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Trifluoromethylated Proline Surrogates as Part of "Pro–Pro" Turn-Inducing Templates

Charlène Gadais[+],[a] Kevin Van holsbeeck[+],[a, b] Samuel L. C. Moors,[c] Dieter Buyst,[b] Krisztina Fehér,[b] Kristof Van Hecke,[d] Dirk Tourwé,[a] Thierry Brigaud,[e] Charlotte Martin,[a] Frank De Proft,[c] Julien Pytkowicz,[e] José C. Martins,[b] Grégory Chaume,*[e] and Steven Ballet*[a]

Proline is often found as a turn inducer in peptide or protein domains. Exploitation of its restricted conformational freedom led to the development of the D-Pro-L-Pro (corresponding to (*R*)-Pro–(*S*)-Pro) segment as a "templating" unit, frequently used in the design of β-hairpin peptidomimetics, in which conformational stability is, however, inherently linked to the *cis–trans* isomerization of the prolyl amide bonds. In this context, the stereoelectronic properties of the CF_3 group can aid in conformational control. Herein, the impact of α-trifluoromethylated proline analogues is examined for the design of enhanced β-turn inducers. A theoretical conformational study permitted the dipeptide (*R*)-Pro–(*R*)-TfmOxa (TfmOxa: 2-trifluoromethyloxazolidine-2-carboxylic acid) to be selected as a template with an increased *trans–cis* rotational energy barrier. NMR spectroscopic analysis of the Ac-(*R*)-Pro–(*R*)-TfmOxa–(*S*)-Val-O*t*Bu β-turn model, obtained through an original synthetic pathway, validated the prevalence of a major *trans–trans* conformer and indicated the presence of an internal hydrogen bond. Altogether, it was shown that the (*R*)-Pro–(*R*)-TfmOxa template fulfilled all crucial β-turn-inducer criteria.

Among the tools developed to decipher biomolecular recognition, protein epitope mimetics (PEMs)[1] aim for improved efficiency, selectivity and specificity, thereby relying on a close conformational mimicry of the exposed secondary structure, including the β-hairpin.[2–4] Chemical strategies were therefore developed to "force" peptide analogues of protein epitopes into the desired hairpin structure. Macrocyclization appeared rapidly as a powerful strategy through the successful mimicry of β-hairpins,[5] which was achieved by grafting epitope sequences onto a semirigid turn-inducing template.[6]

Various pseudopeptidic motifs were reported, such as morpholines **I**[7] and diketopiperazines **II** (Figure 1),[8] but pioneering work in the field by the group of Robinson undoubtedly highlighted the use of heterochiral (*R*)-Pro–(*S*)-Pro dipeptide **III** as one of the most convenient turn inducers.[9,10] Of course, proline is known to give way to amide bond *cis–trans* isomerization,[11] as illustrated by NMR spectroscopy analysis of multiple conformational states involving the (*R*)-Pro–(*S*)-Pro motif,[12,13] and a reduced conformational stability of designed β-hairpin mimetics can be observed. This generated growing interest for the development of tools to allow better modulation of the conformation of proline and control over the isomerization of prolyl amide bonds.[14]

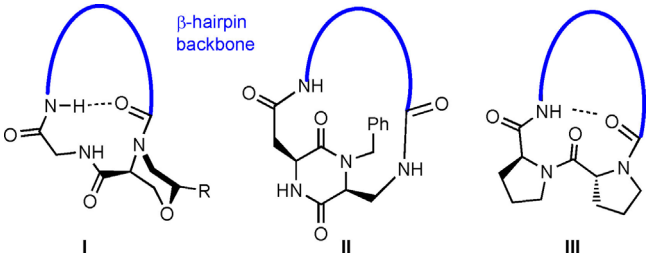

Figure 1. Examples of reported β-turn-inducing templates.

The emergence of fluorine in drug design highlighted the potential of fluorinated amino acids, peptides and mimetics,[15–17] and the possibility to tailor their geometry with such a peculiar element.[18,19] In particular, proline surrogates, which

[a] *Dr. C. Gadais,[+] K. Van holsbeeck,[+] Prof. Em. D. Tourwé, Dr. C. Martin, Prof. S. Ballet*
Research Group of Organic Chemistry
Departments of Bioengineering Sciences and Chemistry, Vrije Universiteit Brussel
Pleinlaan 2, 1050 Brussels (Belgium)
E-mail: steven.ballet@vub.be

[b] *K. Van holsbeeck,[+] Dr. D. Buyst, Dr. K. Fehér, Prof. J. C. Martins*
NMR and Structure Analysis Unit
Department of Organic and Macromolecular Chemistry, Ghent University
Krijgslaan 281, 9000 Gent (Belgium)

