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A temperature-driven, reversible helical handedness inversion in peptaibol analogs tuned by the C-terminal capping moiety

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Abstract:	Trichogin is a natural peptide endowed with antimicrobial and antitumor activity. It belongs to the peptaibol family, characterized by the presence of a C-terminal aminoalcohol. In the past, we substituted that moiety with a methyl ester for synthetic purposes and realized that this apparently slight modification causes great changes in the peptide bioactivity. Aiming at understanding the reasons behind such observation,

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A temperature-driven, reversible helical handedness inversion in peptaibol analogs tuned by the C-terminal capping moiety

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Abstract: Trichogin is a natural peptide endowed with antimicrobial and antitumor activity. It belongs to the peptaibol family, characterized by the presence of a C-terminal aminoalcohol. In the past, we substituted that moiety with a methyl ester for synthetic purposes and realized that this apparently slight modification causes great changes in the peptide bioactivity. Aiming at understanding the reasons behind such observation, we performed a combined spectroscopic study on a number of trichogin analogs. By comparing the data obtained from synchrotron radiation circular dichroism, NMR and fluorescence in organic solvents at cryogenic temperatures with those independently acquired by electron paramagnetic resonance at 80K we were able to reveal and unambiguously identify a clear, reversible, temperature-driven helix screw-sense interconversion from right-handed to left-handed unleashed from the C-terminal capping moiety. Our data demonstrate for the first time the key role of a C-terminal methyl ester in promoting peptide screwsense inversion.

Introduction

Peptaibols are naturally-occurring, membrane-active, antimicrobial peptides with a remarkable resistance to proteolysis.[1] Their sequences feature several C^{α}-tetrasubstituted residues (TAAs) such as α-aminoisobutyric acid (Aib) and a C-terminal 1,2-aminoalcohol. Thanks to their ability to form pores in phospholipid membranes peptaibols and synthetic analogs thereof have been often employed as simple models to study membrane protein behavior.[2,3] In the past, C-terminal methyl ester analogs of peptaibols were commonly used instead of the natural sequences[4] because they were much easier to produce by solution-phase synthesis.[5] Nonetheless, in the last few years an increasing number of *in vitro* biological assays against an array of cancer cell lines revealed significant differences in terms of bioactivity between the natural sequence of a short-length peptaibol (11 residue long) called trichogin GA IV, and its C-terminal modified analogs (Table 1).[6] While trichogin was able to kill cancer cells effectively, its C-terminal methyl ester analog was by far less active. The substitution of trichogin C-terminal Leucinol with a Leucine free carboxylic acid (**Tric-COOH**, Table 1) even led to a complete loss of cytotoxicity.[7].

The present study aims at shedding light on the structure-activity relationship at the basis of such differences through a variety of spectroscopic techniques. We started from a peculiar conformational behavior registered some years ago for some trichogin analogs.[8] It was shown by circular dichroism that a conformational switch was taking place at cryogenic temperatures. We thought of expanding such study to see if any correlation could be drawn between peptide bioactivity and conformational behavior at low temperatures. Conformational studies ranging from physiological to cryogenic temperatures would also help to link results obtained at low temperatures - for instance by electron paramagnetic resonance (EPR)[9] - with peptide behavior in biologically relevant conditions. We thus decided to exploit the unique instrumentations present at the B23 beamline of Diamond Light Source, UK's national synchrotron science facility to acquire both synchrotron radiation circular dichroism (SRCD) and fluorescence spectra for trichogin and several analogs thereof over a wide range of temperatures. We independently applied continuous wave (CW) electron paramagnetic resonance (EPR) spectra and pulsed electron double resonance (PELDOR) at 80K on trichogin analogs bearing different capping moieties. Our results provide an explanation for the differences in peptide bioactivity and selectivity induced by C-terminal capping modifications.

