Amino Acids

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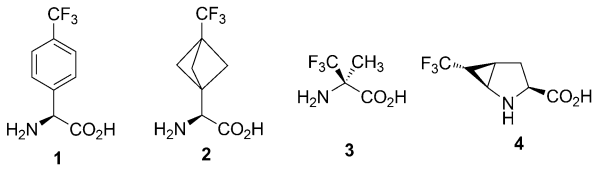
A ^{19}F NMR Label to Substitute Polar Amino Acids in Peptides: A CF_3-Substituted Analogue of Serine and Threonine**

Anton N. Tkachenko, Pavel K. Mykhailiuk, Sergii Afonin, Dmytro S. Radchenko, Vladimir S. Kubyshkin, Anne S. Ulrich,* and Igor V. Komarov**

Structural biology relies heavily on NMR studies of peptides and proteins that are uniformly or selectively labeled with NMR-active isotopes (^{2}H, ^{13}C, ^{15}N, ^{19}F). One of the most informative approaches to analyze membrane-active peptides in their natural membrane-bound form is solid-state ^{19}F NMR spectroscopy in oriented lipid bilayers.[1] The main advantages of this approach are the exquisite sensitivity of fluorine, the lack of any natural abundance background, the simplicity of the NMR experiment and data analysis, especially when CF_3-substituted amino acids are used as labels (CF_3 labels). Numerous applications have demonstrated utility of the designated CF_3 labels **1**–**4**,[2] incorporated into peptides in the place of a bulky hydrophobic amino acid (**1**,**2**), α-aminoisobutyric acid (**3**), or proline (**4**).[3] The ^{19}F NMR parameters from several selectively labeled analogues serve as orientational constraints to yield the backbone conformation, alignment, and dynamic behavior of the peptide in the lipid bilayer. The structure calculation relies entirely on the fact that the CF_3 group must be attached to the peptide backbone with a fixed and well-defined angle, as seen in **1**–**4**.



All the available CF_3 labels **1**–**4** share the common characteristic of having hydrophobic side chains. Therefore, they can only be used as NMR reporters in the place of similarly hydrophobic residues.[2,3] The substitution of any polar amino acid with one of the CF_3 labels **1**–**4** might disturb the conformation and function of a peptide; this is highly undesirable in structural studies.[1b,d,2d,e] The obvious lack of appropriate polar and/or charged CF_3 labels prompted us to develop a novel molecular framework for CF_3-substituted amino acids with functionalized side chains. Here, we report on the design and synthesis of a CF_3-substituted serine/threonine analogue, as well as on the validation of its use in peptide structural studies. To our knowledge, this is the first ^{19}F label for solid-state NMR analysis that possesses a polar side chain.

Serine (Ser) and threonine (Thr) polar uncharged residues are ubiquitous in peptides and proteins.[4] The hydroxy group in the side chain is often involved in very specific protein functions, for example, in posttranslational modifications (phosphorylation, glycosylation), or in catalytic sites of enzymes.[5] In transmembrane segments or in amphiphilic helices of membrane proteins, Ser/Thr-rich motifs are known to form polar binding platforms involved in the recognition of glycans,[6] other proteins,[7] or to be responsible for the sensing of membrane curvature.[8] The latter two functions are even realized in the Ser/Thr-rich segments of stand-alone membrane-active peptides.[7,8] By introducing a suitable CF_3 label to replace a single Ser/Thr residue, new possibilities would open up to study the structure and function of such peptides in membranes by using solid-state ^{19}F NMR spectroscopy. This is especially relevant in cases where the amphiphilic domains contain only a low number of nonpolar amino acids, which can make the entire segment inaccessible to the current labeling scheme based on the known CF_3 reporters **1**–**4**.

The design of a CF_3 label that is suitable to conservatively replace Ser/Thr residues in peptides, and in general, the design of any polar ^{19}F NMR label represents a considerable challenge.[1d,2d,e] The requirement for a CF_3 label not to perturb the structure and function of the peptides (*criterion 1*) is particularly difficult to fulfill. The steric bulk and strong electron-withdrawing character of fluorine-containing sub-

