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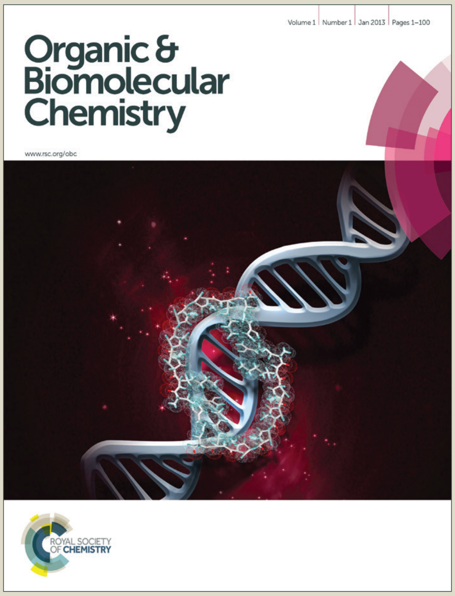

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γ-(*S*)-Trifluoromethyl proline: Evaluation as a structural substitute of proline for solid state ^{19}F-NMR peptide studies

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Abstract

γ-(4*S*)-Trifluoromethyl proline was synthesised according to a modified literature protocol with improved yield on a multigram scale. Conformational properties of the amide bond formed by the amino acid were characterised using N-acetyl methyl ester model. The amide populations (s-*trans* vs. s-*cis*) and thermodynamic parameters of the isomerization were found to be similar to the corresponding values for intact proline. Therefore, the γ-trifluoromethyl proline was suggested as a structurally low-disturbing proline substitution in peptides for their structural studies by ^{19}F-NMR. Indeed, the exchange of native proline for γ-trifluoromethyl proline in the peptide antibiotic gramicidin S was shown to preserve the overall amphipathic peptide structure. The utility of the amino acid as a selective ^{19}F-NMR label was demonstrated by observing the re-alignment of the labelled gramicidin S in oriented lipid bilayers.

Introduction

Proline (Pro) is the only amino acid with a secondary amine function among the canonical α-amino acids. Locally, in the polypeptide backbone Pro forms unique tertiary X-Pro amide bonds which endows a stable s-*cis* conformation (Fig. 1A).[1] The Pro residue is conformationally restricted by the side chain to backbone cyclization, and is therefore the only canonical amino acid with the rigidly constrained phi angle. Due to these features, prolyl residues often play determining roles in the definition of the three-dimensional structures of proteins and limit the folding kinetics.[2] Consequently, proline-rich regions in proteins are predominantly located within the solvent-exposed sections with enigmatic secondary structures such as intra-domain connections, loops, intrinsically disordered segments. In addition, prolines are ample in the folded domains which constitute the inter-molecular interfaces of the protein-protein and protein-drug recognition sites.[3]

The absence of NH not only precludes participation in the secondary structure-stabilizing hydrogen bonds, but also makes Pro sites invisible for many routine NMR methods of the polypeptide structure analysis. Yet, the NMR visibility of Pro can be restored by selective isotope labelling. The labelling *per se* serves enhancing the NMR sensitivity, where among conventional isotope labels, ^{2}H, ^{13}C, ^{15}N, and ^{19}F, the latter is by far the most effective nucleus.[4] However, introduction of the ^{19}F isotope, an unnatural element in the proteinogenic amino acids, requires chemical modification of the side chain, i.e. a mutation. While for large proteins a single mutation does not necessarily interfere with the native fold, for small peptides such a modification may affect significant percentage of the sequence. For ^{19}F-NMR labelling which aims determination of the structure of small peptides, in particular employing anisotropic

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NMR parameters (solid state NMR), the introduction of a fluorine-containing motif is further complicated. In an ideal label, not only should the physico-chemical nature of the side chain be preserved, but simultaneously a rigid molecular connectivity between the NMR-reporter group and the aminocaboxylate must be introduced.[5,6] This challenge is solved in ^{19}F-NMR by designing the residue type-specific substitutive amino acids. These have to be close steric surrogates, which possess conformationally rigid side chains alongside with the NMR-reporter group - a single fluorine atom,[7,8] a CF_2-,[9] or the CF_3-group[10,11] (Fig. 1B). In the case of the intrinsically constricted Pro side chain, design of such analogues is particularly difficult, because the residue steric size always increases, the side chain conformation, unique amide bond isomerization equilibrium and consequently the preferred near-proline backbone conformational angles are strongly influenced by the substituents in the pyrrolidine ring.[12,13]

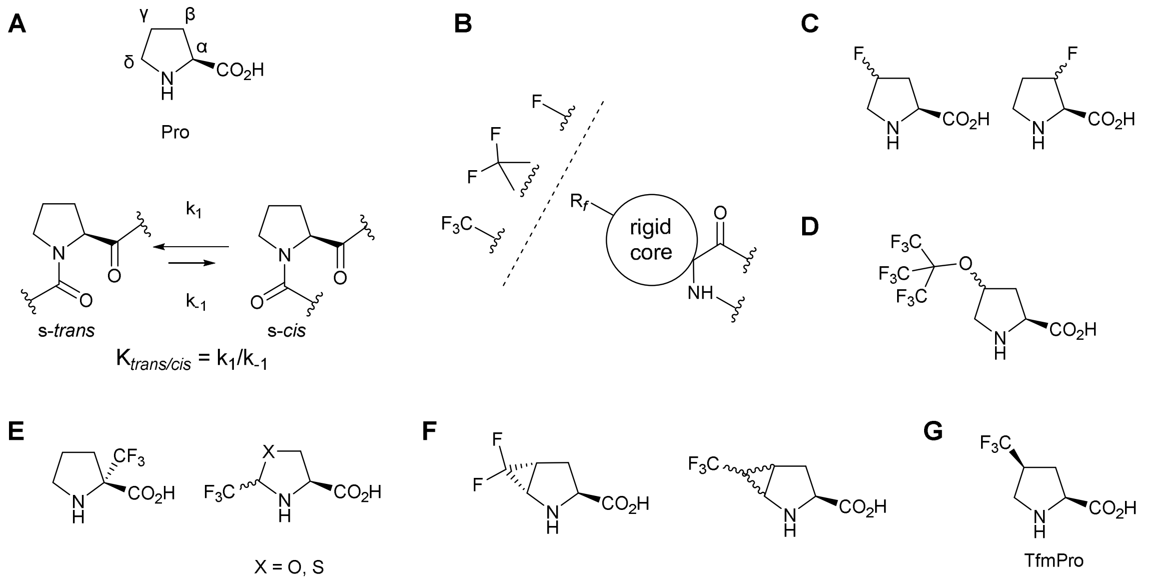

Fig. 1. (A) Structure of proline (Pro) and the *trans-cis* equilibrium in a peptidyl-prolyl fragment; (B) Attachment of a conformationally constrained ^{19}F-NMR reporter to the polypeptide backbone; (C-F) Known fluorine-substituted α-amino acids which could be considered as Pro-replacing ^{19}F-NMR labels: (C) Monofluorine-substituted γ- and β-fluoroprolines; (D) Perfluoro-*tert*-butyl-γ-hydroxyprolines proposed as a ^{19}F-NMR label;[22] (E) Carriers of the CF_3-group: α-CF_3-proline and δ-CF_3-pseudo and thiopseudoprolines; (F) Methanoproline-based difluoro-*trans*-γ,δ-methanoproline and CF_3-γ,δ-methanoproline; (G) γ-CF_3-(*S*)-proline (TfmPro) explored in this study.

There are several potential candidates worth considering for the proline substitutive ^{19}F-labelling in polypeptides. For instance, β-[14] and γ-mono-fluoroprolines,[15,16,17,18] (Fig. 1C) that are among most explored proline modifications. The utility of these amino acids is toughened by their compatibility with the natural ribosomal protein expression machinery.[19,20] However, mono-fluoroprolines are particularly known for the over-stabilization of their preferred ring puckers and both significantly deviate in their *trans-cis* amide bond equilibria from Pro.