Results and Discussion

Synthesis. We produced, by either solution- or solid-phase (SPPS) peptide synthesis, a number of trichogin analogs (Table 1), following published procedures.[10] In particular, the C-terminal methyl ester peptides were synthesized in solution. All peptides were obtained in good yields (up to 50%) and purity (97-99%) and characterized by HPLC and ESI-MS techniques. HPLC profiles and ESI-MS spectra registered for unpublished

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Supporting information for this article is given via a link at the end of the document.

sequences, namely **F0T8-ol**, **L4T8-ol**, and **T18-ol** are reported in the *Supporting Information (SI)*.

Table 1. Peptide sequences

Acronyms	Sequence[a]
Tric-ol	*n*Oct- Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-**Lol**
Tric-OMe	*n*Oct- Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-**Leu-OMe**
Tric-COOH	*n*Oct- Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-**Leu-OH**
T1-OMe	*n*Oct-**Toac**-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-**Leu-OMe**
T4-OMe	*n*Oct- Aib-Gly-Leu-**Toac**-Gly-Gly-Leu-Aib-Gly-Ile-**Leu-OMe**
T8-OMe	*n*Oct- Aib-Gly-Leu-Aib-Gly-Gly-Leu-**Toac**-Gly-Ile-**Leu-OMe**
T8-ol	*n*Oct- Aib-Gly-Leu-Aib-Gly-Gly-Leu-**Toac**-Gly-Ile-**Lol**
T18-OMe	*n*Oct-**Toac**-Gly-Leu-Aib-Gly-Gly-Leu-**Toac**-Gly-Ile-**Leu-OMe**
T18-ol	*n*Oct-**Toac**-Gly-Leu-Aib-Gly-Gly-Leu-**Toac**-Gly-Ile-**Lol**
F0-OMe	**Fmoc**- Aib-Gly-Leu-Aib-Gly-Gly-Leu- Aib-Gly-Ile-**Leu-OMe**
F0T8-OMe	**Fmoc**- Aib-Gly-Leu-Aib-Gly-Gly-Leu-**Toac**-Gly-Ile-**Leu-OMe**
F0T8-ol	**Fmoc**- Aib-Gly-Leu-Aib-Gly-Gly-Leu-**Toac**-Gly-Ile-**Lol**
L4-ol	*n*Oct- Aib-Gly-Leu-**Leu**-Gly-Gly-Leu- Aib-Gly-Ile-**Lol**
L4T8-ol	*n*Oct- Aib-Gly-Leu-**Leu**-Gly-Gly-Leu-**Toac**-Gly-Ile-**Lol**
K56-ol	*n*Oct- Aib-Gly-Leu-Aib-**Lys**-**Lys**-Leu- Aib-Gly-Ile-**Lol**

[a] nOct, 1-octanoyl; Lol, 1,2-aminoalcohol Leucinol; -OMe, methoxy; Fmoc, fluorenylmethyloxycarbonyl; Toac, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid; all protein α-amino acids were of L configuration.

Variable Temperature Synchrotron Radiation Circular Dichroism (vT-SRCD). Several SRCD spectra of representative trichogin analogs have been acquired at the B23 beamline of Diamond Light Source. A Temperature interval between +20°C and -87°C has been investigated. Either methanol or 1-propanol (freezing temperatures -97.6 and -126°C, respectively) were used as solvents.[11] Molar ellipticities were calculated taking into account temperature-induced variations of sample volumes (see *Experimental Section*). At first, the natural trichogin GA IV sequence (**Tric-ol**) and two analogs where the naturally-occurring C-terminal moiety Lol was replaced by either a methyl ester (**Tric-OMe**) or a free carboxylic acid (**Tric-COOH**) were analyzed. The trend of their SRCD spectral profiles vs. temperature are reported in Figure 1.

