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Incorporation of *cis*- and *trans*-4,5-Difluoromethanoprolines into Polypeptides

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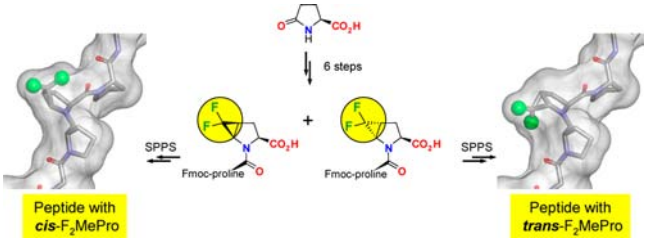
ABSTRACT



Substituted prolines exert diverse effects on the backbone conformation of proteins. Novel difluoro-analogues were obtained by adding difluorocarbene to N-Boc-4,5-dehydroproline methyl ester, which gave the *trans*-adduct as the sole product with 71% yield. Upon cleavage of the N-protection group the free amino acid decomposed rapidly. Its incorporation into the proline-rich cell-penetrating "sweet arrow peptide" was thus accomplished using a dipeptide strategy. Two building blocks, containing either *cis*- or *trans*-4,5-difluoromethanoproline, were obtained by difluorocyclopropanation of the aminoacyl derivatives of 4,5-dehydroproline. The resulting dipeptides were stable under standard conditions of Fmoc solid phase peptide synthesis and, thus, suitable to study conformational effects.

Conformational and electronic effects in the side chain of proline and its substituted analogues can have a significant influence on the three-dimentional structure of proteins. They are known to modify the stability of certain conformations by stucturing proline-containing turns, loops, and/or secondary structure boundaries.[1] Even intermolecular protein assemblies can be influenced this way. Numerous studies have suggested that stereoelectronic effects may result, for example, in a modulation of the triple-helical oligomeric structure of collagen-like peptides. A comprehensive understanding of the interplay between steric and electronic contributions opens the way to rationally control protein structure and thereby function.

During a structural study of the proline-rich cell-penetrating "sweet arrow peptide"[2] (SAP) [(VRLPPP)$_3$],

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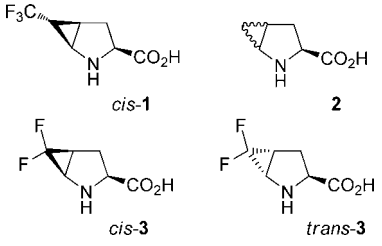
we had modified the peptide with CF_3-*cis*-4,5-methanoproline (*cis*-**1**)[3] substituting the native proline residue at position 11. We noticed an unexpected stabilization of the poly-L-proline type II (PPII) conformation for this SAP analogue in aqueous solution.[4] The change in the conformational balance brought to the peptide by *cis*-**1** was undesired in the structural analysis by solid-state ^{19}F NMR.[5] However, the phenomenal PPII stabilization in a proline-rich peptide by a single residue *cis*-**1**[4] and the influence of the substituents at the proline core in this and analogous amino acids on the amide bond *cis*−*trans* izomerization that can stabilize or destabilize the PPII conformation[1a−m,6] were found to be highly interesting issues and worth studying in more detail.

In the case of amino acid *cis*-**1**, several factors may play a role in stabilizing the PPII conformation of SAP: a steric influence of the cyclopropane ring, its strong π-character, and steric as well as electron-withdrawing effects of the trifluoromethyl group. In order to discriminate these factors, systematic variation of the substituents on proline in positions 4 and 5 was needed. Several new analogues of the parent amino acid 4,5-methanoproline (**2**) should be prepared and incorporated into SAP in order to study the conformational preferences of the peptides and compare the results with literature data.[7] Toward this purpose the novel compound 4,5-difluoromethanoproline **3** (4,5-F_2MePro) was designed. The two fluorine atoms in 4,5-F_2MePro impose an electronic influence on the cyclopropane ring that is much stronger than that of the single trifluoromethyl group in *cis*-**1**. Therefore, **3** may be regarded as a good model to reveal the role of electronic and steric effects in the stabilization of the PPII conformation.



The synthesis of amino acid **3** and its direct use in peptide synthesis turned out to be a nontrivial task, due to its inherent instability. We report in this letter the solution to the fundamental synthetic problems, which may also be useful in similar situations.