[c] *Dr. S. L. C. Moors, Prof. F. De Proft*
Eenheid Algemene Chemie (ALGC)
Department of Chemistry, Vrije Universiteit Brussel
Pleinlaan 2, 1050 Brussels (Belgium)

[d] *Prof. K. Van Hecke*
XStruct Bio-Inorganic Group, Department of Chemistry, Ghent University
Krijgslaan 281, 9000 Gent (Belgium)

[e] *Prof. T. Brigaud, Prof. J. Pytkowicz, Dr. G. Chaume*
Laboratoire de Chimie Biologique, Université de Cergy–Pontoise
5 Mail Gay-Lussac, 95031 Cergy-Pontoise cedex (France)
E-mail: gregory.chaume@u-cergy.fr

[+] *The authors contributed equally to this work.*

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were fluorinated or trifluoromethylated in their lateral chain, were examined as useful conformational tuning tools that allowed fine control of the *cis*- or *trans*-prolyl amide bond population.[20–22] Previous studies on model peptides have established the stereoelectronic effects imparted by the CF_3 group on the rotational energy barrier and puckering of the oxazolidine core.[23,24] Fluorination, especially in the α position, was demonstrated to provide additional *trans*-amide bond stabilization by combining increased steric hindrance and an extra contribution to the intercarbonyl alignment. This *trans*-amide bond preference constitutes a valuable feature for the design of β-turn inducers.

Herein, we demonstrate that α-trifluoromethylated proline analogues constitute a highly attractive alternative to traditional prolines in the (*R*)-Pro–(*S*)-Pro template as β-turn-inducers with improved conformational stability.

To identify the most promising CF_3 proline surrogate, an in silico conformational study was first performed on a set of 11 unnatural variants of the diproline template, including α-CF_3-proline (TfmPro), δ-CF_3 pseudoproline ($\psi^{CF_3,H}$Pro) and 2-trifluoromethyloxazolidine-2-carboxylic acid (TfmOxa), which constitutes an attractive alternative to TfmPro (Figure 2).[25] These di-

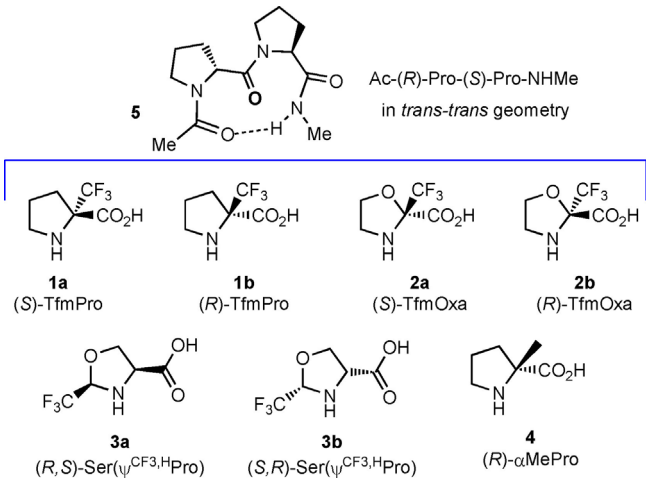


Figure 2. Top: Ac-(*R*)-Pro–(*S*)-Pro-NHMe (**5**) as a model for β-turn formation. Bottom: Set of CF_3 prolines surrogates, including methylated reference residue **4**.

peptide templates were capped with acetyl (Ac) and *N*-methylamide (NHMe) end groups to provide a minimal β-turn model, Ac-XX_1-XX_2-NHMe, with a potential hydrogen-bond acceptor and donor in residues *i* and *i*+3, respectively. For each peptide, all rotameric combinations were generated with the OpenBabel software, by using the MMFF94 force field, and followed by geometry optimization of the lowest energy *trans* and *cis* conformers (see the Supporting Information).