At room temperature (20°C), all three peptides displayed a CD spectrum characterized by the presence of two negative maxima, centered at about 222 and 205 nm, respectively. Such a profile is associated with the onset of a right-handed helical structure. The ratio between molar ellipticities at 222 and 205 nm ($R = [\theta]_{222}/[\theta]_{205}$) depends on the peptide 3_{10}-helical content, that is greater in **Tric-ol** (smaller *R* value) than in its analogs (Figure 1). As the temperature decreased, a general decrease in the helical content (*i.e.*, loss of spectral intensity) was registered for all peptides. At the lowest experimental temperatures, **Tric-ol** displayed a CD spectrum similar to that of **Tric-OMe** at room temperature. For **Tric-OMe** and **Tric-COOH**, the cooling to -87°C appears to invert the helical handedness from right- to left-handed. The two screw senses seem to interconvert as the temperature lowered, with the spectrum at each temperature being the result of an equilibrium between the two.[12]

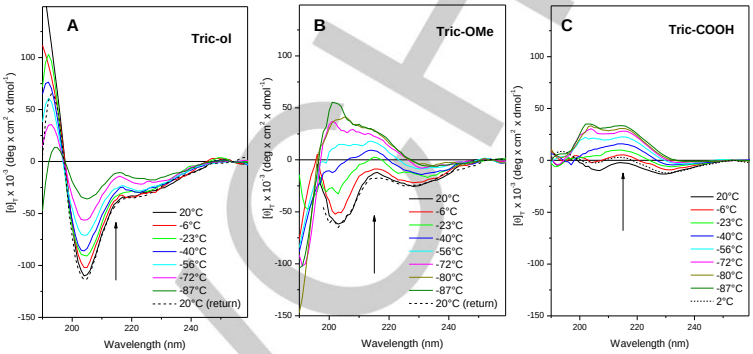

Figure 1. Far-UV SRCD spectra acquired for the naturally-occurring sequence **Tric-ol** (**A**), and its C-terminal methyl ester **Tric-OMe** (**B**) and C-terminal free carboxylic acid **Tric-COOH** (**C**) analogs in methanol solution at different (decreasing) temperatures.[13] Peptide concentration: ca. 1 mM. The arrows indicate the trend of the spectral changes induced lowering the temperature from 20°C to -87°C.

In all cases, the temperature-driven conformational changes were fully reversible when the samples were heated back to 20°C.

Although these three peptides only differ in terms of the C-terminal moiety, this modification has a profound effect on **Tric-ol** behavior. Indeed it did not undergo temperature-dependant CD spectral inversion (Figure 1A). To shed light on the nature of the conformational transition for **Tric-ol** and to determine the residues most involved in the conformational switch, we performed variable temperature SRCD investigation on selected **Tric-ol** analogs. The vT-SRCD analysis performed on **K56-ol**, where two achiral and conformationally flexible Gly residues at position 5 and 6 of the natural sequence were replaced by as many *chiral* Lys residues showed that such a substitution hampered the conformational switch (See Figure S1, *SI*). A structure-stabilizing effect of decreasing temperature was found for **K56-ol**, with an increasing content of right-handed helix. The fact that this behavior was not affected by conducting these low temperature studies at both acidic and basic pH ruled out any contribution from the protonated amines of the Lys side chains (data not shown). The stabilizing effect of additional *chiral* residues indirectly suggests the presence of a helical handedness uncertainty in the native peptaibol sequence. Nonetheless, Lys has a higher helix propensity than Gly,[14] thus a positive effect from the enhanced helix stability is also expected. To further evaluate the influence of helix stability, we performed vT-SRCD on a trichogin analog with Aib at position 4 replaced by a *chiral* and *less* helix-inducer Leu residue (**L4-ol**), which 3D-structure solved by X-ray diffraction analysis,[15] though predominantly helical, has been revealed to be dominated by a kink in the middle of the peptide sequence (helix-loop-helix structure). The SRCD spectrum registered for **L4-ol** at room temperature reflected its high helical content

(Figure S1). On lowering the temperature, **L4-ol** followed a similar trend to that observed for the native **Tric-ol**, but with an enhanced destabilizing effect. In this case, it appears that the loss of helix stability predominates over the chiral gain, favoring the conformational switch. The behavior of **L4-ol** can be seen in between **Tric-ol** and **Tric-OMe** (Figure S1). Based on those data, we concluded that the reversible screw-sense inversion was also promoted by the helical flexibility.