The key chemical transformation in the synthesis of amino acid **3** is the addition of difluorocarbene to the corresponding unsaturated precursor, N-Boc-L-4,5-dehydroproline methyl ester **4** (Scheme 1). Synthesis of enamide **4** from L-pyroglutamic acid (**5**) was performed following the procedures described in the literature.[8] For the success of the next key synthetic step, the proper choice of a method to generate difluorocarbene was of decisive importance. There are a number of published protocols for the difluorocyclopropanation of olefins,[9] which could have been used. In our hands, the first published method of pyrolysis of sodium chlorodifluoroacetate in diglyme[10] performed well under a strictly controlled temperature regime. The difluorocyclopropane derivative, *trans*-**6**, was prepared from alkene **4** as the sole product in 71% yield. Compound *trans*-**6** was purified by column chromatography and then subjected to alkaline hydrolysis, to afford the N-Boc protected amino acid *trans*-**7** in 91% isolated yield. The *trans*-stereoconfiguration of the obtained compounds was determined by X-ray diffraction of *trans*-**7** (Figure 1).

Formation of the single stereoisomer *trans*-**6** was unexpected, as the known similar cycloaddition of the trifluoromethylcarbene to compound **4** had previously led to a mixture of *cis*- and *trans*-isomers of amino acid **1**.[4] The diastereoselectivity of formation of *trans*-**6** can be explained by thermodynamic control of the reaction, which was carried out at high temperature.

When the N-Boc protection in *trans*-**6** and *trans*-**7** was cleaved off by TFA in dichloromethane at room temperature, the corresponding N-deprotected compounds were formed, but they decomposed rapidly (Scheme 1). For example, the half-life ($t_{1/2}$) of the TFA-salt of deprotected *trans*-**6** was ~110 min (in $CHCl_3$) as determined by ^{19}F NMR.

To understand the mechanism by which the amino acid *trans*-**3***TFA gets decomposed, ^{19}F NMR was employed to monitor the mixture obtained by fast cleavage of the N-Boc protection in compound *trans*-**7** and subsequent addition of H_2O (see Supporting Information (SI)). The series of ^{19}F NMR spectra revealed a pseudo-first-order disappearance of the signals of the product, *trans*-**3***TFA

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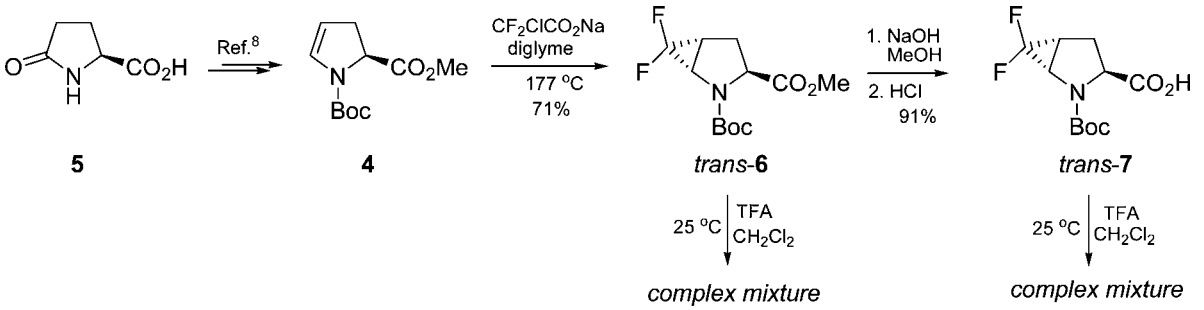
Scheme 1. Attempted Synthesis of Amino Acid **3**



(two doublets at −129.8 and −148.2 ppm, J_{F-F} = 178 Hz), and $t_{1/2}$ was determined to be 39 min. New ^{19}F NMR signals appeared instead: the dominant one (dd at −131.1 ppm, J = 32 and 19 Hz) reached its maximum intensity in 200 min and subsequently faded out steadily. Additional ^{1}H NMR spectra of the reaction mixture suggested structure **9** as the possible intermediate (Scheme 2).[11] The decomposition of *trans*-**3***TFA most likely involves nucleophilic attack of water at C(1), which is highly electrophilic due to the electron-withdrawing effects of the protonated amino group and the two fluorine substituents. Subsequent putative polycondensation of compound **9**, bearing an activated C=C double bond, aliphatic aldehyde function, and the amino group, then lead to a mixture of unidentified products. It is worth mentioning that decomposition of the *trans*-isomers of related amino acid **1** under acidic conditions was previously observed.[4,12] Finally the deprotected free base *trans*-**3** was even more labile than *trans*-**3***TFA, as the substance decomposed completely before the NMR experiment could have been started.