Of the investigated combinations, Ac-(*S*)-TfmPro-(*S*)-Pro-NHMe (**6**), Ac-(*R*)-Pro–(*S*)-TfmPro-NHMe (**14**) and Ac-(*R*)-Pro–(*R*)-TfmOxa-NHMe (**15**) exhibited a higher preference for the *trans–trans* geometry, relative to the "parent" diproline **5** (Figure 3 and Table 1). The rotational energy barrier between the *tt* and *tc* conformers was indeed increased by 3.1, 8.2 and

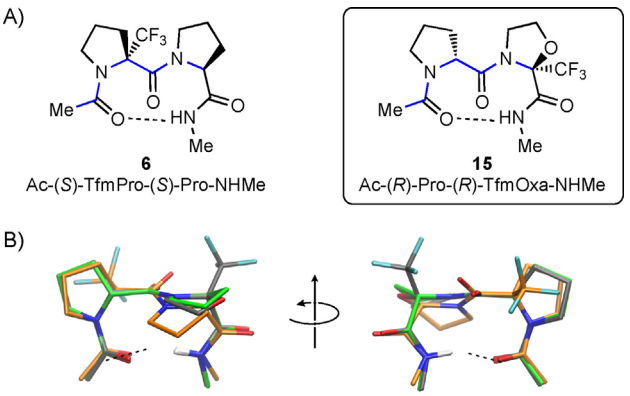


Figure 3. A) CF_3-bearing β-turn inducers **6** and **15** in *trans–trans* (*tt*) geometry (blue). B) Overlay of **5** (green), **6** (orange) and **15** (grey) in front (left) and back orientations (right).

Table 1. Energies relative to the lowest energy *trans–trans* (*tt*) conformer of the investigated Ac-XX_1-XX_2-NHMe analogues [kcal mol^{-1}].[a]

	Ac-**XX_1**-**XX_2**-NHMe	*tt*	*tc*	*ct*	*cc*	H-bond[b]
5	-(*R*)-Pro–(*S*)-Pro-	0	3.6	2.2	7.0	0.3
6	-(*S*)-TfmPro–(*S*)-Pro-	0	6.7	3.8	12.2	0
7	-(*R*)-TfmPro–(*S*)-Pro-	0	1.5	3.9	10.3	3.2
8	-(*S*)-TfmPro–(*S*)-TfmPro-	0	-	5.3	–	0
9	-(*R,S*)-Ser($\psi^{CF_3,H}$Pro)–(*S*)-Pro-	0	2.5	−0.6	3.7	2.5
10	-(*S,R*)-Ser($\psi^{CF_3,H}$Pro)–(*S*)-Pro-	0	12.3	−11.0	−6.5	8.4
11	-(*R*)-TfmOxa–(*S*)-Pro-	0	5.5	4.5	10.5	1.4
12	-(*R*)-α-MePro–(*S*)-Pro-	0	4.3	2.8	8.2	0.6
13	-(*S*)-Pro–(*S*)-TfmPro-	0	3.4	2.8	11.5	3.4
14	-(*R*)-Pro–(*S*)-TfmPro-	0	11.8	3.6	23.4	0
15	-(*R*)-Pro–(*R*)-TfmOxa-	0	8.2	5.6	15.2	0
16	-(*R*)-Pro–(*S*)-TfmOxa-	0	2.4	3.0	8.7	2.3

[a] *t*: *trans*, *c*: *cis*. [b] H-bond stands for the lowest energy conformer with a hydrogen bond between acetyl-O and *N*-methyl-NH (< 2.5 Å).

4.6 kcal mol^{-1}, respectively, relative to **5**, and these motifs still maintained the favourable intramolecular hydrogen bond (the energy of the lowest *tt* conformer featuring a H-bond = 0 kcal mol^{-1}; Table 1). Remarkably, these motifs outperform those of template **12**, bearing (*R*)-αMePro (**4**), supporting a superior *trans*-stabilizing effect of the α-CF_3 group compared with that of α-Me. Notably, the larger steric hindrance imposed by the CF_3 moiety (possessing a van der Waals volume between those of ethyl and isopropyl groups) is anticipated to be the main driving force behind the observed conformational bias. The lowest energy conformations of **6** and **15** are visualized in Figure 3 (overlaid with **5** for comparison). Interestingly, unlike **15**, diastereoisomer **16** bearing (*S*)-TfmOxa (**2a**) displayed energetic values close to those of **5**. It can be assumed that steric hindrance created by the CF_3 group in the *S* configuration does not promote the *trans–trans* conformation and disrupts the stabilizing hydrogen bond. A similar observation can also be made for (*S*)-TfmPro (**1a**) and its *R* enantiomer **1b** in the corresponding models **6** and **7**. Finally, it can be noted that pseudoproline-bearing templates **9** and **10** both display negative energetic values for their *ct* and *cc* (**10** only) conformers;

