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Incorporation of labile *trans*-4,5-difluoromethanoproline into a peptide as a stable label for ^{19}F NMR structure analysis

Vladimir S. Kubyshkin [a], Pavel K. Mykhailiuk [b,c,*], Sergii Afonin [d], Stephan L. Grage [d], Igor V. Komarov [b,c], Anne S. Ulrich [a,d,**]

[a] Karlsruhe Institute of Technology (KIT), Institute of Organic Chemistry, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany
[b] Taras Shevchenko National University of Kyiv, vul. Volodymyrska 64, 01601 Kyiv, Ukraine
[c] Enamine Ltd., vul. Oleksandra Matrosova 23, 01103 Kyiv, Ukraine
[d] KIT, Institute of Biological Interfaces (IBG-2), POB 3640, 76021 Karlsruhe, Germany

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ABSTRACT

Trans-4,5-Difluoromethano-proline was incorporated into the cyclic antimicrobial peptide gramicidin S in place of a native proline residue. Introduction of this intrinsically unstable amino acid into the polypeptide backbone was achieved using a dipeptide strategy. The stable dipeptide building block with the N-acylated 4,5-difluoromethano-proline fragment was obtained by direct difluorocyclopropanation of an unsaturated precursor. The influence of the unnatural amino acid on the conformation and function of gramicidin S was evaluated using circular dichroism and biological assays. The application of *trans*-4,5-difluoromethano-proline as a new label for solid state ^{19}F NMR structure analysis of membrane-active peptides was tested on gramicidin S and compared with previous labeling schemes.

1. Introduction

Fluorine-substituted amino acids are widely used for engineering and tuning the properties of peptides and proteins [1]. The presence of a fluorine substituent can dramatically alter the chemical and conformational behavior of amino acids and thus may also, when incorporated as ^{19}F NMR label into peptides, influence their structure. For example, stereoelectronic effects of the fluorine atom in 4-fluoroproline were shown to increase the conformational stability of collagen-mimicking peptides [2]. 4-Fluoroproline has also been incorporated into proteins biosynthetically, thereby affecting their folding [3]. The enhanced hydrophobicity of the fluoro-substituted aliphatic groups has further been used for stabilizing the secondary structure of peptides [4]. Substitutions of a peptide bond with trifluoroethylamine lead to peptide analogues with enhanced metabolic stability [5].

On the other hand, when it is possible to minimize the steric and electronic impact of the fluorine substitution, it can serve as an excellent NMR label. Fluorine-19 benefits from the high magnetogyric ratio, 100% natural isotope abundance, absence of biological background, and high sensitivity of the NMR signals to the local environment. Fluorine-substituted amino acids have thus been used as powerful reporter groups in structural and functional studies of peptides [6], both *in vitro* [7] and *in vivo* [8]. In particular, conformationally restricted ^{19}F-amino acids have been used to study membrane-active peptides (MAPs) in artificial and native lipid membranes by solid state ^{19}F NMR [9]. This technique provides information not only on the peptide conformation, but also on its membrane alignment and mobility when embedded in a lipid bilayer, and hence sheds light on the functionally relevant interactions of MAPs with membranes. These interactions are the key for understanding the molecular mechanisms of MAPs action as antimicrobial or cell-penetrating peptides [10]. The synthesis of the specific amino acids that are suitable for such studies is challenging, and requires careful exploration of the conformational and functional consequences upon incorporating the ^{19}F-amino acid into a peptide [9(a),11].

* Corresponding author at: Taras Shevchenko National University of Kyiv, vul. Volodymyrska 64, 01601 Kyiv, Ukraine. Tel.: +38 066 2666399; fax: +38 066 2666399.
** Corresponding author at: Karlsruhe Institute of Technology (KIT), Institute of Organic Chemistry, Fritz-Haber-Weg 6, 76131 Karlsruhe, Germany.
E-mail addresses: Pavel.Mykhailiuk@gmail.com, Pavel.Mykhailiuk@mail.enamine.net (P.K. Mykhailiuk), Anne.Ulrich@kit.edu (A.S. Ulrich).

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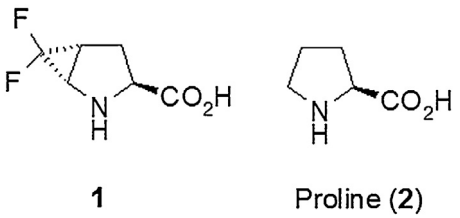

Fig. 1. Structures of the proline analogue (1, CF_2-Pro) and natural L-proline (2, Pro).

Recently, we have designed a proline analogue (compound **1** in Fig. 1) as a ^{19}F NMR label to study the conformational properties of the cell-penetrating peptide SAP (sweet arrow peptide), capable of folding as a poly-*L*-proline type-II helix [12]. The free amino acid **1** quickly decomposed, being very prone to three-membered ring opening *via* nucleophilic attacks. Acylation of the nitrogen atom, however, was shown to give stable derivatives. In the case of SAP, incorporation of **1** into the linear polypeptide sequence was achieved using the stable dipeptide building block Fmoc-Pro-**1**-OH.

In this work, we addressed the question as to whether the proline analogue **1** (CF_2-Pro) can be used as a ^{19}F NMR label for structural studies of membrane-active peptides. We selected the antimicrobial peptide gramicidin S (GS) as a model system, because it contains proline in a β-turn and has been extensively studied using other ^{19}F-labels before [13].

