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Amino Acids

Synthesis of Trifluoromethyl-Substituted Proline Analogues as ^{19}F NMR Labels for Peptides in the Polyproline II Conformation**

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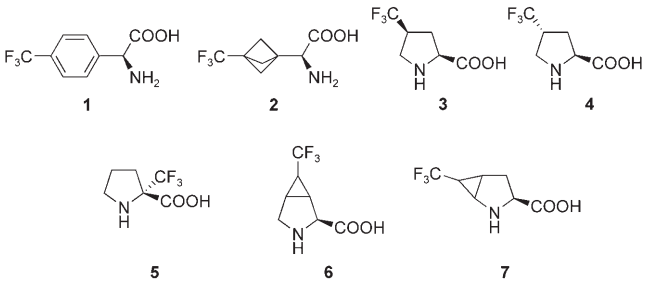
The location of proline (Pro) in proteins is peculiar: it is most frequently found in loops, turns, and flanking positions of stable secondary-structure elements.[1] Such positioning is a consequence of the structural properties of Pro: first, the lack of a second hydrogen atom on the α-amino group prevents hydrogen bonding within a polypeptide, and second, the cyclic nature of the side chain reduces the conformational freedom and typically restricts the torsion angle, φ, to $(-63 \pm 15)°$.[1a] Polyprolines, $(Pro)_n$, in aqueous solvents exist in a "poly-L-proline II" (PPII) conformation;[2] this conformation is also readily adopted by various proline-rich peptides (PRPs) and protein segments, which are important as structural or recognition motifs.[3–5]

Major obstacles in identifying and studying the native PPII conformation are 1) the lack of intramolecular hydrogen bonds, 2) the close similarity to the random coil (RC) conformation when observed by conventional spectroscopic methods, and 3) the frequent coexistence with other extended conformations.[3c,5d] One of the very few ways to address such ambiguities experimentally is to use solid-state NMR spectroscopy on selectively isotope-labeled peptides (by using ^{15}N, ^{13}C, ^{2}H, or ^{19}F isotopes). ^{19}F NMR spectroscopy in particular benefits from the high sensitivity of the ^{19}F nucleus and does not suffer from any natural-abundance background either.[6] This approach is particularly well suited to study the structure, alignment, and dynamics of peptides embedded in lipid membranes.[7] It requires the selective incorporation of a suitable ^{19}F-substituted amino acid into the peptide backbone to serve as an NMR fluorine label (FL). Such FLs should 1) be conformationally rigid to place the ^{19}F reporter group (preferably CF_3) in a well-defined position, 2) be compatible with standard solid-phase peptide-synthesis protocols, 3) be chemically stable in a polypeptide under the conditions of study, and 4) not perturb the native structure/function of the peptide.

^{19}F-labeled amino acids with nonpolar side chains (**1**, **2**) have already been successfully used as FLs.[7,8] Unfortunately, the unique properties of Pro rule out the use of **1** or **2** as a replacement for this residue. At first glance, the known CF_3-substituted proline analogues (**3**–**5**) may be considered as FLs. Though, in **3** and **4**,[9] criterion (1) from above is not fulfilled. Amino acid **5**[10] has no such deficiency because the CF_3 group is directly attached to the Cα carbon atom. However, the CF_3 moiety could severely alter the steric and electronic environment of the peptide backbone and could reduce the chemical reactivity of the amino acid.[11]



Our present research was motivated by the structural and functional importance of proline in proteins and by the wish to expand the repertoire of available FLs. We have designed and synthesized the isomeric CF_3-substituted 3,4- and 4,5-methanoproline analogues **6** and **7** and compared them in order to select the best FL candidate to be used in place of Pro. Metal-catalyzed trifluoromethyl cyclopropanation of C=C bonds, by using CF_3CHN_2, was recently shown to be practically beneficial.[12] We utilized this approach in the construction of **6** and **7**.