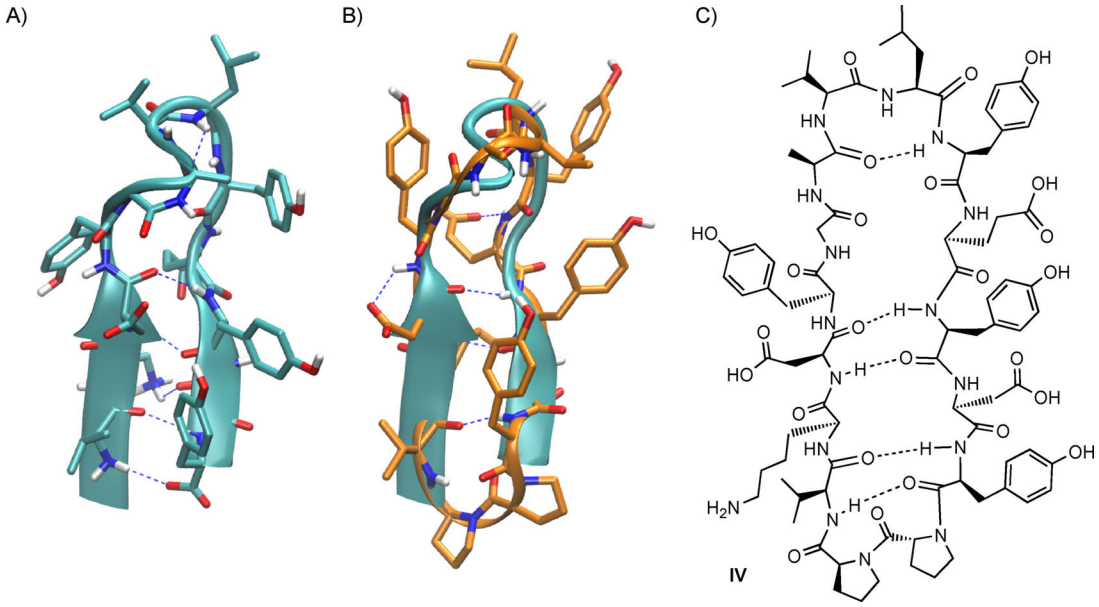


Figure 4. A) Structure of the Nb80 CDR3 domain. B) Overlay of the best fitting molecular dynamics (MD) conformation (Cα root-mean-square deviation (RMSD) = 1.4 Å) of mimetic **IV** (orange) and the secondary structure of the CDR3 loop region in Nb80. C) Detailed structure of **IV**.[26]

these data support a preference for the *cis* conformation, as previously reported.[24]

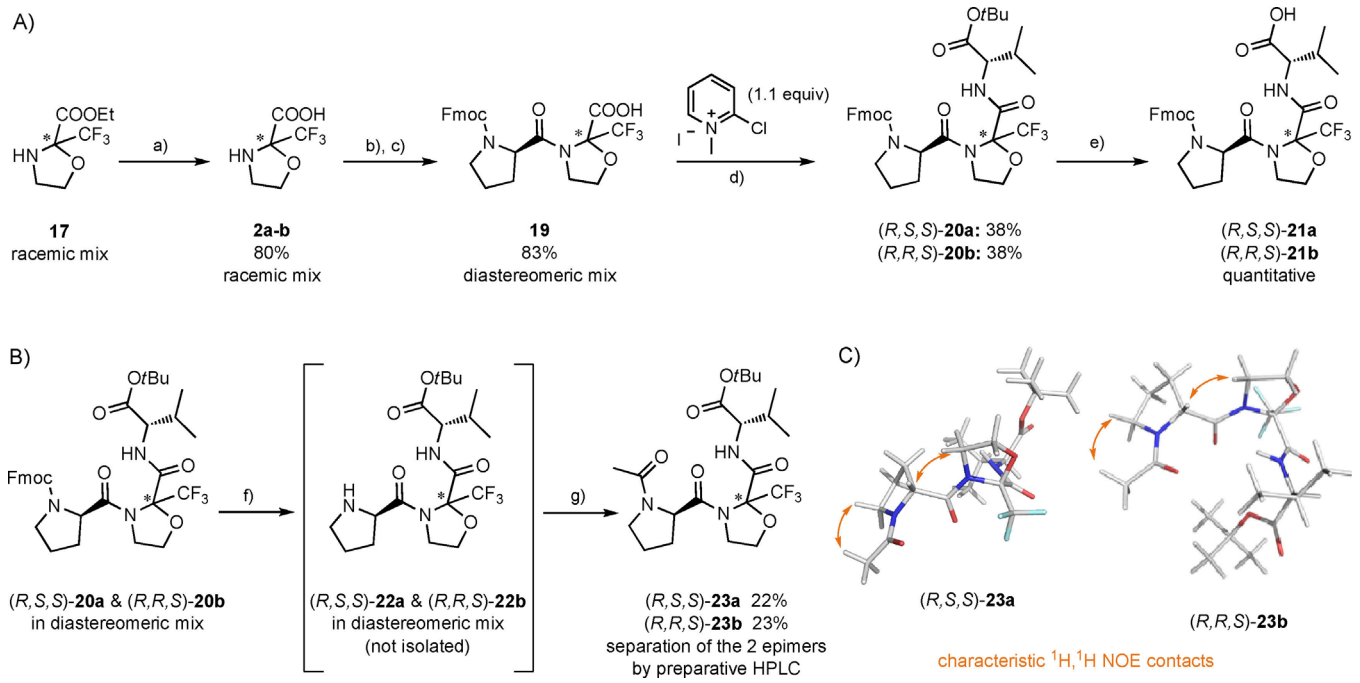
Considering the general synthetic accessibility of building block **2a**,**b**, which is synthesized as a racemic mixture in two steps,[25] compound **15** was selected over **6** and **14** for incorporation into tripeptide sequences PG-(*R*)-Pro–(*R*)-TfmOxa–(*S*)-Val-OH, in which PG is an acetyl group for conformational studies and a fluorenylmethyloxycarbonyl (Fmoc) group for subsequent solid-phase peptide synthesis (SPPS) incorporation. Valine was selected as the TfmOxa-flanking residue because it directly correlated with the CDR3 sequence of previously reported nanobody Nb80 mimetic peptide **IV** (Figure 4).[26] This mimetic was designed by grafting the CDR3 loop on a (*R*)-Pro–(*S*)-Pro template. Disappointingly, this cyclic peptide displayed an extensive conformational heterogeneity, as determined by NMR spectroscopy (see the Supporting Information), and it was therefore considered as an adequate model inclusion of the (*R*)-Pro–(*R*)-TfmOxa motif, as an improved version of the (*R*)-Pro–(*S*)-Pro templating unit.