[*] A. N. Tkachenko, Dr. P. K. Mykhailiuk[+]
Organic Chemistry Department
Taras Shevchenko National University of Kyiv
Volodymyrska 64, 01601 Kyiv (Ukraine)
and
Enamine Ltd.
Matrosova 23, 01103 Kyiv (Ukraine)
Dr. S. Afonin,[+] Prof. A. S. Ulrich
Institute of Biological Interfaces (IBG-2)
Karlsruhe Institute of Technology (KIT)
POB 3640, 76021 Karlsruhe (Germany)
E-mail: sergiy.afonin@kit.edu
anne.ulrich@kit.edu
Homepage: http://www.ibg.kit.edu/nmr/
Dr. D. S. Radchenko, Prof. I. V. Komarov
Institute of High Technologies
Taras Shevchenko National University of Kyiv
Volodymyrska 60, 01601 Kyiv (Ukraine)
E-mail: ik214@yahoo.com
Homepage: http://418lab.chem.univ.kiev.ua
V. S. Kubyshkin, Prof. A. S. Ulrich
Institute of Organic Chemistry and CFN, KIT
Fritz-Haber-Weg 6, 76131 Karlsruhe (Germany)
[+] These authors contributed equally to this work.

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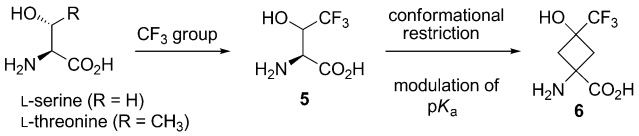
stituents[9] inevitably cause changes in the properties of the adjacent functional groups. The labeled amino acid must thus carry the CF_3 moiety sufficiently distant from its amino and carboxylic groups and from any functional groups in the side chain. In addition, the structural rigidity already mentioned above (*criterion* 2), as well as resistance to racemization and compatibility with standard peptide synthesis procedures (*criterion 3*) must also be implemented in the design. One of the possible logical routes towards designing the new CF_3-substituted Ser/Thr analogue that we report here is illustrated in Scheme 1.

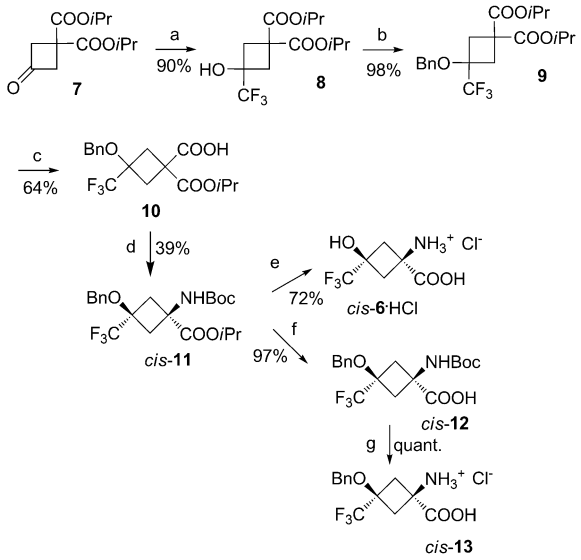


Scheme 1. Design of CF_3 label **6**, an analogue of serine or threonine.

To minimize any electronic and structural perturbations of the polypeptide, the bulky electron-withdrawing CF_3 reporter should be placed at the most distal position to the aminocarboxylate function, that is, at the C_β atom of the parent Ser/Thr to construct structure **5**. This compound, however, is still a poor CF_3 label. First, molecule **5** is conformationally flexible, which is undesirable for an NMR label (*criterion 2*). Second, the CF_3 group is expected to increase the acidity of the hydroxy function in **5** compared to that of Ser/Thr. Furthermore, the amino acid (2*S*,3*S*)-**5**[10] substituted for Thr in an enkephalin-derived hexapeptide had been shown to lead to a conformational alteration in the main chain of the peptide owing to the strong electron-withdrawing effect of the CF_3 group.[11]

A comparison of the reported pK_a values for the OH groups of methanol (15.9),[12] 2,2,2-trifluoroethanol (12.4),[13] and of Ser/Thr (ca. 13), suggests that the influence of the CF_3 group on the acidity of the side chain hydroxy group is almost equal to that of the aminocarboxylate moiety. Therefore, if the electron-withdrawing effect of the aminocarboxylate could be eliminated, then the acidity of the hydroxy group would be expected to remain comparable to that in Ser/Thr. To implement this idea in our design of the label, we decided to place a cyclobutane ring between the aminocarboxylic moiety and the OH group. This should also diminish further the electronic and steric influence of the CF_3 group on the aminocarboxylate moiety, and provide the necessary rigidity (*criterion 2*) to the CF_3 label **6** thus designed.