Another beneficial possibility is a straight incorporation of the CF_3-group. The latter carries three times more ^{19}F spins per residue and allows utilization of the strong homonuclear F-F dipolar coupling. In the solid state ^{19}F-NMR of peptides, the F-F coupling is a structural restraint which is superior to the chemical shift anisotropy.[6,7,10] However, the trifluoromethylation not only impose significant steric demand and potentially modulate electronic configuration of the pyrrolidine ring, but is also known to render template structures becoming more lipophilic,[21] which directly affects function of the membrane-active peptides, for instance. The steric demand and the increase in

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lipophilicity will be very large when multiple CF_3-groups are present, e.g. like in the perfluoro-*tert*-butyl-γ-hydroxyproline (Fig. 1D) as was recently proposed for ^{19}F-NMR labelling.[22] Furthermore, in the solid state a stongly coupled homonuclear 9-spin system will produce NMR spectra with a very complex multiplicity, which might obscure the data analysis and cancel the initial sensitivity advantage. Therefore an isolated CF_3-group should be preferred in the solid state ^{19}F-NMR studies.

Synthetically, a sole CF_3-group can be introduced into proline or the pseudoproline template in different positions. In particular, it has been described that α-CF_3 proline[23] and δ-CF_3 oxazolidine-based pseudoprolines[24] (Fig. 1E) could be incorporated in peptides. Still, in both Pro surrogates with the CF_3-substituent adjoining the aminocarboxylate, conformational freedom within the adjacent backbone swerves the parent Pro structure. Besides, incorporation of these analogues into a peptide chain is still a challenge as the amino acid reactivity in the peptide synthesis is predictably lowered.[25] Poor compatibility with the standard solid-phase peptide synthesis (SPPS) and sophisticated synthesis are the major utilization problem also for the recently described difluoro-[26] and trifluoromethyl-γ,δ-methanoprolines[11c] (Fig. 1F). In contrast, simple γ-CF_3-prolines (Fig. 1G) are chemically stable and carry the CF_3-group in the most remote position relative to both amino acid functionalities and correspondingly from the future polypeptide backbone. Therefore they may be the most balanced candidates for an intact proline substitution.

The conformational impact of a large number of γ-substituents has been addressed within ample mutagenesis investigations. Curiously, such studies for a single CF_3-group in the γ-position have been not described so far. We fill this knowledge gap and determine herein conformational and thermodynamic parameters of the γ-(*S*)-CF_3-proline (TfmPro) amide bond in a model compound. Judging by these parameters, we demonstrate TfmPro being close to proline, and further test the amino acid utility as a structural NMR label, in particular in the solid state ^{19}F-NMR study of the membrane-active peptide gramicidin S.

Results and Discussion

Synthesis of TfmPro

The synthesis of TfmPro was described in several publications about a decade ago.[27,28,29,30] Among these approaches we choose the simplest and the most economic (to our opinion) synthesis of the N-Boc derivative **1** reported by Qui&Qing,[29] which starts from hydroxyproline **2**.

Instead of using the benzyl ester which was obtained in the original study in a poor 45 % yield we employed methyl-ester protection and got the protected hydroxyproline **3** in 96 % yield (Scheme 1). In the following, hydroxyl group was converted to the keto-function (leading tot **4**) with 76 % yield (7 gram) using chromium (VI) oxide – pyridine (Collins reagent). However, for the scaled up synthesis we altered to the Parikh-Doering oxidation and increased the yield of **4** to 90 % (50 gram). Installation of the CF_3-group and subsequent water abstraction worked well using the original protocols. This way we obtained **5** in a 94% yield. The compound **5** heated with thionyl chloride in pyridine for 20 min under reflux gave 20 gram of the alkene **6** in a satisfactory 60 % yield. The second stereocenter was conventionally generated by hydrogenation of the double bond. Resulting TfmPro derivative **7** was obtained in 94 % yield. The carboxyl-group was then deprotected by saponification giving **1** (86 % yield, 9 gram). The overall yield of **1** was therefore 39 %, almost twice higher than the 21 % reported by Qui&Qing.

For the following conformational study the model compound Ac-TfmPro-OMe (**8**) was prepared (*vide infra*). In addition, the Boc-protection in **1** was exchanged to Fmoc (**9**) with quantitative yield. The Fmoc amino acid was later used for the SPPS.

NMR spectra, in particular ^{19}F-NMR of compounds **1**, **7**, **8**, **9** were consistent with only one diastereomeric form of TfmPro in our synthesis. Furthermore, the $[\alpha]_D$ value of -77º determined for **1** was

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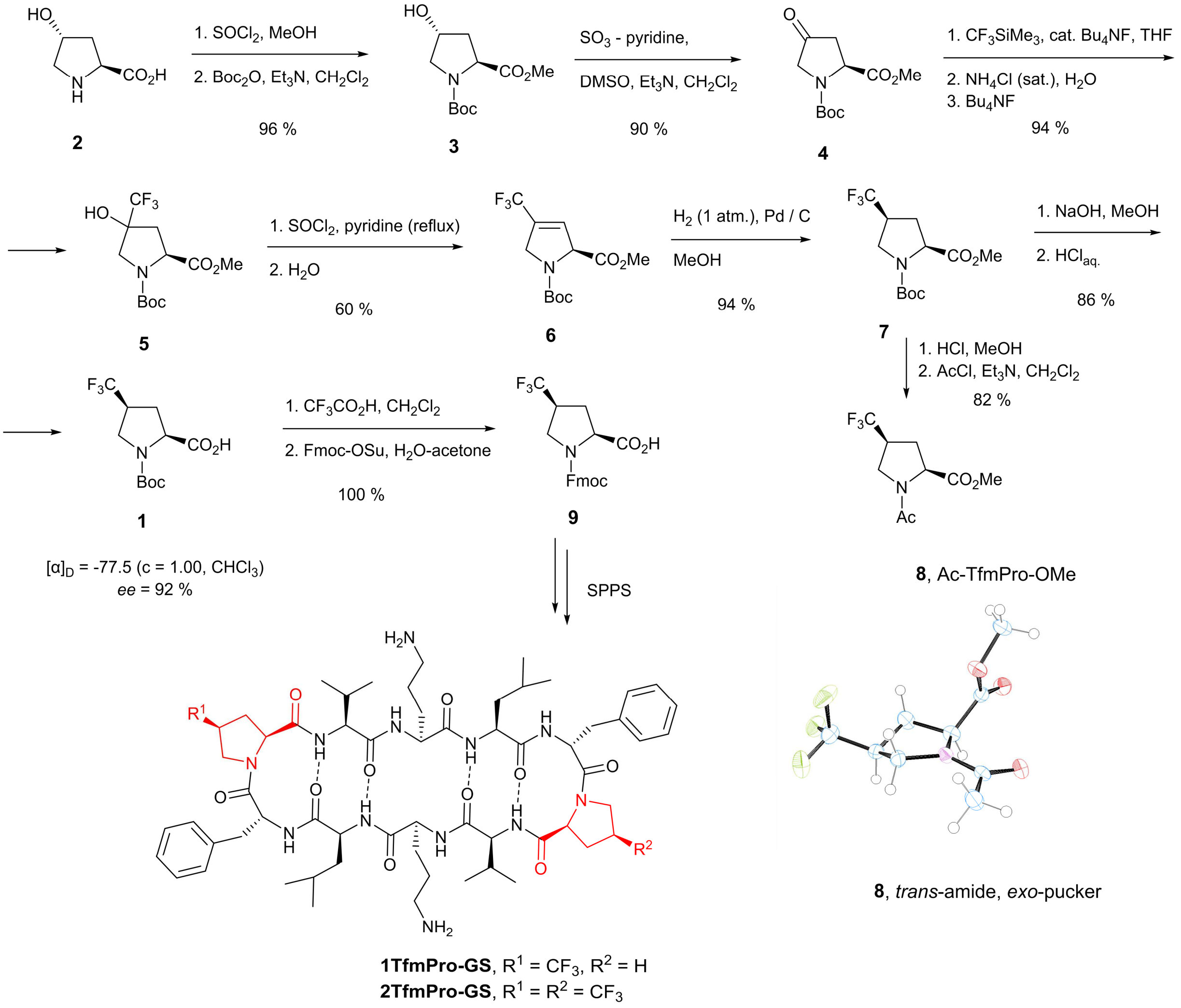
in full agreement with the -77.6° reported by Qui&Qing. We therefore found that in our modification the induction of the second stereo center under hydrogenation of **6** yields TfmPro of the correct *cis* relative stereochemistry despite the induction step was not coupled with the Bn-ester hydrogenolysis as described in the initial report.