The N-terminal coupling to α-trifluoromethylated amino acids is a challenging exercise because the CF_3 group strongly deactivates the nucleophilicity of the amino group, which prevents easy couplings and the general use of automated SPPS. However, activation by acyl chloride or mixed anhydride formation proved to be highly efficient.[27] Access to the tripeptides Fmoc-(*R*)-Pro–(*R*/*S*)-TfmOxa–(*S*)-Val-OH (**21a**,**b**) was hence developed in solution by means of a four-step synthesis (Scheme 1 A). Prepared as a racemic mixture,[25] and after saponification of **17**, H-TfmOxa-OH (**2**) was coupled to readily available Fmoc-(*R*)-Pro-Cl **18**. After hydrolysis, unreacted Fmoc-(*R*)-Pro-OH was esterified directly in situ, whereas **2a**,**b** remained untouched due to the low reactivity of their carboxylic acid functionality. In the absence of such a workup, remaining Fmoc-(*R*)-Pro-OH systematically coeluted with dipeptides **19**, which reduced the yield drastically, while increasing the amount of deletion peptide in subsequent steps.

As previously described, C-terminal coupling of the dipeptide Fmoc-Gly-TfmOxa-OH onto glutamic acid was successfully achieved by using bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP-Cl).[25] Surprisingly, the use of this reagent for the activation of **19a**,**b** in the synthesis of **21a**,**b** did not give any desired conversion. Similarly, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), or isobutyl chloroformate (IBCF) led to the same observation. A screening of coupling reagents was thus extended to less conventional agents. The Mukaiyama reagent was efficiently employed in the activation of hindered carboxylic acids and amines,[28,29] and revealed to be particularly efficient in the C-terminal coupling of **2**, and hence, compounds **20a**,**b** could be accessed through the smooth coupling between **19** and HCl·H-Val-O*t*Bu. The two diastereomeric tripeptides **20a**,**b** were separated and isolated in good yield (38%), after which they were treated with TFA to quantitatively give the desired tripeptides (*R*,*S*,*S*)-**21a** and (*R*,*R*,*S*)-**21b**, ready for SPPS assembly.

In view of subsequent conformational analysis, access to the acetylated analogues **23a**,**b** was then elaborated by Fmoc removal of the readily synthesized tripeptides **20** as a diastereomeric mixture or as separated compounds, by using DBU/1-octanethiol as deprotecting reagents.[30] The resulting deprotected tripeptides **22a**,**b** were triturated in diethyl ether and directly treated with acetyl chloride, without further purification steps, to finally isolate the two epimers **23a**,**b** after preparative HPLC (Scheme 1 B).