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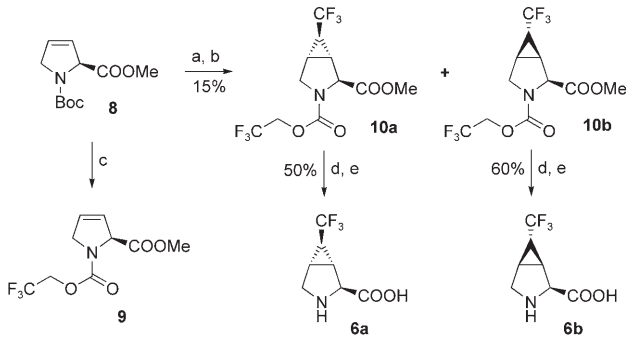
The synthesis of **6** started from optically pure 3,4-dehydroproline derivative **8**.[13] Extensive experimentation was needed to find the optimal reaction conditions with suppression of electrophilic attack of the carbene at the O*t*Bu group (Scheme 1). The latter reaction prevailed if CuCl was

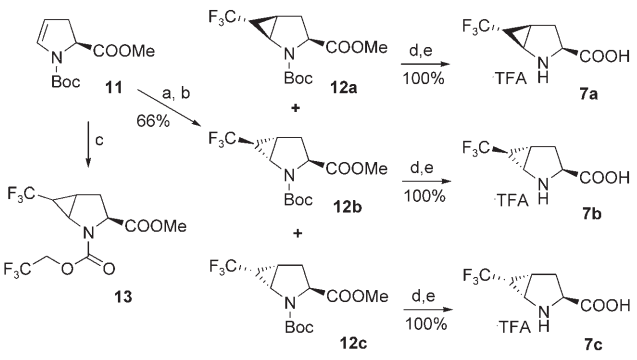


Scheme 1. Reagents and conditions: a) excess CF_3CHN_2, CuOTf, RT; b) column chromatography on silica gel Merck 60; c) excess CF_3CHN_2, CuCl, RT; d) HBr (48%), reflux, 6 h; e) chromatography on Dowex-50. Tf: trifluoromethanesulfonyl, Boc: *tert*-butoxycarbonyl.

used as a catalyst; the only isolated product in that case was **9**. By increasing the reaction time and applying CuOTf, we managed to isolate two diastereomeric products, **10a**,**b**, in a modest yield (15%). The deprotected amino acids **6** were obtained after **10** was heated to reflux in 48% HBr, and the products were purified by ion-exchange column chromatography.

The synthesis of **7** turned out to be more fruitful. The reaction of **11** with 2,2,2-trifluoromethyldiazomethane in the presence of catalytic amounts of CuCl produced trifluoromethyl-substituted proline analogues **12a–c** in an overall yield of 66% (Scheme 2). The molar ratio of **12a**/**12b**/**12c** was 1.0:0.9:0.7. In contrast to the corresponding reaction with **8**, the formation of side products **13**[14] was observed only with prolonged reaction times. The three isomers of **7** were obtained by standard deprotection methods.

The relative configurations of **6a**,**b** and **7a–c** were determined by NMR spectroscopy (see the Supporting



Scheme 2. Reagents and conditions: a) CF_3CHN_2, CuCl, RT; b) column chromatography on silica gel Merck 60; c) excess CF_3CHN_2, CuCl, RT; d) NaOH, MeOH, RT; e) TFA/CH_2Cl_2, RT. TFA: trifluoroacetic acid

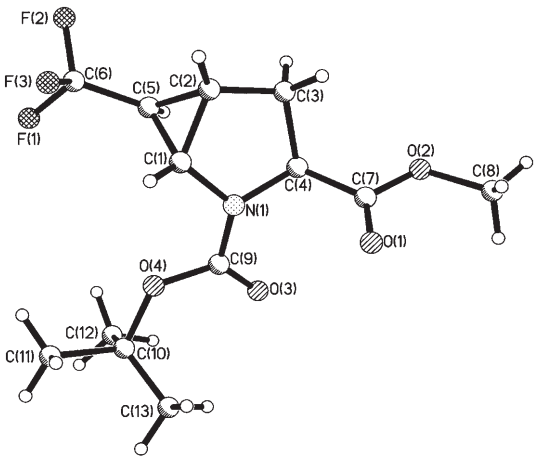


Figure 1. Molecular structure of **12b**.

Information), and the assignment was also confirmed for **7b** by X-ray analysis of the precursor **12b** (Figure 1).

In principle, each isomer of **6** and **7** could now be used as an FL. They all possess a CF_3 group, this group is sufficiently distant from the backbone to prevent perturbation, and the side chains are conformationally more restricted than in **3** and **4**. However, amino acids **6a**,**b** are not preferable, because of the poor total yield of their synthesis. In the case of **7b** and **7c**, the cyclopropane ring was found to be unstable at low pH values. By contrast, **7a** is stable at both high and low pH values, and it was therefore selected as a promising FL to replace Pro in peptides.