Scheme 1. The synthesis of 4-(*S*)-trifluoromethylproline, as N-Boc (**1**), its derivative Ac-TfmPro-OMe (**8**) used for the conformational analysis, and the gramicidin S analogues used in solid state ^{19}F-NMR. The X-ray crystal structure of **8** (carbon – blue, oxygen – red, fluorine – green, nitrogen – purple).

We also obtained crystals of **8** which were then taken for X-ray analysis. To our surprise, the substance crystallized as racemic mixture in the orthorhombic cell containing four (*2S,4S*) and four (*2R,4R*) enantiomeric molecules. The main part of the substance **8** remained liquid and did not crystallize. The fact that the substance was diastereomerically pure strongly indicates that the partial epimerization of the proline α-(*S*)-chirality indeed occurred before the induction of the second stereo center. We presume that partial epimerization happened at the water elimination step (**5** to **6**). Finally by derivatisation with N-(2,4-dinitrophenyl)proline and subsequent ^{19}F-NMR we determined the enantiomeric excess of **1** being 92 %.

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Conformation analysis of the Ac-TfmPro-OMe model

For detailed conformational characterization of TfmPro in a quasi-peptide context, we studied the properties of its tertiary amide bond and the pucker in a small derivative. We selected Ac-TfmPro-OMe model as a well characterised framework in which many Pro analogues were studied.[1,15]

It is generally known that proline prefers s-*trans* amide rotamer, and the amide bond *trans*-*cis* interconversion could be conveniently characterized by the $K_{trans/cis}$ constant or by corresponding free energy difference. For instance, for Ac-Pro-OMe model in aqueous solution, the $\Delta G_{300\ K}$ was reported being -3.90 kJ/mol, which corresponds to the "native" Pro $K_{trans/cis}$ of 4.8.[19] It is also known that substituents in the γ-position influence this ratio mainly through stabilization of particular pyrrolidine ring γ-puckers. Comparative studies[12] suggest that generally, an electron withdrawing substituent in the position γ-(*S*) shifts the equilibrium towards the s-*cis* configuration; whereas an electron-donating group alleviates the s-*trans* rotameric form due to the *gauche*-effect. At the same time, a sterically demanding moiety in the γ-(*S*) configuration should also lead to the s-*trans* stabilization. Thus, the electronegative[31] and sterically demanding trifluoromethyl group might exhibit two counteracting effects: an influence on the pyrrolidine ring conformation and on the amide bond configuration in TfmPro. Should these effects be mutually compensated, TfmPro residue may be rendered as an almost intact proline analogue, i.e. to possess backbone conformational propensities very close to the ones in Pro.

The electronegative substituent effect was already seen in the pK_a value for TfmPro which we determined to be 8.5±0.1 close to that of γ-(*S*)-fluoroproline (9.2[32]). In order to test the conformational impact, we performed first the van't Hoff type of analysis for **8** in aqueous medium employing ^{19}F-NMR, which experimentally gives more accurate results than could be obtained from the crowded ^{1}H-NMR spectra. The chemical shift difference between the two amide bond rotamers in the ^{19}F spectra was 0.35 ppm at 25°C and changed to 0.22 ppm at 90°C. In agreement with our hypothesis, we found (Tab. 1) the equilibrium constant $K_{trans/cis}$ for Ac-TfmPro-OMe being equal 4.0. The γ-(*S*)-CF_3-group thus only marginally shifts the overall equilibrium towards the *cis*-amide bond in the contrast to the large perturbation a single fluorine atom causes at the same ring position ($K_{trans/cis}$ = 2.5 for γ-(*S*)-fluoroproline). In addition, we have determined kinetic parameters of the isomerisation process. In particular, the activation energy of the *cis*-to-*trans* rotation process (81.9 kJ/mol) was slightly lower but overall similar to that of proline (84.5 kJ/mol). This effect is potentially related to the higher acidity of the imonium function likewise in γ,γ-difluoroproline, where corresponding activation barrier value was even lower, 80.8 kJ/mol.[19] We also observed *cis*-*trans* and *trans*-*cis* isomerization processes to be both driven enthalpically (amide conjugation), the same way as this is known for proline.

The 1D ^{1}H-NMR spectra of Ac-TfmPro-OMe have also been analysed to assess the preferred conformation of the pyrrolidine ring. Since in our model, the α-CH couples to both protons of the adjacent β-CH_2-group, two characteristic patterns are expected. For the *exo*-puckers the two $^{3}J_{\alpha\beta}$ couplings should be in the range of 7-11 Hz, whereas for the *endo*-configuration one coupling should be in the range of 6-10 and the other of 2-3 Hz.[33] For the major s-*trans* isomer of Ac-TfmPro-OMe we observed two equal $^{3}J_{\alpha\beta}$ values of 8.3 Hz, thus confirming its preference for the *exo*-pucker. Remarkably, the same α-CH triplet shape for the major s-*trans* amide rotamer has been reported for s-*trans* rotamer of glycosylated γ-(*S*)-hydroxyproline which exhibits the *exo*-pucker.[34] The splitting of 8.3 Hz also persisted in water, chloroform and DMSO solutions of **8**. The *exo*-pucker configuration for the s-*trans* was also confirmed in the solid state (X-ray structure, Scheme 1). In contrast, the minor s-*cis* form exhibited two distinct $^{3}J_{\alpha\beta}$ values of 3.8 and 9.8 Hz (in water), having therefore no qualitative pucker preference. Following the same NMR criteria, proline itself should be classified as preferring an *endo*-pucker in the s-*cis* form and a mixed pucker in the s-*trans* form.[35]

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Table 1. The thermodynamic parameters, conformational equilibrium and the tertiary amide bond rotational rates determined in Ac-TfmPro-OMe (**8**), compared to proline and γ-fluoroprolines (Flp).

equilibrium	$K_{trans/cis\ 300K}$	ΔH, kJ/mol	ΔS, J/mol·K	ΔG_{300K}, kJ/mol
Ac-TfmPro-OMe	4.0	-4.86 ± 0.11	-4.72 ± 0.33	-3.44 ± 0.21
Ac-Pro-OMe[19]	4.8	-5.04 ± 0.05	-3.82 ± 0.16	-3.90 ± 0.10
Ac-(*R*)-Flp-OMe[19]	6.8	-7.73 ± 0.26	-9.81 ± 0.81	-4.78 ± 0.50
Ac-(*S*)-Flp-OMe[19]	2.5	-3.04 ± 0.03	-2.47 ± 0.11	-2.30 ± 0.07
cis-to-*trans*	k_{300K}, s^{-1}	$\Delta H^{\neq}$, kJ/mol	$\Delta S^{\neq}$, J/mol·K	$E_{a\ 300K}$, kJ/mol
Ac-TfmPro-OMe	0.034	78.3 ± 0.3	-11.9 ± 1.0	81.8 ± 0.6
Ac-Pro-OMe[19]	0.012	87.2	9.00	84.5
Ac-(*R*)-Flp-OMe[19]	0.026	84.2	5.37	82.6
Ac-(*S*)-Flp-OMe[19]	0.015	84.7	2.42	84.0
trans-to-*cis*	k_{300K}, s^{-1}	$\Delta H^{\neq}$, kJ/mol	$\Delta S^{\neq}$, J/mol·K	$E_{a\ 300K}$, kJ/mol
Ac-TfmPro-OMe	0.009	81.4 ± 0.5	-12.6 ± 1.6	85.2 ± 1.0
Ac-Pro-OMe[19]	0.003	92.3	12.8	88.4
Ac-(*R*)-Flp-OMe[19]	0.004	91.8	14.8	87.4
Ac-(*S*)-Flp-OMe[19]	0.006	87.5	4.17	86.3

TfmPro represents therefore a proline substitute which in summary has the pucker favouring the *exo*-conformer, whereas its amide bond thermodynamic preference is slightly shifted towards s-*cis*. From the structural labelling perspective, despite differences in the pucker preferences, the two amino acids can be considered mutual analogues since the overall amide bond configuration in TfmPro is very close to the one in proline.