In the resulting tripeptides, the stereochemical assignment of the TfmOxa α-carbon was achieved by means of crystallization and X-ray analysis of epimer (*R*,*S*,*S*)-**20a** (see the Supporting Information), which existed in the solid form at room tem-



Scheme 1. A) Synthetic pathway developed to access Fmoc-protected tripeptides **21 a,b**. B) Synthetic pathway developed to access acetylated tripeptides **23 a,b**. C) Lowest energy *tt* conformers of **23 a,b** and their characteristic ^{1}H,^{1}H NOE contacts. a) LiOH aq. (1.6 M, 1.1 equiv), THF/H_2O (72:28), 0 °C, 1.5 h; b) Fmoc-(*R*)-Pro-Cl **18** (1.2 equiv), NEt_3 (1.0 equiv), dry DMF, [Ar], RT, 2 h; c) MeOH, H_2SO_4 (cat.), RT, overnight; d) HCl·Val-O*t*Bu (1.1 equiv), *N*,*N*-diisopropylethylamine (DIPEA; 3.2 equiv), dry CH_2Cl_2, RT, 1 h; e) CH_2Cl_2/trifluoroacetic acid (TFA)/triisopropylsilane (TIS; 5:4.8:0.2), RT, 1.5 h; f) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 1 equiv), 1-octanethiol (10 equiv), THF, Ar, RT, 1 h; g) AcCl, (10 equiv), CH_2Cl_2, Ar, RT, 40 min.

perature, whereas (*R*,*R*,*S*)-**20 b** displayed a highly viscous oily state. Having identified the configuration of these two compounds, we established the stereochemistry of **21 a,b** and **23 a,b** by conducting the depicted synthetic steps on separated diastereoisomers (*R*,*S*,*S*)-**20 a** and (*R*,*R*,*S*)-**20 b**. To enable a comparison with the (*R*)-Pro–(*S*)-Pro model, the reference peptide Ac-(*R*)-Pro–(*S*)-Pro–(*S*)-Val-O*t*Bu (**24**) was also prepared by following a similar synthetic pathway (see the Supporting Information).

The conformational properties of tripeptides **24**, (*R*,*S*,*S*)-**23 a** and (*R*,*R*,*S*)-**23 b** were investigated by means of NMR spectroscopy, assisted by molecular modelling. Whereas in the 1D ^{1}H spectrum recorded in $CDCl_3$ and [D_6]DMSO reference peptide **24** shows three and four sets of resonances, respectively, compound **23 a,b** only shows the presence of two sets of resonances. Based on 2D ^{1}H,^{1}H NOESY experiments, the different conformers could be identified by NOE interactions between either CH_3 Ac-H_δ (*R*)-Pro (*t*) or CH_3 Ac-H_α (*R*)-Pro (*c*) and H_α (*R*)-Pro-H_δ TfmOxa/Pro (*t*) or H_α (*R*)-Pro-H_α TfmOxa/Pro (*c*; see the Supporting Information). In addition, all conformations were found to be in slow exchange with each other, as evidenced by exchange cross peaks. From the corresponding populations, it is apparent that only the *tt* and *ct* conformers are observed for **23 a,b** (Table 2).

Switching from relatively nonpolar $CDCl_3$ to the strong hydrogen-bond acceptor [D_6]DMSO showed a strong destabilization of the *tt* conformer of **24**, in contrast to **23 a,b**, which still populated the *tt* conformer in 70 and 71 %, respectively, in the latter solvent. In addition, upon going from $CDCl_3$ to [D_6]DMSO, the amide NH chemical shift is only moderately influenced for the *tt* conformers of **24** and (*R*,*R*,*S*)-**23 b**, which supports the solvent-shielding of their amide protons, and thereby the possibility of forming an intramolecular hydrogen bond. This solvent-shielding effect was also observed for the *tt* conformer of (*R*,*R*,*S*)-**23 b** upon performing a titration of [D_6]DMSO to $CDCl_3$, which showed only a small downfield shift (0.03 ppm; see the Supporting Information).[7] The ability of the *tt* conformers of **24** and (*R*,*R*,*S*)-**23 b** to form a hydrogen bond is further supported by their temperature coefficients, as determined in [D_6]DMSO, which show a significantly higher value than those of the other conformers.[23] Altogether these data show that (*R*,*R*,*S*)-**23 b** only populates a *trans*-amide bond

Table 2. Population of different conformers of **24** and **23 a,b** in $CDCl_3$ and [D_6]DMSO, and the solvent and temperature dependence of the amide protons.