In order to address the suitability of **7a** as an FL and to examine its influence on the PPII conformation, the proline-rich cell-penetrating peptide SAP (**14**, $(VRLPPP)_3$) was selected.[15] This peptide is known to self-assemble above 50 μM in aqueous solutions, and it is believed to exist as a mixture of the PPII and RC conformations below this concentration. The peptide VRLPPPVRLP–**7a**–PVRLPPP (**15**), containing a Pro/**7a** substitution, was synthesized. Neither degradation nor low reactivity of **7a** was observed. No racemization was detected either.

We have also synthesized the SAP analogue VRLPPPVR-**2**-PPPVRLPPP (**16**), labeled with the previously reported FL **2**[8c,d] in the position of Leu9. Comparison of the CD spectra of peptides **14**–**16** at different concentrations (Figure 2) revealed that FL **2** did not affect the SAP structure, whereas FL **7a** unexpectedly stabilized the PPII conformation of SAP. It can be seen that **14** and **16** exhibit spectral changes up to 50 μM, indicating oligomerization beyond this point, while **15** oligomerized at a concentration that was twice as high. Moreover, for all concentrations of **15**, a positive band was observed at 223 nm, the hallmark of the PPII conformation. The same result is seen in the temperature series of **15** (Figure 2D). The positive band at 223 nm persisted even at 50°C, in contrast to the results with native SAP.[15]

In summary, **7a** was demonstrated to be a promising label for solid-state ^{19}F NMR analysis of PRPs in the light of its chemical stability, compatibility with solid-phase peptide synthesis, and propensity to maintain, and even to stabilize,

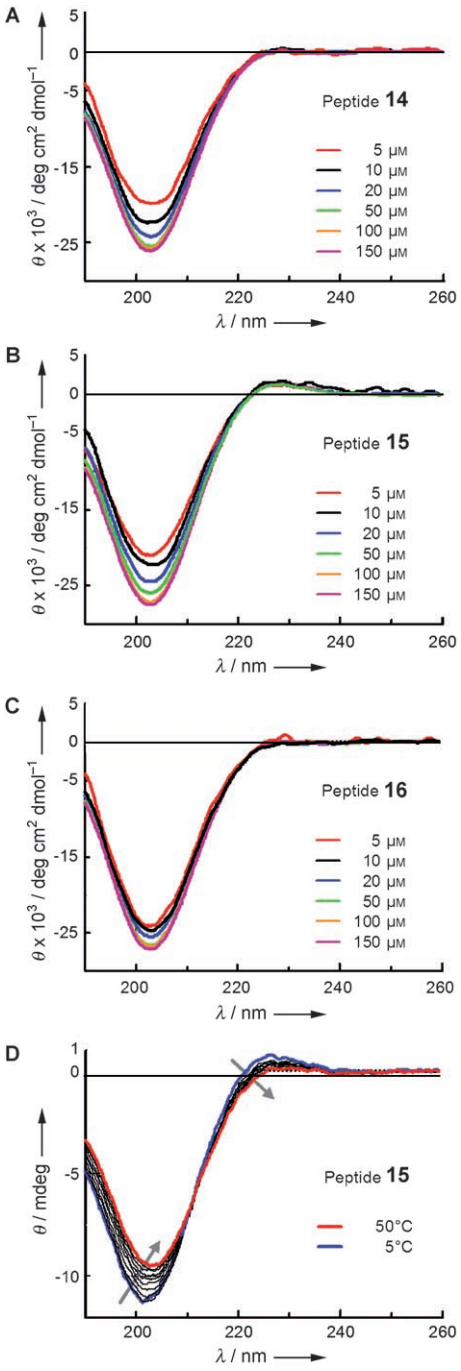


Figure 2. CD spectra of A) wild-type SAP peptide **14**, B) CF_3MePro-substituted **15**, and C) **7a**-substituted **16** over a concentration range of 5–150 μM. The spectra were measured in 10 mM phosphate buffer pH 7.4 at 20 °C. D) Temperature dependence of the CD spectrum for **15** (10 μM solution in phosphate buffer). Arrows indicate direction of spectral changes.

the PPII conformation; this stabilization is probably a consequence of the conformational properties of **7a**.

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