To assess the role played in the screw-sense switch by Aib residues, we performed the low temperature SRCD studies of trichogin analogs where a single Aib residue at position 1, 4 or 8 was replaced by the more helicogenic, tetrasubstituted amino acid TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid). TOAC is a spin-labeled amino acid commonly exploited to acquire information on peptides/peptaibols by EPR, usually at very low temperatures.[16] Low temperature SRCD can be successfully coupled with EPR to detect changes in peptide 3D-structure under similar experimental conditions (see below). The complete peptide methyl ester series (**T1-OMe**, **T4-OMe**, and **T8-OMe**) were studied, Figure S3 (*SI*), showing that in **T8-OMe** the helix-inducer effect of TOAC remarkably reduced the peptide ability to undergo the reversible screw-sense interconversion. No effect was observed when TOAC was inserted at the N-terminus or in the middle of the sequence (Figure S3). The corresponding **T8-ol** analog (bearing the native, C-terminal amino alcohol) did not undergo any temperature-dependent, CD-detectable conformational changes (Figure S3). To confirm the triggering effect of the C-terminal Aib, the peptide **L4T8-ol**, where Aib residues at positions 4 and 8 where substituted with Leu and Toac, respectively, was synthesized and analyzed [see Figure S4, *SI*, where a comparison with the corresponding **T8-ol** analog (see Table 1) is also drawn]. It turned out that the presence of a TOAC residue at position 8 completely counteracts the formerly described switch-promoting effect of Leu at position 4.

The presence of an isodichroic point (Figure 1) is indicative of the equilibrium between two states and is consistent with a thermally-driven helical handedness switch from right-handed (more stable at room temperature) to left-handed (more stable at low temperatures). A *melting temperature* (T_m) for the conformational transition could be estimated by applying a two-component Boltzmann fitting (Table 2). The melting curve for **Tric-OMe** is reported in Figure 2 (all others can be found in the *Supporting Information*).

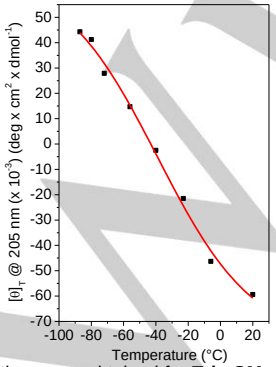

Figure 2. Melting curve obtained for **Tric-OMe** from the molar ellipticity values registered at 205 nm.

Table 2. Conformational transition Temperature (T_m) extrapolated from the two-component Boltzmann fitting of the molar ellipticity values acquired at 205 nm for selected peptides (curves reported in SI).

Acronyms	T_m (°C)
L4-ol	-80 ± 20
T8-OMe	-49 ± 3
Tric-COOH	-47± 5
Tric-OMe	-39 ± 6

A lower conformational transition temperature (T_m, Table 2) is associated to a higher stability of the right-handed structure. Data listed in Table 2 indicate that the presence of a C-terminal methyl ester destabilize the right-handed helix, thus promoting the screw-sense switch.

In conclusion, the SRCD study performed on several trichogin analogs as a function of temperature in the range +20 to -87°C, revealed the occurrence of a reversible, temperature-driven conformational transition from right-handed to left-handed helix promoted by the presence of a C-terminal methyl ester or free carboxylic acid. Although examples of temperature-driven screw-sense helical switch in peptides were previously reported in the literature,[17] this is the first time - to the best of our knowledge - that the promoting effect of a C-terminal methyl ester (or carboxylic acid) has been unambiguously demonstrated. The involvement of the C-terminal Aib residue in triggering this switch in **Tric-ol** analogs was also proven and confirmed by the vT-NMR study discussed below.

vT-NMR. To independently investigate the residues most involved in the temperature-driven conformational switch we performed a vT-NMR titration on **Tric-OMe** in CD_3OH. The chemical shift variation as a function of decreasing temperature for each NH signal (unambiguously assigned by means of 2D-NMR analysis) was measured in the range 22°C to -63°C (295-210 K) and is reported in Figure 3.