TfmPro in gramicidin S

To observe TfmPro in a real peptide and to later evaluate the amino acid as an NMR label for peptide studies we incorporated TfmPro in the cyclic antimicrobial peptide gramicidin S (*cyclo*[Pro-Val-Orn-Leu-^{D}Phe]$_2$, where Orn stands for ornithine; **GS**). The two possible peptides **1TfmPro-GS** (one TfmPro to Pro substitution, mono-substituted) and **2TfmPro-GS** (two TfmPro to Pro substitutions, doubly-substituted) were produced by conventional Fmoc-SPPS as reported earlier.[9,36] The peptides were synthesised in two steps: first a linear sequence was constructed on the solid support, followed by the cyclisation in solution. Since the linear precursors have to be pre-organized for the cyclisation, success of the reaction *per se* indirectly confirms the compatibility of TfmPro with a turn conformation of the native Pro-containing segment. The peptides were standardly purified using TFA-free RP-HPLC[7], performing which we observed an expected stepwise increase in the retention times (compared against **GS**) as hydrophobicity rose upon successive introduction the CF_3-groups (Fig. 2). In the view of GS being the membrane-active peptide, this suggests a potential modulation of the peptide functional activity, but may not necessarily be a sign of the structural deviation.

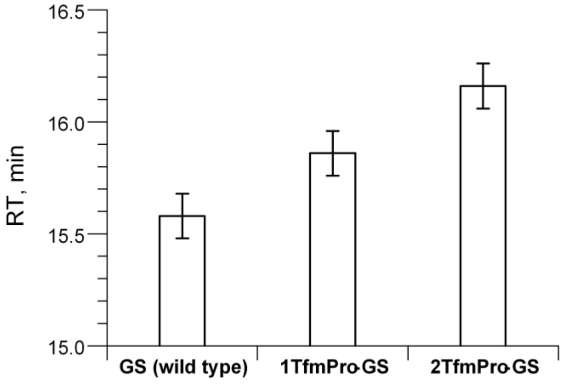

Fig. 2. RP-HPLC retention times of **GS** and its analogues containing TfmPro. The error bars represent deviations of the values measured in triplicate.

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Since we address only structural aspect, in the following, we only focused on the studies of potentially more perturbed doubly-substituted analogue **2TfmPro-GS.**

As it is usually done in the label-validation studies to verify global conformational impact of a substitute, the new analogue was compared against **GS** by circular dichroism spectroscopy (CD) in membrane-mimicking environment (Fig. 3). Practically the same spectral shapes for both molecules suggest convergence of the modified backbone conformation to the parent **GS** structure in a membrane-mimicking environment. Formally, since we have demonstrated TfmPro possesses proline-like intrinsic amide conformational preferences and not likely to influence overall geometry of the **GS** skeleton, we therefore could call TfmPro a reasonable ^{19}F-label to substitute proline.[6,11c]

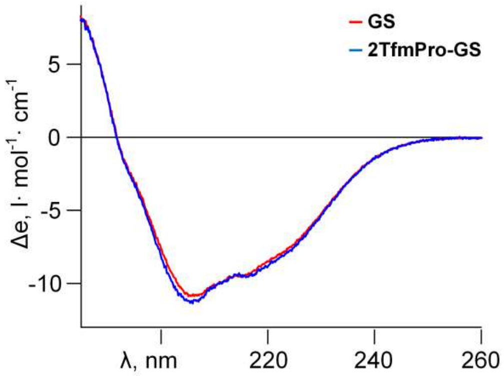

Fig. 3. Circular dichroism spectra of **GS** and its analogue **2TfmPro-GS** in aqueous buffer–trifluoroethanol mixture (phosphate buffer (PB), 10 mM, pH 7.4; TFE - 30%) at 25 °C.

To validate the use of TfmPro in the solid state NMR structural studies of peptides, we used the **GS** system, solid state NMR behaviour of which is comprehensively studied. In particular, we benefited from the knowledge of the conditions in which the peptide changes its membrane-bound state. Using conventional ^{19}F-labels it was earlier shown that in the oriented lipid bilayers molecules of **GS** change their alignment from the surfacially-bound basic state (“S-state”, Fig. 4A) to an upright orientation (“**I**-state”), the latter being attributed to the formation of homooligomeric pores relevant to the biological activity.[9,37]

We knew that the amount of molecules in the **I**-state increases: i) with the increase of the concentration, (ii) is more pronounced at temperatures close to the *gel*-to-*fluid* phase transition (T_m) of the bilayers and (iii) in the short-chain saturated glycerophospholipids.[38] To satisfy these conditions we prepared **2TfmPro-GS**–containing oriented proteobilayers using 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (DLPC) as a lipid matrix and 1/40 as a peptide/lipid ration. From these samples a series of solid state ^{19}F-NMR spectra were collected as a function of temperature. The temperature range was chosen to include the T_m of DLPC. Notably, the *gel*-to-*fluid* transition in DLPC is a complex process, characterised by conversions of several polymorphic mesophases and is rather broad (spans between -2°C and +7°C).[39] As can be seen in the Fig. 4C, the oriented peptide in *fluid* lipids revealed single ^{19}F-NMR signal (F-F dipolar coupling of +2.3 kHz), which was no longer resolved in the more rigid *gel*-phase bilayers. Such behaviour was expected and in both cases the peptide molecules should reside in the same orientation (the S-state), but should have different mobilities. The re-orientation to the **I**-state was indeed observed upon cooling at around T_m (the triplet with ca. -6.0 kHz splitting). Thus we reproduced temperature-dependent re-alignment of **GS** and demonstrated a qualitative application

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of TfmPro to monitor this process, but also showed this process for the first time by using F-F dipolar couplings, not the chemical shift.[37,40]

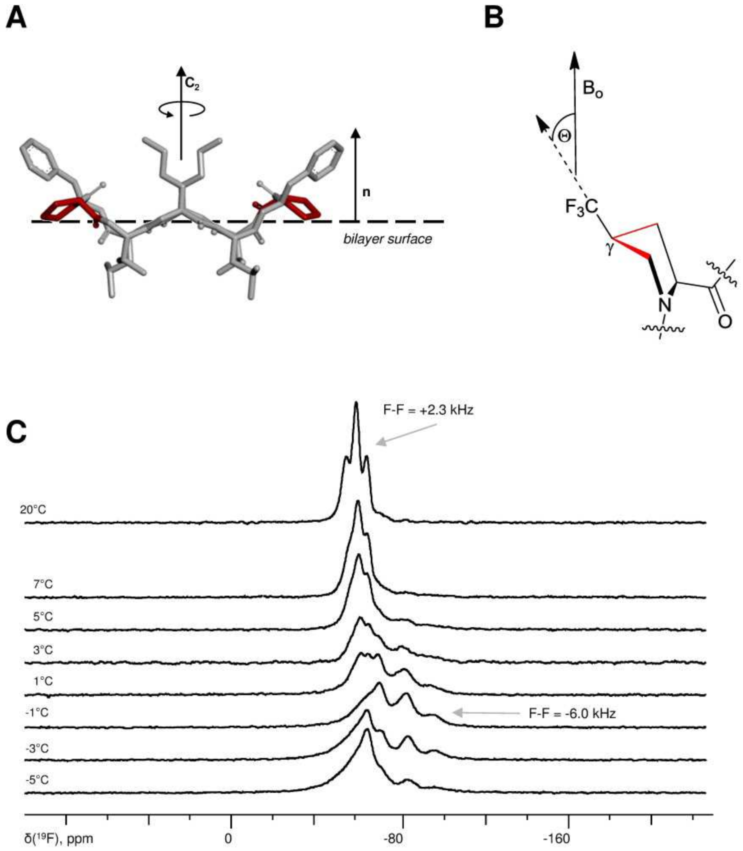

Fig. 4. (A) Schematic representation of **GS** in its membrane-bound S-state viewed from the bilayer plane; definition of the membrane normal (n), and rotational axis of C_2 symmetry in **GS** molecule. Backbone structure as determined by Xu *et al*.[41] The side-chains are taken as statistically most probable rotamers, protons are not shown for clarity, the prolines are highlighted in red. (B) Definition of the angle Θ (pucker is arbitrarily selected) which can be determined from solid state ^{19}F-NMR from the dipolar splitting F-F according to the relation $\text{F-F}_{obs} = \text{F-F}_{max} \cdot S_{mol} \cdot (3\cos^2\Theta - 1)/2$. (C) Temperature-dependent re-alignment of **2TfmPro-GS** in DLPC as followed by solid state ^{19}F-NMR. The sample is oriented with the membrane normal parallel to the magnetic field, temperatures and characteristic F-F values are indicated.

