyl ring. However, to fulfill criterion 1, we had to prevent any further flexibility of the side chain in amino acid **7** by blocking the rotation around χ_1. To achieve this aim, one could introduce the conformationally rigid cyclopropane (**8**) or restricted cyclobutane (**9**) moieties in place of the C_α–C_β bond. In principle, both compounds **8** and **9** should be suitable for ^{19}F NMR spectroscopy, but in our opinion amino acid **9** is more preferable, because a cyclopropane ring that is activated by electronegative substituents tends to be susceptible to nucleophilic attack.[23] Hence, amino acid **8** could decompose during SPPS. Moreover, in contrast to **8**, compound **9** is achiral and does not require the time-intensive and costly separation of enantiomers and/or asymmetric synthesis, which often limits the multigram preparation and subsequent application of chiral compounds.[24]

Synthesis of amino acid **9** commenced from the readily available styrene **10** (Scheme 2). Ketone **12** was obtained from compound **10** in two steps following a modified literature protocol.[25] A [2+2] cycloaddition with dichloroketene generated in situ from Cl_3CCOCl and Cu/Zn, gave cyclobutane **11**. Subsequent removal of the chlorine atoms in **11** with Zn in acetic acid afforded ketone **12**. Next, a Strecker reaction with benzylamine ($BnNH_2$) and $(CH_3)_3SiCN$ yielded aminonitrile **13** as a 4:1 mixture of stereoisomers. Our attempts to separate the compounds by column chromatography on silica gel led to decomposition of the product. Therefore, the amino function in **13** was protected with an acetyl group to produce, after recrystallization, amide *cis*-**14** as a single isomer. The stereoconfiguration of *cis*-**14** was determined by X-ray diffraction (Figure 1).[26] Acidic hydrol-



Scheme 2. Ar = 4-$CF_3C_6H_4$. Reagents and conditions: a) Cl_3CCOCl, $POCl_3$, Zn/Cu, Et_2O, reflux; b) Zn, AcOH, reflux; c) $(CH_3)_3SiCN$, $BnNH_2$, MeOH, 0–25 °C; d) CH_3COCl, Et_3N, CH_2Cl_2, 0–25 °C; e) crystallization from *i*PrOH; f) 6 N HCl, reflux; g) Pd/H_2, MeOH, 20 bar, 25 °C; h) $(CH_3)_3SiCl$, $NEt(iPr)_2$, Fmoc-Cl, CH_2Cl_2, 0–25 °C.

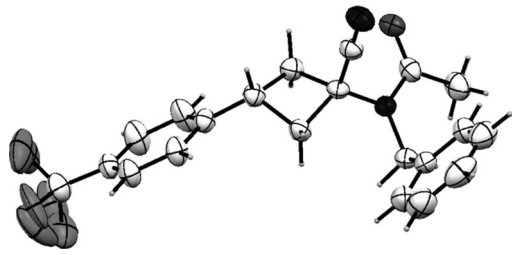


Figure 1. X-ray crystal structure of compound *cis*-**14**. Thermal ellipsoids are shown at 50% probability levels (see the Supporting Information for details). F: light grey (both equally populated positions observed for the CF_3 group are shown), O: dark grey, N: black.

ysis of the amide and nitrile groups in *cis*-**14** gave acid *cis*-**15**·HCl in 92% yield. Finally, the desired amino acid *cis*-**9**·HCl was obtained from *cis*-**15**·HCl by Pd-catalyzed hydrogenolysis of the N–benzyl bond.

N-Fmoc protection of amino acid *cis*-**9**·HCl with either Fmoc-Cl or Fmoc-OSu (9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide)[27] afforded, in less than 10% yield, the desired compound *cis*-**16** suitable for SPPS. Therefore, *cis*-**9**·HCl was instead treated with $(CH_3)_3SiCl$/Fmoc-Cl,[28] which gave the desired product in a much higher yield of 37%. It is worth mentioning that we have previously observed a similar behavior for amino acid **3** upon Fmoc protection.[6]

To evaluate the practical applicability of the obtained amino acid *cis*-**9** as a label in solid-state ^{19}F NMR structure analysis, we incorporated it into the well-known antimicrobial peptide Magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS) from *X. laevis*.[29] The three native phenylalanine residues were each replaced with **9** to produce three peptides: **17**, in which Phe^5 has been replaced with *cis*-**9**, **18**, in which Phe^{12} has been replaced, and **19**, in which Phe^{16} has been replaced. Importantly, amino acid *cis*-**9** was readily incorporated into the polypeptide sequence by using a standard Fmoc SPPS protocol with TBTU as the coupling agent.[30] Neither degradation nor low reactivity of *cis*-**9** was observed. All

compounds **17**–**19** were obtained as single diastereomers and were stable during subsequent storage, thus fulfilling criterion 2.

Magainin 2 is a representative antimicrobial peptide that changes from a random coil in aqueous solutions to an amphiphilic α-helix in a membrane environment.[31] The ^{19}F-labeled peptides **17**–**19** underwent the same conformational transition (Figure 2 A) as the parent peptide. The biological activities of **17**–**19** were tested in bacterial-growth inhibition assays (see the Supporting Information for details). Like the parent peptide,[32] compounds **17**–**19** were active against Gram-negative *E. coli* and Gram-positive *S. aureus* strains with minimal growth inhibition concentrations in the micromolar range (5–30 μM). These results demonstrate that amino acid *cis*-**9** also satisfies fully criterion 3 with regard to an unperturbed peptide structure and function.