GS is a cyclic decapeptide (*cyclo*[Val-Orn-Leu-^{D}Phe-Pro]$_2$) with prominent antimicrobial [14] and hemolytic [14(b),15] activities, which can be readily tested to assess whether the new proline analogue would impair the biological functions. The native proline residues that can be replaced with **1** are located at the β-turns of the GS ring [16]. Since the cyclization of the linear precursor of GS requires a structural pre-organization of the backbone with a β-turn [17], synthesis of the GS modified by a proline replacement would be severely hampered if the conformational preference of the proline analogues differs significantly from that of proline itself. Therefore, a successful cyclization reaction already provides a test of the structural suitability of the new proline analogue. In addition, any potential conformational changes induced by **1** can be readily evaluated using circular dichroism and NMR spectroscopy.

GS has been previously studied by solid state ^{19}F and ^{15}N NMR in lipid membranes, which showed that the peptide undergoes a concentration and temperature-dependent re-alignment [13(b)]. As the first application of difluoromethano-proline for an NMR structural study of the peptide, we thus tested whether these characteristic changes in the orientation of GS can also be observed with the peptide analogue labeled by one CF_2-Pro.

2. Results and discussion

2.1. Synthesis

The synthesis of GS containing **1** in place of a proline residue was started from *tert*-butyl (S)-pyroglutamate **3** (Scheme 1). Compound **3** was coupled with O-nitrophenyl-activated N-Boc-D-phenylalanine (**4**) to produce the dipeptide derivative **5** in 89% yield [18]. Next, **5** was subjected to one-pot reduction-dehydratation of the pyrrolidone ring [19]. Notably, in the presence of one equivalent of $LiBHEt_3$ the reaction proceeded regioselectively involving only the endocyclic amide moiety [20]. Alkene **6** was obtained after silica gel column chromatography as a sole product in 76% yield. Enamide **6** was difluorocyclopropanated by sodium chlorodifluoroacetate in refluxing diglyme [21]. It is highly important to use a very large excess of the carbene-generating salt (~25 equivalents) in order to completely convert alkene **6** into the products. The target compound **7** was obtained as a single stereoisomer in 29% yield, along with a side product, which was isolated from the reaction mixture as a minor component in 7% yield. The structure of the latter compound was deduced from the spectroscopic data. The ^{13}C and ^{19}F NMR data were in full agreement with compound **8** bearing a ClF_2C–C(=O)–NH moiety [22]. Presumably, at the high temperature of the reaction, an excess of sodium chlorodifluoroacetate acted as the chlorodifluoroacetylating agent of the Boc-NH fragment followed by elimination of the Boc-group. Notably, we did not observe the formation of the corresponding side product earlier during the synthesis of Boc-Pro-**1**-OBu^t [12] where the Boc-protected nitrogen did not contain hydrogen atom.

The dipeptide **7** was transformed into the corresponding N-Fmoc derivative **9** in two steps. The building block **9** was then used for solid phase peptide synthesis of the linear decapeptide **10** on a 2-chlorotrityl chloride resin pre-loaded with D-phenylalanine [17]. A mixture of 6Cl-HOBt/HCTU/DIPEA was used to activate the carboxylic group of each amino acid in the coupling step. The linear peptide was cleaved off the resin using a hexafluoroisopropanol/dichloromethane cocktail under conditions, which left the ornitine Boc-protection intact [23]. GS analogue **11** was obtained by cyclization of **10**, performed in dichloromethane under high dilution, and using PyBOP/HOBt/DIPEA to activate the carboxylic function. The N-Boc protecting groups on both ornithine residues were removed by TFA/TIS. Purification of the crude product by reverse phase HPLC afforded the pure peptide *cyclo*[Val-Orn-Leu-^{D}Phe-**1**-Val-Orn-Leu-^{D}Phe-Pro] (**11**). The reasonable overall yield of 20% of the peptide synthesis served as an indirect indication that the substitution of **1** for natural proline did not significantly affect the structure of the linear precursor **10**, and hence, the final GS analogue **11**.

2.2. $^1H\{^{19}F\}$ NOE experiments

The diastereoselectivity of the difluorocyclopropanation reaction was deduced from one-dimensional $^1H\{^{19}F\}$-NMR NOE difference experiments of the products (**7** and **8**). In the control experiment saturation of the *exo*-fluorine gave almost equal enhancement of two spectral lines of H(1) and H(5) as expected (Fig. 2, lower traces). Next, saturation of the *endo*-fluorine in both compounds resulted in nearly equal NOE values for H(1) and H(3) (Fig. 2, upper traces). The NOE value of H(3) was only 2% larger or 8% smaller than that of H(1) in the case of **7** and **8**, respectively. Therefore, one can assume that the distances between the *endo*-F atom and both H(1) and H(3) are almost identical in **7** and **8**, which is in good agreement with the crystal structure of an analogous compound, *trans*-Boc-**1**-OH [12] (3.25 Å and 3.22 Å, for H(1) and H(3) respectively). The observed NOEs thus indicated a *trans*-disposition of the carboxylic and difluorocyclopropane moieties in both dipeptide derivatives.

2.3. Circular dichroism analysis

A conformational pre-organization of the linear decapeptide **10** plays an important role for its cyclization into GS [17]. The two Val-Orn-Leu chains form an antiparallel β-sheet in the cyclic GS molecule, and the ^{D}Phe-Pro moieties constitute β-turns of type II′. The reasonable yield of compound **11** suggested that the incorporation of amino acid **1** in the place of a proline residue was compatible with the intrinsic peptide conformation. Essentially identical CD spectra of the wild type peptide and its analogue **11** in liposomes also proved that the secondary structure was not significantly perturbed (Fig. 3). These results demonstrated that an