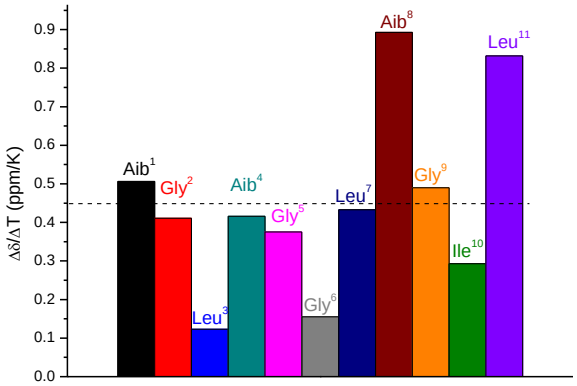

Figure 3. Temperature coefficients for NH chemical shifts (temperature range 295-210 K) of **Tric-OMe** in CD_3OH. The dashed line is drawn at 0.45 ppm/K, i.e., the commonly accepted value dividing intramolecularly hydrogen-bonded from free amide NHs.[18,19]

Figure 3 reports the temperature coefficients (Δδ/ΔT) measured for amide NHs of **Tric-OMe** in CD_3OH. A high Δδ/ΔT value is expected at the helix N-terminus: Aib[1] and Gly[2] NHs are exposed to the solvent regardless of the presence of a helical conformation and therefore can be affected by a temperature change. The rest of the NHs are involved in the intramolecular H-bond network. Hence, the high Δδ/ΔT values exhibited by the C-terminal residues Aib[8] and Leu[11](OMe) indicate that they are in some way solvent-exposed and most likely involved in the conformational switch. A flexibility at the level of the H-bonds involving Aib[8] and Leu[11] NHs may well induce a change in the screw sense of the whole helical structure. Such conclusion is in agreement with the results obtained by SRCD, namely that the structural change is triggered by the C-terminal methyl ester, with a special role played by Aib[8]. We note that a previous study[20] reported a left-handed helical CD profile at room temperature for the short peptide methyl ester *n*Oct-Aib-Gly-Ile-Leu-OMe, comprising just the four C-terminal residues of trichogin.

Our vT-SRCD and NMR studies gave strong evidence in support of a screw-sense inversion occurring at cryogenic temperatures. Nonetheless the CD spectra registered at the minimum and maximum experimental temperatures were never exact mirror images. To get further insights on the nature of the endpoint 3D-structure and to evaluate the possible contribution from other peptide 3D-structures, two independent studies were conducted, by means of EPR and fluorescence spectroscopy.

Electron Paramagnetic Resonance (EPR). EPR can be employed to measure spin-spin distances in bis-labeled molecules(peptides). To explore peptide length variations possibly associated with the observed conformational switch, two bis-labeled trichogin analogs, namely **T18-ol** and **T18-OMe** (see Table 1), were synthesized and analyzed by continuous wave (CW) and pulsed EPR. A screw-sense inversion should not change the peptide length significantly. An initial study by CW EPR was performed under a variety of experimental conditions, namely glassy CH_3OH/C_2H_5OH 95:5 mixture, C_2H_5OH and CF_3CH_2OH (TFE) (*SI*). The CW EPR spectra (Figure S6, *SI*) show the triplet lineshape typical of diluted solid-state nitroxide free radicals and are almost superimposable for the two peptides in the same solvent. Pulsed electron double resonance (PELDOR) time traces were acquired for both analogs, again in glassy CH_3OH/C_2H_5OH 95:5 mixture, C_2H_5OH and TFE. The *semi*-logarithmic plots of PELDOR signal time traces (Figure S8, *SI*) are those typical of biradicals: a fast decay with noticeable signal oscillations is followed by a slow decay with almost linear dependence. The former is caused by *intra*-molecular interactions between the two radicals, $V_{INTRA}(T)$, with oscillation frequencies depending on spin-spin distances; the latter, $V_{INTER}(T)$, results from weak *inter*-molecular interactions. Those two interactions influence the PELDOR signal *V(T)* according to the formula: [21]