Based upon the above confirmation that **2TfmPro-GS** has the same membrane alignment as **GS**, we attempted to validate the membrane-bound structure of the molecule. Intrinsically symmetric **GS** molecules in the S-state (Fig. 4A) should have both Pro residues identically oriented with respect to the membrane normal. We indeed observed only one signal, thus confirmed the C_2 symmetry of the **2TfmPro-GS** in the lipid-bound situation. The angle Θ (Fig. 4B) between the ^{19}F-reporter group (the vector which connects the Cγ and the carbon of the $CF_{3,}$) and the external magnetic field (B_0) can be directly determined from the value of the orientation-dependent F-F dipolar coupling.[11] The observed F-F splitting (+2.3 kHz), considering the order parameter S_{mol} of 0.34[37] and the maximum F-F of the rotating CF_3 group of 15.8 kHz, gives Θ of ~37°. When we docked the TfmPro crystal geometry (*exo*-pucker, *s*-*trans*) onto NMR-determined backbone structure of **GS**[41] and align the model in the S-state as it was done in the earlier studies[37,38], to our dissatisfaction, we obtain Θ of 45-50°. However, when we do the same using one of the reported crystal **GS** structures,[42] Θ assumes the range of 25-30°. Remarkably, our

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experimental value of ~37° lays in-between these two values, but this appears to be a pure coincidence as clearly the major uncertainty in membrane-bound structure determination is the assumed backbone conformation of **GS**.

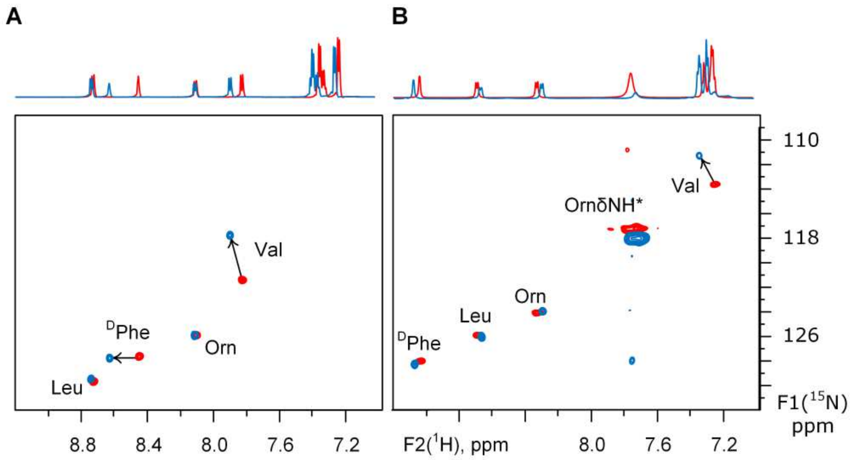

Fig. 5. $^1H^{15}N$ sofastHMQC spectra on the **GS** and its analogue **2TfmPro-GS** in: (A) aqueous buffer-trifluoroethanol and (B) DMSO. The ornithine δ-NH resonance is a folded signal.

The uncertainty in the peptide backbone urged us to re-evaluate the degree of conformational perturbance TfmPro provides on the **GS** backbone. From CD analysis above we have concluded **2TfmPro-GS** does not interfere with the **GS** backbone in the rough approximation only. To have a more detailed conformational insight we have additionally characterized the structure of **2TfmPro-GS** by NMR. The ^{19}F-NMR solution spectra of the **2TfmPro-GS** showed single resonances in DMSO, water-trifluoroethanol and aqueous SDS micelles at -69.5, -71.4 and -70.8 ppm, respectively, confirming the overall C_2 symmetry in solution. Conformation of backbone amides was examined by $^1H^{15}N$ single bond correlation NMR in 30% trifluoroethanol (Fig. 5A), i.e. exactly at conditions of CD (Fig. 3). In addition we inspected **2TfmPro-GS** in DMSO environment (Fig. 5B), which is a better membrane mimic[43], but a condition inaccessible to CD spectroscopy. In the first case the ^{D}Phe NH signals of were affected only in the ^{1}H dimension implying a different solvent exposure, which may come from the deviations within the β-turn atom arrangements. The Val NH, which is a part of the Val-TfmPro peptide bond, exhibited in contrast a prominent shift in the ^{15}N dimension which persisted in both solvents. This is a potential indication of a local conformational effect of the γ-CF_3-substituent in the proline analogue. Alternatively, an electrophilic influence of the CF_3-substituent to the proline carbonyl could cause such a shift. Nevertheless, the rest of the signals remained in the modified peptide at same or close positions as they were in the wild type peptide. NMR data therefore corroborates the CD spectra-based conclusion that the TfmPro is compatible with the global secondary structure of **GS** and could affect only the local arrangement. The former finding is critically important to conclude solid state ^{19}F-NMR data consistency with conditions-dependent states of the native peptide in membrane environment. Whereas the correspondence of the local atomic arrangements is a technical prerequisite for correct conversion of the experimental anisotropic parameters (F-F dipolar couplings) into alignment angles for the wild type peptide molecule.

In the DMSO solution we found also, that the Pro ring conformation in **GS** (α-CH as d, $J = 7.2$ Hz) was different to the TfmPro containing analogue (α-CH as dd, $J = 9.8$ and 4.5 Hz). In **2TfmPro-GS**, the α-CH multiplicity of the imino acid is no longer consistent with the preference for the *exo*-pucker seen in the *trans*-amide of the Ac-TfmPro-OMe. This observation alludes additional reason to the uncertainties in the **2TfmPro-GS** structure analysis from the solid state NMR (*vide supra*). Besides, it demonstrates that the

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substituted prolines may not necessarily maintain their "free" conformations (Ac-TfmPro-OMe model) in the restrained context of a structured polypeptide. Just as in the recent reports for γ-mono-fluoroprolines in the thiredoxin fold[44] and γ-hydroxyprolines in the cyclotide kalata B1[45].

Conclusions

In summary we describe herein the properties of a peptide bond formed by the γ-(*S*)-CF_3-proline as an analogue of proline. The CF_3-group in the γ-position of the pyrrolidine ring showed minimal influence on both the *trans-cis* amide equilibrium ratio (slightly shifted towards *cis*) and the rotational barrier (minimally reduced). The energy parameters of the γ-(*S*)-CF_3-proline amide were described in details. In the β-turn of gramicidin S, the proline surrogate did not change the overall peptide structure. Used as a solid state ^{19}F-NMR label, γ-(*S*)-CF_3-proline confirmed the changes of the peptide alignment in lipid membranes in a qualitative way. Straightforward synthesis, marginal perturbation of the amide bond conformation, remote position from the peptide bond, and excellent NMR properties make γ-(*S*)-CF_3-proline a good choice for a broad range of ^{19}F-NMR proline labelling studies in peptides and proteins.