The labeled peptides were incorporated into macroscopically aligned bilayers of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) on solid supports, as previously described in detail.[10a] In these fully hydrated samples, Magainin 2 is known to form an amphipathic α-helix and to align parallel to the membrane plane, that is, with a helix tilt angle of about 90° relative to the membrane normal.[33] The reconstituted samples of **17**–**19** were well behaved and no signs of aggregation were detected. Each peptide revealed a clear dipolar splitting, indicating a well-defined orientation of each CF_3 reporter group (Figure 2 B). The obtained splittings were fitted against the typical secondary structures: a continuous α-helix and a β-strand. Even though more constraints from additional labels would be needed for a precise structural analysis including the dynamical behavior,[2,34] the ^{19}F NMR data from **17**–**19** allows us to conclude that a) the conformation of Magainin 2 in DMPC is not a β-strand but an α-helix, b) the α-helix extends at least from position 5 to position 16, and c) it is consistent with a tilt angle of about 90° for a surface-bound helix. These results obtained here with the new ^{19}F-label *cis*-**9** are in a full agreement with the known structural features of Magainin 2 determined independently by solid-state ^{15}N NMR spectropscopy.[33]

In summary, we have synthesized the novel conformationally restricted trifluoromethyl-substituted α-amino acid *cis*-1-amino-3-[4-(trifluoromethyl)phenyl]-cyclobutanecarboxylic acid (*cis*-**9**), designed as a label for the structure analysis of membrane-active peptides by solid-state ^{19}F NMR spectroscopy. The compound was validated as a suitable substituent for phenylalanine by incorporating it into the membrane-active antimicrobial peptide Magainin 2. All criteria required for a ^{19}F NMR label were fulfilled by amino acid *cis*-**9**, when it was used to replace either of the three native phenylalanine residues. Thus, a growing arsenal of available ^{19}F labels can now be used to replace specific residues in peptides and study them by solid-state ^{19}F NMR spectroscopy.

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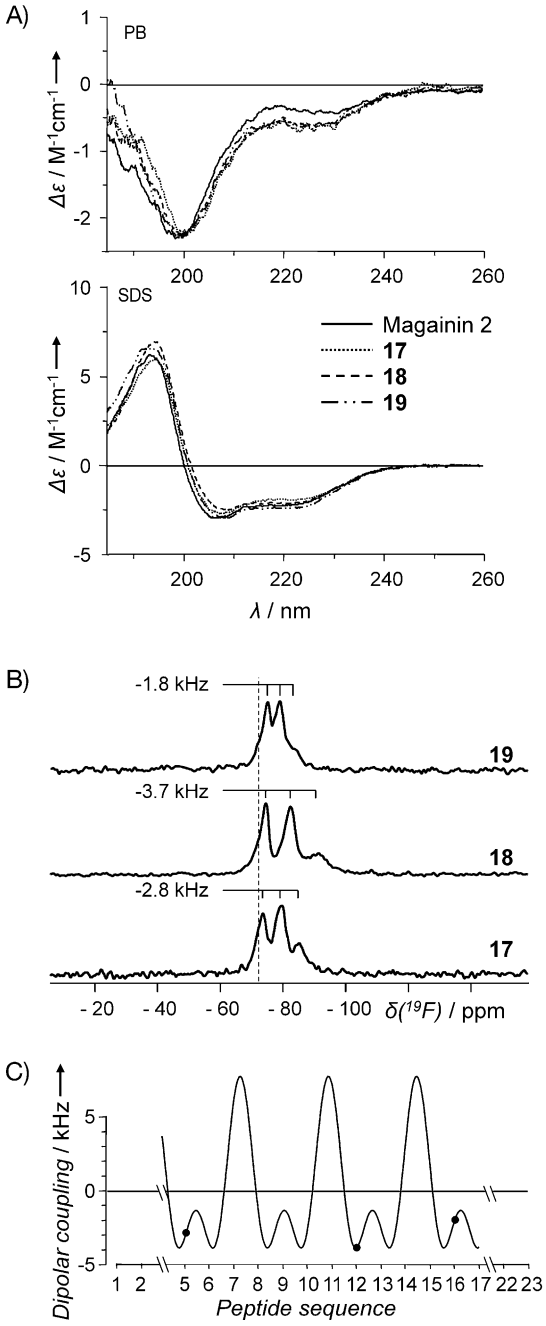

Figure 2. Characterization of amino acid *cis*-**9**: A) Circular dichroism spectra of Magainin 2 and its ^{19}F-labeled analogues **17**–**19** in phosphate buffer at pH 7 (PB, top) and in sodium dodecyl sulfate micelles (SDS, bottom). B) Solid-state ^{19}F NMR spectra of **17**–**19**, each incorporated into oriented DMPC bilayers (peptide/lipid = 1/10 mol mol^{-1}, 40 °C). The observed values of the dipolar couplings are indicated above the signals, and the dashed line shows the isotropic chemical shift. C) Best-fit dipolar wave plot for a continuous α-helix in the lipid bilayer.

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[26] CCDC 909069 *cis*-**14** contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

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