$$V(T) = V_{INTER}(T)V_{INTRA}(T)$$

For a random pair distribution in the sample, the theory predicts that

$$V_{INTER}(T) = \exp(-\alpha CT)$$

where α is a coefficient and C is the volume spin concentration. The theoretical expression for $V_{INTRA}(T)$ is given by averaging over the pair distribution function, P(*r*), as

$$V_{INTRA}(T) = 1 - p_B + p_B \int P(r)dr \sin\theta d\theta \cos\frac{g^2\mu_B^2}{\hbar}\frac{(1-3\cos^2\theta)}{r^3} \quad (1)$$

where p_B is the efficiency of the partner spin inversion in the pair (commonly denoted as *B*-spin) caused by the pumping microwave pulse ($0 < p_B < 1$); g is the *g*-factor; μ_B is the Bohr magneton; r is the interspin distance; and θ is the angle between vector $\boldsymbol{r}$ and the external magnetic field. The $P(r)$ function can thus be extracted from experimental data by solving this integral equation.

The p_B value obtained from our data coincides (within the experimental error) with the calculated one.[10c,22] The theoretical Eq. (1) is to be compared with the normalized *intra*-molecular time trace contribution determined as:

$$V_n(T) = \frac{V_{INTRA}(T) - V_{INTRA}(\infty)}{V_{INTRA}(0) - V_{INTRA}(\infty)} \quad (2)$$

The $V_n(T)$ time traces are plotted in Figure 4.

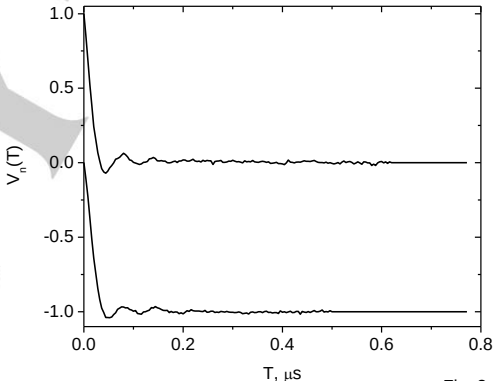

Figure 4. Normalized *intra*-molecular PELDOR decays for **T18-ol** (top) and **T18-OMe** (bottom) in glassy CF_3CH_2OH at -195°C (78 K). Time kinetics are shifted downwards for convenience.

Figure 4 shows that $V_n(T)$ decays slightly slower for **T18-OMe** than for **T18-ol**. Such difference is more apparent in the frequency domain (Pake pattern, Figure S10, *SI*) where it appears as a broader lineshape for the latter analog. The distance distribution functions *P(r)* derived from the Multi-Gaussian Monte-Carlo fitting[23] of the PELDOR data gathered in TFE and shown by points, are plotted in Figure 5.

Both peptides display a main interspin (TOAC⋯TOAC) distance in glassy TFE (Figure 5) centered at a value of about 1.65 nm. The measured distance roughly corresponds to that expected on the basis of a structural model (Figure 5) built on the mixed, 3_{10}-/α-helix of the natural trichogin (**Tric-ol**) solved by X-ray diffraction analysis.[24] The distance distribution function for **T18-OMe** is characterized by an additional peak centered at about 1.75 nm, suggesting that conformational flexibility can indeed be associated with the presence of a C-terminal methyl ester. Such a distance is compatible with the onset of the rare

2.2_7-helix, as already reported in the literature.[25] The ratio between the two peaks is almost the same in all solvents, with always a clear prevalence of the main conformation (1.65 nm). A certain percentage (about 25%) of molecules adopting rather elongated conformations (with interspin distances of about 2.2-2.5 nm) was also detected for both peptides in all solvents.