The pronounced instability of compound *trans*-**3** under both acidic and basic conditions severely hampered the use of *trans*-**7** in Boc-solid phase peptide synthesis (Boc-SPPS), as much as its transformation into the N-Fmoc-protected form required for Fmoc-SPPS. To avoid the use of N-deprotected amino acid **3** in the synthesis of SAP analogues, another strategy thus had to be followed. Voigt et al. have reported that the incorporation of an intrinsically unstable 1-aminocyclopropane-2-carboxylic acid into peptidomimetics could be achieved by using the corresponding N-protected tripeptide derivatives.[13] Keeping in mind the stability of the N-protected compounds *trans*-**6** and *trans*-**7**, we used aminoacyl derivatives of L-4,5-dehydroproline as the starting compounds for the difluorocyclopropanation, in order to prepare dipeptide building blocks containing the 4,5-F_2MePro residue at the C-terminus.

(11) The structure of compound **9** was assumed based on the signals in ^{1}H NMR (d, J = 19 Hz at 9.21 ppm; dt, J = 32 and 7 Hz at 6.31 ppm; t, J = 7 Hz at 4.26 ppm and m at 3.02 ppm) and ^{19}F NMR spectra, which evolved synchronously (see Supporting Information).

(12) Difluoromethylcyclopropanes are often unstable: (a) Kobayashi, Y.; Taguchi, T.; Morikawa, T.; Takase, T.; Takanashi, H. *J. Org. Chem.* **1982**, *47*, 3232. (b) Nowak, I.; Cannon, J. F.; Robins, M. J. *Org. Lett.* **2004**, *6*, 4767.

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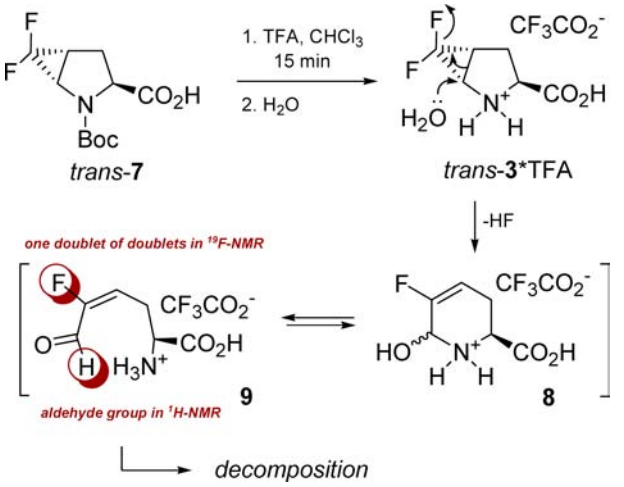
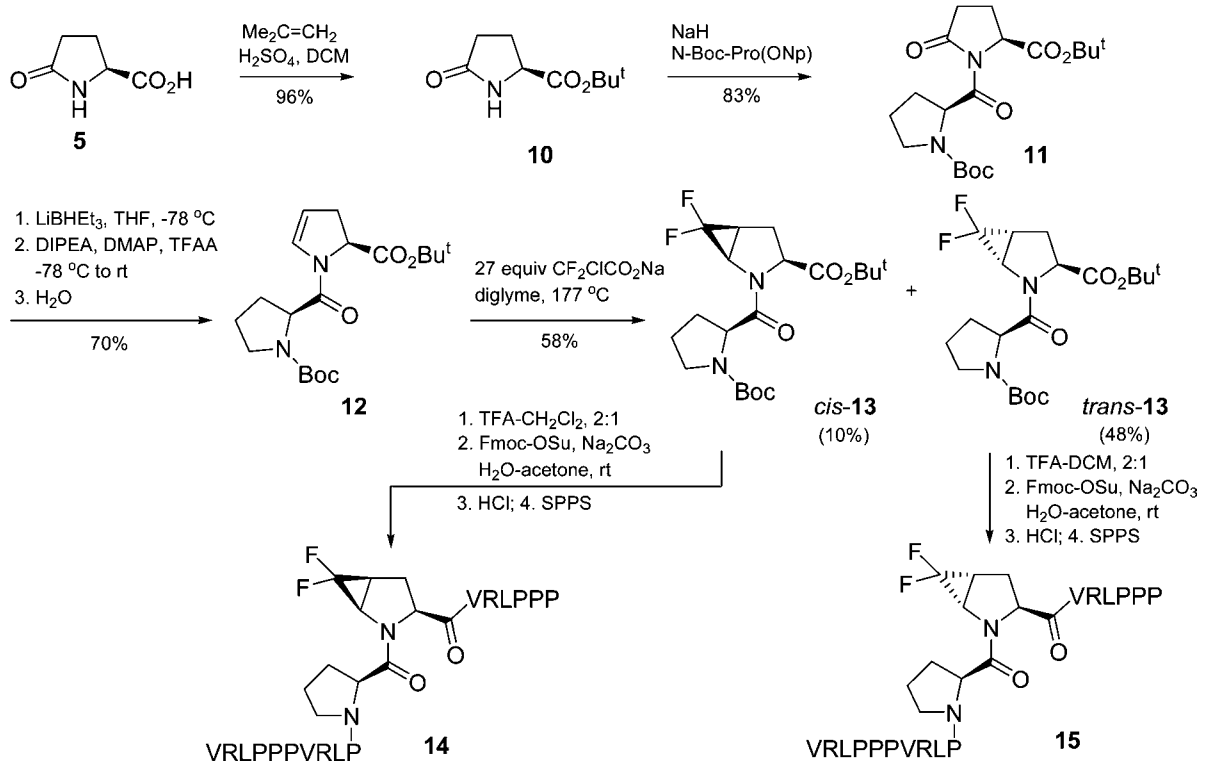
Scheme 2. Proposed Mechanism for Decomposition of *trans*-**3***TFA in Water