Experimental part

The chemical shifts are given in δ scale according to the conventional internal deuterium referencing. The standards are TMS (Me_4Si) and Freon-11 (CCl_3F).

Methyl esther N-Boc hydroxyproline (**3**) was obtained from hydroxyproline (**2**, 10.5 g) according to the literature protocol (19.0 g, 96 % yield).[46] 4-ketoproline (**4**) was then prepared as following. **3** (50 g) was dissolved in dichloromethane (700 ml), triethylamine (91 ml) was added and the solution was cooled down in an ice bath under argon atmosphere. Suspension of pyridine·SO_3 complex (63.5 g, 2 eq.) in DMSO (250 ml) was added portionwise within 8 min. The mixture was stirred for 18 hours at ambient temperature. It was then poured in 1 kg of ice. Organic layer was separated, washed with citric acid solution (10 %, 2 x 500 ml), hydrochloric acid (4 %, 1 x 500 ml), sodium hydrogencarbonate (saturated, 1 x 500 ml), citric acid solution (10 %, 1 x 500 ml) and sodium hydrogencarbonate (saturated, 1 x 500 ml), dried over sodium sulphate, filtered and concentrated under reduced pressure. **4** was obtained as yellowish oil (46.9 g, 90 % yield). ^{1}H NMR data was consistent with the literature.[47]

Compound **5** was prepared as following. **4** (44.72 g, 184 mmol) was dissolved in THF (300 ml) under argon atmosphere. The solution was cooled down in an ice bath. CF_3TMS (28.5 ml, 193 mmol) was added within 5 min followed by TBAF (1M in THF, 1 ml). The ice was allowed to melt down, and the mixture was stirred at ambient temperature for 22 hours. Saturated ammonium chloride (300 ml) was added, the mixture was stirred for 20 min, then TBAF solution (1M in THF, 190 ml) was added. The mixture was stirred for 1 hour. Organic layer was separated, and aqueous layer was extracted with diethyl ether (2 x 150 ml). Combined organic fractions were washed with water (1 x 300 ml), brine (1 x 300 ml), dried over sodium sulphate, filtered and concentrated under reduced pressure. **5** (54.1 g, 94 %) was obtained as glassy oil. ^{1}H-NMR ($CDCl_3$, 400 MHz), δ, ppm (two rotamers): 4.52 and 4.43 (two d, *J* = 4.5 Hz, 1H), 4.37 and 4.22 (two br s, 1H), 3.77 and 3.75 (two s, 3H), 3.75-3.62 (m, 2H), 2.53 (m, 1H), 2.19 (t, 1H, *J* = 13 Hz), 1.44 and 1.39 (two s, 9H). ^{19}F-NMR ($CDCl_3$, 376 MHz), δ, ppm (two rotamers 1:1): -81.1 and -81.2 (two s, CF_3).

2-tert-butyl 2-methyl (2S)-trifluoromethyl-3-pyrrolin-1,2-dicarboxylate (6)

In a 2 l reactor **5** (33.9 g, 108 mmol) and dry pyridine (1 l) were placed under argon. Thionyl chloride (100 ml) was added and the mixture was heated and refluxed. The reflux was continued for 20 min and then the mixture was allowed to cool down to ambient temperature. The mixture was poured into iced water (0.5 kg). Aqueous layer was extracted with diethyl ether (4 x 300 ml)

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and ethyl acetate (1 x 1 l). Combined organic fractions were concentrated under reduced pressure to have the volume of ~ 300 ml. Resulting organic solution was washed with hydrochloric acid (5 %, 2 x 150 ml), sodium hydrogenecarbonate (saturated, 1 x 150 ml) and brine (1 x 150 ml), dried over sodium sulphate and concentrated in vacuum. The black crude material was filtered through a short silicagel (70 g) column in hexane – ethyl acetate 2:1 mixture. Final product **6** (19.2 g, 60 %) was obtained as yellowish oil.

^{1}H-NMR ($CDCl_3$, 400 MHz), δ, ppm (two rotamers): 6.19 (td, *J* = 19 and 2 Hz, 1H, CH=C), 5.08 (two m, 1H, N-CH-CO_2Me), 4.33 (m, 2H, N-CH_2), 3.71 (two s, 3H, OCH_3), 1.42 and 1.37 (two s, 9H, $C(CH_3)_3$). ^{13}C-NMR ($CDCl_3$, 101 MHz), δ, ppm (two rotamers): 169.2 and 168.8 (two s, CO_2Me), 153.4 and 152.8 (two s, N-C(=O)-O), 132.8 and 132.6 (two q, *J* = 36 Hz, C=CH), 128.3 and 128.1 (two q, *J* = 5 Hz, C=CH), 120.7 (two q, *J* = 270 Hz, CF_3), 81.1 (two s, O-$C(CH_3)_3$), 66.5 and 66.3 (two s, CH-CO_2Me), 52.8 and 52.6 (two s, OCH_3), 51.0 and 50.8 (two s, N-CH_2), 28.3 and 28.2 (two s, $C(CH_3)_3$). ^{19}F-NMR ($CDCl_3$, 282 MHz), δ, ppm: -65.3 (d, J_{F-H} = 19 Hz, CF_3). IR bands: 3093, 2983, 2876, 1748, 1703, 1667 rest of the peaks below 1460 cm^{-1}. Mass-spectrum (EI), m/z: 236 $[M-Bu^t]^+$, 195 $[M-Boc]^+$. $[\alpha]_D^{20}$ = -202 ($CHCl_3$, c = 0.55). CHN, found. C, 49.03; H, 5.30; N 4.99. $C_{12}H_{16}F_3NO_4$ requires C, 48.81; H, 5.46; N 4.47.

Methyl (2*S*,4*S*)-N-tert-buthoxycarbonyl-4-trifluoromethylprolinate (7)

6 (13 g; 44 mmol) and Pd/C (5 %, 3 g) in methanol (100 ml) were stirred under hydrogen (1 atm) for 5 hours. The mixture was filtered and concentrated under reduced pressure to give **7** (12.3 g, 94 %) as white solid.

^{1}H-NMR ($CDCl_3$, 400 MHz), δ, ppm (two rotamers): 4.42 and 4.33 (2t, *J* = 8 Hz, 1H), 3.92 and 3.84 (2t, *J* = 10 Hz, 1H), 3.77 (s, 3H), 3.51 (t, *J* = 10 Hz), 2.96 (m, 1H), 2.56 (m, 1H), 2.14 (m, 1H), 1.49 and 1.43 (2s, 9H). ^{13}C-NMR ($CDCl_3$, 101 MHz), δ, ppm (two rotamers): 172.3 and 172.0 (2s, CO_2Me), 153.8 and 153.2 (2s, N-C(=O)-O), 126.0 (q, *J* = 277 Hz, CF_3), 80.8 (s, O-$C(CH_3)_3$), 58.5 and 58.2 (2s, N-CH-CO_2Me), 52.4 and 52.2 (2s, OCH_3), 45.8 and 45.7 (2m, N-CH_2), 41.9 and 41.1 (q, *J* = 30 Hz, CH-CF_3), 30.1 and 29.1 (2s, CH_2-CH-CO_2Me), 28.3 and 28.2 (2s, $C(CH_3)_3$). ^{19}F-NMR ($CDCl_3$, 282 MHz), δ, ppm: -70.4 (d, J_{H-F} = 8 Hz, CF_3). IR bands: 2987, 2870, 1747, 1695, further peaks below 1481 cm^{-1}. Mass-spectrum (EI), m/z: 297 $[M]^+$, 240 $[M-But]^+$, 239 $[M-CO_2Me]^+$, 224, 196 $[M-Boc]^+$. T_{melt} = 50 °C, $[\alpha]_D^{20}$ = -70 ($CHCl_3$, c = 2.1). CHN, found. C, 48.59; H, 6.05; N 4.85. $C_{12}H_{18}F_3NO_4$ requires C, 48.48; H, 6.10; N 4.71.