The synthesis of **6** started from the ketodicarboxylate **7**[14] (Scheme 2), which was transformed into the trifluoromethyl alcohol **8** by using the Ruppert–Prakash reagent[15,16] in a reasonable yield. As a convenient OH protecting group needed for further transformations we chose the benzyl group, considering its ease of introduction and orthogonality to the further planned *N*-Fmoc protection. The OBn derivative **9** was subjected to monohydrolysis followed by a Curtius rearrangement and *N*-Boc protection. This set of reactions led to a mixture of diastereomers, in which *cis*-**11** prevailed (*cis*/*trans* ca. 2:1); it was isolated in 39% yield after fractional



Scheme 2. Synthesis of OH-protected *cis*-**13**. a) CF_3SiMe_3, CsF, THF, 25 °C, 15 h; b) NaH, BnBr, DMF, 25 °C, 18 h; c) KOH, *i*PrOH, reflux, 3 h, then HCl; d) $(PhO)_2P(O)N_3$, Et_3N, toluene, 80 °C, 5 h, *t*BuOH, 80 °C, 15 h; e) 6 N HCl, reflux, 6 h; f) KOH, EtOH, reflux, 2 h; g) 2 N HCl in Et_2O. Boc = *tert*-butoxycarbonyl.

crystallization and hydrolyzed to *cis*-**12**. The molecular structure of *cis*-**11** was confirmed by X-ray analysis (Figure 1). Notably, all the protecting groups, including the

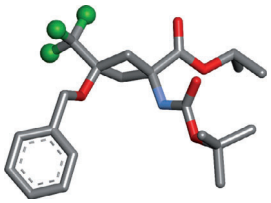
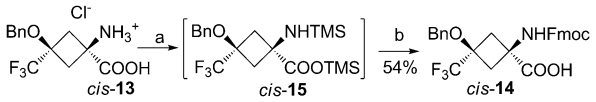


Figure 1. Molecular structure of *cis*-**11**.[20] For clarity, individual conformers are selected for the *i*Pr and benzyloxy substituents (see the Supporting Information for details). F green, O red, N blue.

benzyl group, were readily removable (yielding *cis*-**6**·HCl) under reflux of *cis*-**11** in 6 N HCl. Later on, we advantageously used this acid lability of the OBn protection in the final stages of solid-phase peptide synthesis (SPPS). Deprotection of *cis*-**12** under mild conditions gave the OBn derivative, *cis*-**13**.

Installment of the *N*-Fmoc protecting group onto *cis*-**13**, to prepare the building block for use in SPPS, required some experimentation. The standard methods,[17] which employ Fmoc-Cl or N-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu) under Schotten–Baumann conditions, gave very poor yields of the protected compound *cis*-**14**. The two-step approach,[18] without isolation of the putative bis-silylated intermediate *cis*-**15** resulted in acceptable 54% yield of *cis*-**14** (Scheme 3).

To evaluate the compatibility of *cis*-**14** with the SPPS (*criterion 3*), we first synthesized a model tripeptide Ac-Gly-(*cis*-**6**)-Gly-NH_2 (**16**). The OBn protecting group was removed by prolonged (ca. 7 days) treatment with the



Scheme 3. Synthesis of OBn-*N*-Fmoc-protected *cis*-**14**. a) $SiMe_3Cl$, DIEA, CH_2Cl_2, 0 °C, 30 min; b) Fmoc-Cl, 25 °C, 3 h. DIEA = diisopropylethylamine.

cleavage cocktail during the obligatory cleavage off the resin. The synthesis of **16** also served an additional purpose, namely, to provide a polypeptide framework for studying the acid–base properties of the *cis*-**6** side chain. The pH-dependent ionization was studied by liquid-state NMR spectroscopy. The ^{19}F NMR chemical shift of the CF_3 group was sensitive to the protonation state of the OH group, thus allowing us to determine the titration curve for **16**, giving a pK_a value of 11.7 ± 0.1 (23 °C). The value is close to the corresponding pK_a value in both Ser and Thr (ca. 13), thus justifying our design. Hence, from a chemical perspective amino acid *cis*-**6** could serve as a replacement of these natural amino acids. It resembles them not only by size, polar character of the side chain, and spatial position of the hydroxy group, but also by the acid–base properties of this functional group.

To validate the use of *cis*-**6** as a ^{19}F NMR label in structural studies, we chose the natural membrane-active antimicrobial peptide Temporin A (TA)[19] as a model compound. We synthesized the wild-type sequence (FLPLIGRVL**S**GIL-NH_2, TA-**17**-wt), and two CF_3-labeled analogues, by using either the novel *cis*-**6** (FLPLIGRVL-*cis*-**6**-GIL-NH_2, TA-**18**), or the known hydrophobic CF_3 label **2** (FLPLIGRVL-**2**-GIL-NH_2, TA-**19**). In each case the native Ser at position 10 was replaced. In accordance with our published approach,[1] the peptides were compared in terms of their functional activity, structure, and behavior in the solid-state ^{19}F NMR experiment.