The implementation of this strategy is illustrated in Scheme 3. The unsaturated dipeptide precursor **12** was prepared in three laboratory steps from L-pyroglutamic acid (**5**). Enamide **12** was then subjected to the difluorocyclopropanation reaction under conditions analogous to those used in the synthesis of compound *trans*-**6**. The only important difference was the use of a large excess of sodium chlorodifluoroacetate (27 equiv) in order to achieve complete conversion of the starting compound. Formation of two isomeric products, *trans*-**13** and *cis*-**13**, in the ratio of 5:1 was observed. Configuration of the obtained products was first determined by heteronuclear ^{19}F−^{1}H NOE experiments (see SI for details). The structure of *cis*-**13** was subsequently confirmed by X-ray crystallography (Figure 1).

Both of the dipeptides, *cis*-**13** and *trans*-**13** (after hydrolysis and exchange of the N-Boc protection for N-Fmoc), were subsequently used in standard Fmoc-SPPS to afford the target SAP analogues VRLPPPVRLP-(*cis*-**3**)-PVRLPPP (**14**) and VRLPPPVRLP-(*trans*-**3**)-PVRLPPP (**15**). Both MALDI-TOF mass spectra of the final peptides and their analytical HPLC showed that the SPPS had proceeded smoothly and cleanly. The peptides **14**, **15**

Scheme 3. Synthesis of SAP Analogues **14**, **15** Containing the *cis*-**3** and *trans*-**3** Analogues of Proline



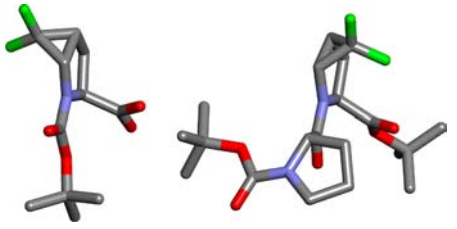


Figure 1. Molecular structures of the protected amino acid *trans*-**7** (left) and of the dipeptide *cis*-**13** (right), as determined by X-ray crystallography (see SI for details).

turned out to be fully stable upon cleavage from the resin and under purification conditions.

In summary, the novel amino acid N-Boc *trans*-**3** was successfully synthesized. The compound decomposed rapidly upon N-deprotection, which hindered its direct use in SPPS. Therefore, dipeptide building blocks *cis*-**13** and *trans*-**13** were prepared to enable the incorporation of amino acid **3** into the proline-rich SAP peptide. Both the *cis*- and *trans*-forms of the synthesized peptides **14**, **15** were completely stable. Conformational analysis of the obtained SAP analogues specifically focusing on the phenomenon of PPII stabilization/destabilization is being pursued in our laboratory and will be reported in due course.

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Supporting Information Available. Experimental procedures, spectral and analytical data for all new compounds, details of the peptide synthesis, X-ray data on *trans*-**7** and *cis*-**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.