The compound **1** was prepared as following. To **6** (10.88 g, 36.6 mmol) in methanol (100 ml) sodium hydroxide (1M in methanol, 40 ml) was added. The mixture was stirred for 2 hours at the room temperature and then concentrated in vacuum (temperature in bath ≤ 40 °C). The residue was dissolved in water (200 ml) and washed with diethyl ether (2 x 40 ml) and organic fractions were discarded. The water fraction was then acidified with hydrochloric acid (13 %, 17 ml) until acidic pH was reached (~ 1-2). Aqueous layer was extracted with dichloromethane (4 x 60 ml), the organic fractions were dried over sodium sulphate, filtered and concentrated under reduced pressure. **1** (8.98 g, 86 %) was obtained as white solid. ^{1}H-NMR ($CDCl_3$, 500 MHz), δ, ppm (two rotamers): 6.75 (br s, 1H), 4.43 and 4.36 (2m, 1H), 3.93 and 3.85 (2m, 1H), 3.50 (m, 1H), 2.97 (m, 1H), 2.61 and 2.54 (2m, 1H), 2.41 and 2.22 (2m, 1H), 1.49 and 1.43 (2s, 9H). Spectral data was consisted with the one published by Qiu&Qing.[29] Also, the $[\alpha]_D$ values varied in different repetitions of the full synthesis from -72 to -77 indicating different levels of racemization on the previous steps. In this particular approach described in this experimental section we obtained the product with $[\alpha]_D$ -77 (c = 1.0, $CHCl_3$).

(2*S*,4*S*)-N-(9-fluorenylmethoxycarbonyl)-4-trifluoromethylproline (9)

To a solution of **1** (569 mg, 2.0 mmol) in dichloromethane (10 ml) trifluoroacetic acid (3 ml) was added, the solution was stirred at the room temperature for 2 hours. Liquids were removed under reduced pressure (temperature in bath 30 °C), then additional dichloromethane (10 ml) was added

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and evaporation was repeated. Water (10 ml) and sodium carbonate solution (10%, 7 ml) was added to reach pH ≈ 8-9 and acetone (5 ml) was subsequently added until clear solution was afforded. Resulting mixture was cooled down in an ice bath. Suspension of Fmoc-OSu (745 mg, 2.2 mmol) in acetone (2 ml) was added within 1 min upon stirring, and then about 10 ml of acetone was added to the reaction mixture. After 4 hours the ice bath was removed and stirring was continued for 25 hours at ambient temperature. Acetone was removed under reduced pressure (temperature in bath ≤ 31 °C), the transparent solution was poured into water (100 ml), and resulting suspension was washed by diethyl ether (4 x 30 ml). The aqueous layer was acidified by hydrogen chloride solution (1M) until pH ≈ 1;, resulting aqueous fraction was extracted with ethyl acetate (4 x 50 ml). The ethyl acetate fractions were dried over magnesium sulphate, filtered and concentrated under reduced pressure. The residue was dissolved in acetonitrile-water mixture, the solution was frozen in liquid nitrogen and lyophilized overnight to give **9** (813 mg, 100%) as beige powder (amorphous).

^{1}H-NMR ($CDCl_3$, 700 MHz), δ, ppm (two rotamers 3:2): 7.89 (br s, 1H, CO_2H), 7.79 (major) and 7.73 (minor) (2d, *J* = 7 Hz, 2H, aromatic CH), 7.58 (major) and 7.54 (minor) (m, 2H, aromatic CH), 7.43 (major) and 7.37 (minor) (m, 2H, aromatic CH), 7.34 (major) and 7.30 (minor) (m, 2H, aromatic CH), 4.55-4.40 (m, 2H, CH_2-O), 4.50 (major) and 4.31 (minor) (2t, *J* = 8, 1H, α-CH), 4.27 (major) and 4.15 (minor) (2t, *J* = 7 Hz, C$\underline{H}$-CH_2-O), 3.54 (m, 1H, δ-CH), 2.98 (major) and 2.94 (minor) (m, 1H, γ-CH), 2.61 (m, 1H, β-CH), 2.28 (major) and 2.21 (minor) (2m, 1H, β-CH). ^{13}C-NMR ($CDCl_3$, 176 MHz), δ, ppm (two rotamers): 176.1 and 175.2 (CO_2H), 155.0 and 154.1 (N-C(=O)-O), 143.8, 143.5, 141.4 and 141.3 (aromatic C), 127.9, 127.7, 127.2, 127.1, 125.0, 124.8, 120.04 and 120.0 (aromatic CH), 125.8 (CF_3), 68.2 and 67.8 (CH_2-O), 58.5 and 57.7 (α-CH), 47.13 and 47.10 ($\underline{C}$H-CH_2-O), 46.2 and 45.8 (δ-CH_2), 41.9 (major) and 41.0 (minor) (q, *J* = 30 Hz, $\underline{C}$H-CF_3), 30.1 and 28.8 (β-CH_2). ^{19}F-NMR ($CDCl_3$, 659 MHz), δ, ppm, (two rotamers): -70.34 (major) and -70.38 (minor) (d, *J* = 8 Hz). IR bands: 3600-2150 broad peak with a maximum at 2957, 1705 broad peak, further peaks below 1430 cm^{-1}. Mass-spectrum (EI), m/z: 405 $[M]^+$, 179 $[Fmoc]^+$. $[\alpha]_D^{20}$ = -50.7 ($CHCl_3$, c = 1.04). CHN, found. C, 62.02; H, 4.40; N 3.60. $C_{21}H_{18}F_3NO_4$ requires C, 62.22; H, 4.48; N 3.46.

Methyl (2*S*,4*S*)-N-acetyl-4-trifluoromethylprolinate (8)

Acetyl chloride (0.45 ml, 6.3 mmol) was mixed to dry methanol (25 ml) and resulting acidic methanol was added to **7** (1.57 g, 5.3 mmol) in methanol (75 ml). The mixture was stirred at the room temperature for 2 hours and then refluxed for the next 4 hours. The mixture was concentrated under reduced pressure. Dichloromethane (70 ml) and triethylamine (1.62 ml, 11.6 mmol) were added to the residue and the mixture was stirred for 5 min, then acetyl chloride (0.41 ml; 5.8 mmol) was added and the mixture was stirred at the room temperature for 3 days. The mixture was then concentrated under reduced pressure, the obtained residue was rinsed with diethyl ether (2 x 50 ml, 2 x 20 ml) and the organic fractions were concentrated in vacuum to give pure matter. This product was additionally purified on a silica gel column using ethyl acetate elution to give **8** (1.03 g, 82 %) as colourless oil.

^{1}H-NMR (D_2O, 700 MHz), δ, ppm (two rotamers): 4.81 (dd, *J* = 9.7 and 3.7 Hz, minor) and 4.50 (t, *J* = 8.2 Hz, major, 1H, CH-CO_2Me), 4.00 (dd, *J* = 8.7 and 9.7 Hz, 1H major, the minor rotamer resonance lays underneath, 1H, N-C$\underline{H}$H), 3.75 (s, minor) and 3.71 (s, major, 3H, OCH_3), 3.69 (t, *J* = 10.0 Hz, major) and 3.42 (dd, *J* = 12.7 and 6.3 Hz, minor, 1H, N-CH$\underline{H}$), 3.28 (m, major) and 3.18 (m, minor, 1H, CH-CF_3), 2.75 (m, minor) and 2.63 (m, major, 1H, C$\underline{H}$H-CH-CO_2Me), 2.42 (m, minor) and 2.11 (m, major, 1H, CH$\underline{H}$-CH-CO_2Me), 2.07 (s, major) and 1.98 (s, minor, 3H, CH_3-C=O). ^{13}C-NMR ($CDCl_3$, 176 MHz), δ, ppm (two rotamers): 173.62 (s, major, CO_2Me), 173.60 (s, minor, N-C=O), 173.3 (s, minor, CO_2Me), 172.9 (s, major, N-C=O), 126.7 (q, *J* = 277 Hz, minor) and 126.0 (q, *J* = 276 Hz, major, CF_3), 59.7 (s, minor) and 58.6 (s, major, $\underline{C}$H-CO_2Me), 53.4 (s, minor) and 53.1 (s, major, OCH_3), 47.2 (q, *J* = 3 Hz, major) and 45.6 (q, *J* = 3 Hz, minor, N-CH_2),