		Population [%]		$\Delta\delta_{NH}$[a]	$\Delta\delta_{NH}/\Delta T$[a]
		$CDCl_3$	[D_6]DMSO	relative to $CDCl_3$	[ppb K^{-1}]
24	*tt*	67	26	0.20	−4.2
	ct	7	33	1.33	−6.9
	tc	26	34	1.88	−6.7
	cc	/	7	/	−5.9
(*R*,*S*,*S*)-**23 a**	*tt*	90	70	0.75	−12.5
	ct	10	30	0.94	−13.2
(*R*,*R*,*S*)-**23 b**	*tt*	96	71	0.23	−4.3
	ct	4	29	1.21	−14.1

[a] In [D_6]DMSO.

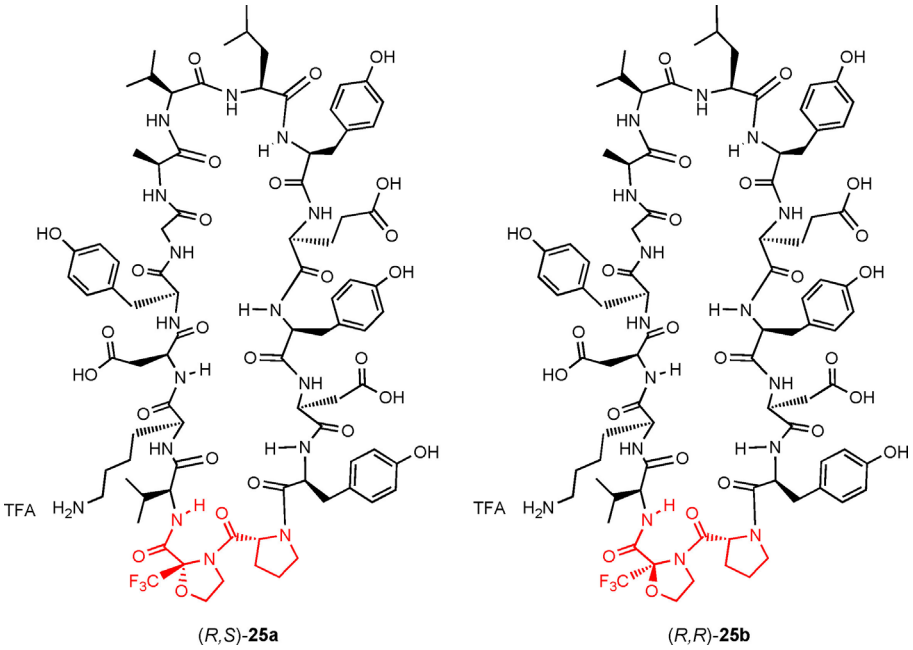


Figure 5. Chemical structures of (*R*,*S*)-**25 a** and (*R*,*R*)-**25 b** CDR3 Nb80 cyclic mimetics incorporating TfmOxa in the turn region.

between (*R*)-Pro and (*R*)-TfmOxa, with its major *tt* conformer having the possibility of forming a hydrogen bond, and thereby showing a stronger propensity to adopt a β-turn relative to **24**.

In view of the results observed with the above tripeptides, the Fmoc-protected tripeptide building blocks (*R*,*S*,*S*)-**21 a** and (*R*,*R*,*S*)-**21 b** were incorporated by SPPS to replace the (*R*)-Pro–(*S*)-Pro template in the Nb80 CDR3 mimetic **IV** (see the Supporting Information). The two resulting cyclic peptides, (*R*,*S*)-**25 a** and (*R*,*R*)-**25 b**, were subsequently analysed by means of NMR spectroscopy to investigate the impact of TfmOxa on their conformational behaviour (Figure 5).

An initial screening of both cyclic peptides (*R*,*S*)-**25 a** and (*R*,*R*)-**25 b,** in 80/20 H_2O/CD_3CN revealed a single set of amide signals, and subsequent full spectral assignment of ^{1}H, ^{13}C, ^{15}N and ^{19}F resonances confirmed this (see the Supporting Information). Special attention was then given to the assignment of NOE contacts not used in the $C\alpha H_i$–NH_{i+1} sequential walk. Although a few additional through-space contacts could be identified, these were mostly limited to the nearest neighbours of the proton signals in question, while there was a general lack of the characteristic long-range backbone ^{1}H–^{1}H distances present in β-sheets.[31] In addition, the chemical shifts of the samples in question were submitted to the CSI 3.0 web server[32] as a second source of information for the presence or absence of any secondary structure. In this specific analysis, the chemical shift data of the TfmOxa residue was not included because this is a custom amino acid and, hence, not recognized by the web server. Its outcome suggested the absence of a defined and stable conformation of the peptide as a whole. This remained true upon using chemical shifts obtained from a 70/30 solvent mixture of H_2O/2,2,2-trifluoroethanol-d_3 ([D_3]TFE), which was intended to mimic conditions more similar to those of the hydrophobic binding pocket of the Nb80 CDR3 domain. In this case, only a random coil or small α-helical fragment could be found (see the Supporting Information). The latter is believed to be an anomaly due to sparser chemical shift information for that specific sample in TFE because it could not be confirmed by any other spectral information.