In a standard antimicrobial assay (two-fold dilution series to obtain the minimal inhibitory concentration, MIC), all three peptides were active against Gram-positive *S. aureus* and showed low activity against Gram-negative *E. coli*, so the modified peptides TA-**18** and TA-**19** displayed the same selectivity as the wild-type TA. The MIC values, however, varied significantly depending on the mutation at the Ser position. While for both TA-**17**-wt and TA-**18** the MIC values were comparable ($8\ \mu g\,mL^{-1}$), in the case of TA-**19** the activity decreased to a MIC of $32\ \mu g\,mL^{-1}$. Furthermore, the circular dichroism spectra (Figure 2A) showed that both TA-**17**-wt and TA-**18** adopt a random coil conformation in aqueous solutions but fold as a helix in membrane-mimetic environments. Peptide TA-**19**, in contrast, was helical only in the presence of TFE but not SDS, and exhibited a significant tendency to aggregate in aqueous buffer and in the presence of detergent micelles. These data demonstrate the superiority of *cis*-**6** over **2** as a CF_3 label for Ser.

Both the labeled peptides, TA-**18** and TA-**19**, were readily reconstituted in oriented lipid bilayers despite the solubility differences, and remained nonaggregated and uniformly aligned. The bilayer integrity was preserved in both samples and did not change with time (as judged from ^{31}P NMR spectra, data not shown). Under these conditions, TA-**18** and TA-**19** showed a synchronous concentration-dependent change of the ^{19}F NMR signal (Figure 2B), with a threshold at approximately 2 mol % peptide. This change in splitting is indicative of a realignment of the helical peptide in the lipid membrane, as previously observed for several other peptides as well.[1,3b,d–h] Remarkably, the dipolar splitting of the CF_3 group in the peptide containing **2** was large when the *cis*-**6**-labeled peptide showed a minimal value (ca. 0 kHz), and vice versa. Thus, the CF_3 groups of the two labels appear to be aligned orthogonally with respect to each other. This relationship is useful, as one could obtain two independent NMR constrains from the same site of the peptide (as in reference [3h]), provided that it can be nonperturbingly substituted with **2** and *cis*-**6**. The full structural analysis of the membrane-bound Temporin A peptide will require several such NMR constraints[1] and is currently ongoing.

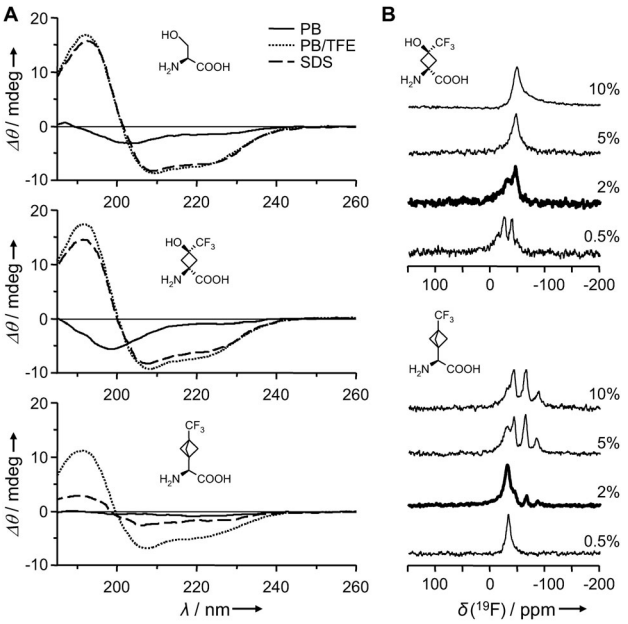


Figure 2. Evaluation of *cis*-**6** as a new, polar CF_3 label. A) Circular dichroism analysis (0.05 $mg\,mL^{-1}$ peptide concentration, 25 °C) of TA-**17**-wt (top), TA-**18** (middle), and TA-**19** (bottom). B) Solid-state ^{19}F NMR spectra of TA-**18** (top) and TA-**19** (bottom) in oriented dimyristoyl phosphatidylcholine bilayers, with varying peptide content (in mol %). PB = phosphate buffer, TFE = 2,2,2-trifluoroethanol, SDS = sodium dodecyl sulfate.

In conclusion, we have designed, synthesized, and confirmed the compatibility of *cis*-1-amino-3-hydroxy-(trifluoromethyl)cyclobutanecarboxylic acid (*cis*-**6**) as the first polar CF_3 label for solid-state ^{19}F NMR structure analysis of membrane-active peptides.

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[20] CCDC-890358 (**1**) 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