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41.3 (q, J = 29 Hz, major) and 39.4 (q, J = 29 Hz, minor, $\underline{C}H-CF_3$), 29.4 (q, J = 2 Hz, minor) and 28.3 (q, J = 3 Hz, major, $\underline{C}H_2-CH-CO_2Me$). ^{19}F-NMR (470 MHz), δ, ppm (two rotamers): in D_2O -71.0 (major, J_{F-H} = 9 Hz) and -71.3 (minor, J_{F-H} = 10 Hz); in DMSO-d6 -69.4 (major, J_{F-H} = 9 Hz) and -69.9 (minor, J_{F-H} = 10 Hz); in $CDCl_3$ -70.7 (major, J_{F-H} = 8 Hz) and -71.1 (minor, J_{F-H} = 9 Hz). IR bands: 2959, 2892, 1743, 1652, further peaks below 1438 cm^{-1}. Mass-spectrum (EI), m/z: 239 [M^+], 180, 139. $[\alpha]_D^{20}$ = 78 ($CHCl_3$, c = 0.56). CHN, found. C, 45.34; H, 5.30; N 6.05. $C_9H_{12}F_3NO_3$ requires C, 45.19; H, 5.06; N 5.86. X-ray crystall structure can be found in the Cambridge Crystallographic Data Center under ID CCDC 1042476.

^{19}F-NMR parametrization of the amide rotation

The ^{19}F-NMR spectra were measured on Bruker Avance III 500 spectrometer (470.7 MHz) equipped with a BBFO probe. The variable temperature unit was calibrated using sample with acidified glycerol. A temperature series of conventional 1-pulse spectra was run in the range 30-90 °C with solution of Ac-TfmPro-OMe (**8**, 75 mg) in deuterium oxide as 12 experiments with the temperature pre-equilibration delay of 5 min. Resulting spectra were baseline corrected (5th order) and integrated. Corresponding rotameric ratios ($K_{trans/cis}$) were calculated and then converted to ΔG according to $\Delta G = -RT\ln K$. The ΔG values were plotted against the temperature, ΔH and ΔS values were then extracted using $\Delta G = \Delta H - T\Delta S$ equation.

The amide rotation rate constants were determined in ^{19}F cross-relaxation experiments (EXSY) with the following setup: “noesygpph” experiment from the standard Bruker library, with the mixing time of 1 s and 20 ms for referencing. 18 experiments (9 for exchange and 9 referencing) were acquired at 30-55 °C. Resulting 2D spectra were baseline corrected in both dimensions and integrated. The exchange rates (exchange rate matrices) were calculated with EXSYCalc® (Mestrec) freeware. In particular, detected at 55 °C the exchange rates were $k_{cis-trans}$ = 0.617 and $k_{trans-cis}$ = 0.182 s^{-1}. Standard linearisation according to the Eyring equation (in ln(k/T) – 1/T coordinates) delivered the values of ΔH and ΔS.

Peptide synthesis

Linear peptides were synthesised on a Val-preloaded 2-chlorotrytyl resin. The amino acids were taken as N-Fmoc (ornithine side chain amine was Boc-protected) in 4 eq. along with 4 eq. of 6Cl-HOBt, 3.9 eq. of HCTU, 8 eq. of DIPEA premixed in 2 ml of DMF before adding to the resin for a coupling step which was performed for 2 hours. The Fmoc-removal was done with 22 % piperidine in DMF for 20-30 min. The linear peptides were cleaved from the resin by treatment with 25 % hexafluoroisopropanol in dichloromethane for 15 min. Cyclisation was performed under high dilution conditions in dichloromethane (0.15 mmol / 1 l) with 3 eq. of HOBt and 3 eq. PyBOP and 6 eq. of DIPEA, which were pre-mixed in DMF (1.5 ml) before addition. The reaction was continued for 12 hours. The Boc-protection groups from the ornithine side chains were removed by treatment with TFA:TIS:water 92.5:5:2.5 cocktail for > 15 min. The crude matters were then purified on the semi-preparative RP-HPLC C18 column (10 x 250 mm) with the water-acetonitrile gradient elution. 5 mM hydrogen chloride concentration in the eluent was used as an ion-paring agent. Analytical RP-HPLC was done as described[26] on a biphenyl analytical column (4.6 x 250 mm).

1TfmPro-GS: ^{19}F-NMR (1:1, vol/vol, acetonitrile/water), 282 MHz), δ, ppm: -70.5 (d, J_{F-H} = 10 Hz, CF_3). Mass-spectrum (MALDI-TOF), m/z, found/calcd.: 1211.2/1209.5.

2TfmPro-GS: ^{19}F-NMR (1:1, vol/vol, acetonitrile/water), 282 MHz), δ, ppm: -70.5 (d, J_{F-H} = 9 Hz, CF_3). Mass-spectrum (MALDI-TOF), m/z, found/calcd.: 1278.3 /1277.5.

Solution NMR of peptides

The spectra were recorded on Bruker Avance III 700 spectrometer (^1H 700.2 MHz; ^{15}N 71.0 MHz) equipped with a TXI probe at 25 °C. The phosphate buffer was of pH 6.0 (22 °C) and 15 mM concentration containing 10 vol % D_2O for deuterium lock. The spectra in DMSO-d6 were

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referenced using deuterium lock signal, while the spectra in PB-TFE were referenced using TPS internal standard. Assignment of the proton spectra was done using ^{1}H TOCSY experiments (dipsi2 spin lock of 60 ms). The ^{1}H^{15}N single bond correlations were detected in the sofast-HMQC experiments with the recycling delay of 100 ms.

Found HN resonances (^{1}H/^{15}N). **GS** in DMSO-d6: 9.08/128.1 (^{D}Phe), 8.72/125.8 (Leu), 8.34/124.0 (Orn), 7.24/113.3 (Val); **2TfmPro-GS** in DMSO-d6: 9.12/128.0 (^{D}Phe), 8.70/125.8 (Leu), 8.32/123.4 (Orn), 7.34/110.5 (Val); **GS** in PB-TFE: 8.76/129.6 (Leu), 8.48/127.5 (^{D}Phe), 8.12/125.8 (Orn), 7.83/121.2 (Val); **2TfmPro-GS** in PB-TFE: 8.77/129.4 (Leu), 8.66/127.7 (^{D}Phe), 8.13/125.8 (Orn), 7.91/117.4 (Val).

CD spectra of the peptides

The spectra were measured on Jasco J-720 spectropolarimeter. The spectra were measured at 25 °C in the same buffer as was taken for NMR at 90 μM peptide concentrations.

Solid state NMR

The solid state ^{19}F-NMR spectra were recorded on Bruker Avance III 500 spectrometer (470.6 MHz) equipped with wide bore magnet and the home-built HF lowE flat-coil probe.

An oriented sample was prepared from 0.6 mg **2TfmPro-GS** and 11.7 mg of DLPC ((12:0/12:0)PC) at the peptide-to-lipid molar ratio of 1/40. Dry mixtures were co-dissolved in methanol, spread over 16 rectangular glass plates (18 x 7.5 mm; Marienfeld, Germany), were dried in vacuum (> 4 hours), stacked and hydrated at 96 % relative humidity (saturated potassium sulfate at 48°C) for 26 hours. A fresh sample was wrapped in Nescofilm® and Sarogold® films for prevention of drying. The proper orientation of the lipid bilayers was checked by ^{31}P-NMR to have at least 80% bilayer lipids being coplanar with the slide surface.

The ^{19}F-NMR spectra in oriented samples were measured using aring composite pulse sequence (for background suppression) with proton decoupling during acquisition. Accurate temperature series (in particular, 1 °C step in the range +5 ÷ -5 °C) was performed with 10 min temperature pre-equilibration time at each temperature. The temperature was calibrated using acidified methanol sample. The spectra were processed with Lorentzian window function (LB 250 Hz).

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