The beneficial contribution of the (*R*)-Pro–(*R*)-TfmOxa template was eventually assessed by means of a comparative NMR spectroscopic analysis of the two Nb80 CDR3 cyclic mimetics: the (*R*,*R*)-**25 b** peptide, including the fluorinated (*R*)-Pro–(*R*)-TfmOxa motif, and cyclic peptide **IV** (Figure 4C). As observed from the spectral comparison (see the Supporting Information), the spectra of (*R*,*R*)-**25 b** showed little to no minor signals and the peptide signals themselves were clearly defined with completely resolved multiplicity. This is in contrast to peptide **IV**, which showed the clear presence of other (minor) conformations in addition to the main signals. Furthermore, the signals of **IV** show line broadening, which indicates that there is at least one conformational exchange process active for this specific sequence. The presence of these multiple conformations effectively prohibits the assignment of the peptide sequence by using a classical set of 2D spectra. This is clearly not the case for (*R*,*R*)-**25 a**,**b**, for which a dominant conformation is present and can be assigned without any problem. Hence, TfmOxa-modified building blocks enable the goal of reducing the conformational *ct* heterogeneity, previously observed in the (*R*)-Pro–(*S*)-Pro congeners, to a single well-defined *tt* conformation.

Conclusion

Herein, α-trifluoromethylated proline surrogates were considered as part of potent β-turn inducers with enhanced confor-

mational stabilizing features. A computational study showed that TfmPro **1** and TfmOxa **2** constituted highly promising proline alternatives. In particular, the (*R*)-Pro–(*R*)-TfmOxa derivative exhibited a remarkably improved rotational energy barrier compared to the (*R*)-Pro–(*S*)-Pro template, and was therefore selected for further experimental studies. Starting from racemic TfmOxa **2**, original two- to four-step syntheses, involving an unprecedented use of the Mukaiyama reagent in the coupling of α-trifluoromethylated amino acids, were developed to access the desired Fmoc-(*R*)-Pro–(*R*)-TfmOxa–(*S*)-Val-OH tripeptide (*R*,*R*,*S*)-**21 b**. This tripeptide was readily employed for SPPS incorporation into the Nb80 CDR3 β-hairpin cyclic mimetic sequence, whereas Ac-(*R*)-Pro–(*R*)-TfmOxa–(*S*)-Val-OH (*R*,*R*,*S*)-**23 b** was used for an in-depth conformational analysis by means of NMR spectroscopy. Both the desired *tt* configuration at the prolyl amide bonds and an intramolecular acetyl–O and NH–valine hydrogen bond, characteristic of a β-turn motif, were clearly established, thereby strongly supporting the added value of the proline surrogate TfmOxa **2** in these β-turn motifs.

Experimental Section

Synthetic procedures, spectral data and NMR spectroscopy analysis are detailed in the Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: backbone constraints · beta-turn inducers · isomerization · NMR spectroscopy · peptidomimetics

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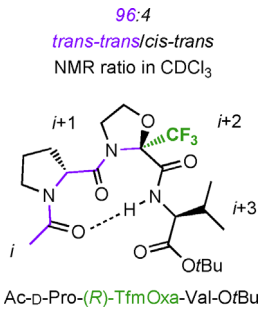
Stabilizing twists and turns: The amino acid 2-trifluoromethyloxazolidine-2-carboxylic acid (TfmOxa) induces an enhanced conformational stability in diproline turn-inducing templates. An in silico and NMR spectroscopy based conformational study has demonstrated a strong prevalence for a *trans–trans* geometry and typical $i,i+3$ hydrogen bond requirement in peptidic β-turn structures.



C. Gadais, K. Van holsbeeck, S. L. C. Moors, D. Buyst, K. Fehér, K. Van Hecke, D. Tourwé, T. Brigaud, C. Martin, F. De Proft, J. Pytkowicz, J. C. Martins, G. Chaume, S. Ballet**

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Trifluoromethylated Proline Surrogates as Part of "Pro–Pro" Turn-Inducing Templates

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