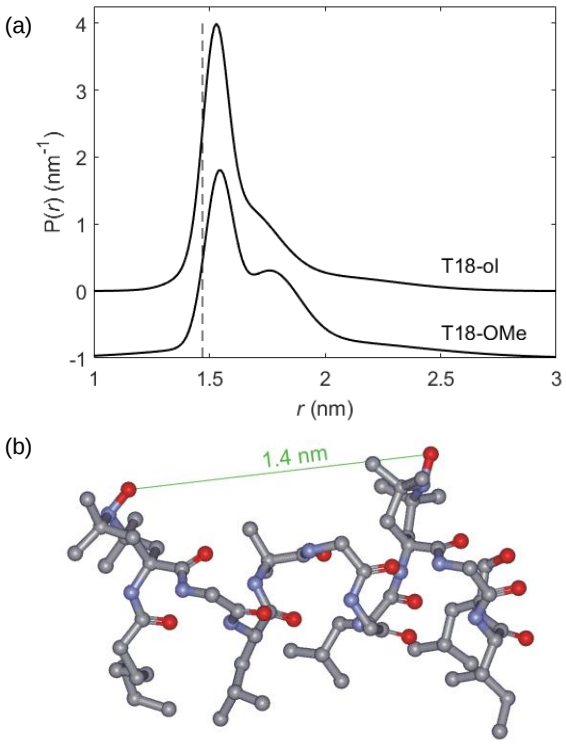

Figure 5. (a) Distance distribution function *P(r)* for **T18-ol** and **T18-OMe** in glassy TFE (-195°C, 78 K). The dashed line shows the interspin distance corresponding to a 3_{10}-helix. (b) Model for **T18-ol** created on the basis of the X-ray 3D-structure of natural trichogin. Hydrogen atoms are omitted for clarity. The interspin distance calculated from the model (1.4 nm) is reported in green.

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In conclusion, our EPR analysis showed that the main 3D-structure adopted by the analogs at cryogenic temperatures is associated with a similar peptide length as the conformation found at room temperature for the natural peptaibol trichogin. Nonetheless, a clear contribution from more elongated structure(s) was revealed in the presence of a C-terminal methyl ester. The EPR study showed further differences between the two trichogin analogs **T18-ol** and **T18-OMe**, that could be attributed to a different degree of structural flexibility caused by the different C-terminal moieties.

vT-Fluorescence spectra. To characterize the structural differences induced by the C-terminal modification by an additional, independent technique, we exploited the distance-dependant, fluorescence-quenching ability of TOAC. Two trichogin analogs bearing an N-terminal fluorene moiety and a TOAC residue at position 8 (analogs **F0T8-OMe** and **F0T8-ol**, Table 1) were synthesized. A fluorene-containing trichogin analog devoid of TOAC (**F0-OMe**, Table 1) was used as reference. The fluorescence intensities and absorbance spectra were acquired in the 180-300K temperature range (*i.e.*, from -97 to 27°C). The variation in molar concentration due to temperature-induced changes in solvent density was corrected by normalizing all fluorescence values through the measured fluorescence/absorbance at the excitation wavelength.

Absorbance spectra of all analogs and vT fluorescence spectra of the reference compound **F0-OMe**. are reported in the *SI*. A decrease in fluorescence intensity with increasing temperature is observed for **F0** (even after taking into account the absorbance variations), as expected from the temperature dependence of non-radiative processes. Figure S14, *SI* reports the quantum yields determined using a 20 μM aqueous solution of Trp (pH 6.0) at 298 K as a standard (quantum yield 0.15±0.1[28]).

The vT fluorescence spectra of **F0T8-OMe** and **F0T8-ol** registered in the temperature range 180-300K (*i.e.*, from -97 to 27°C) are reported in Figure 6.

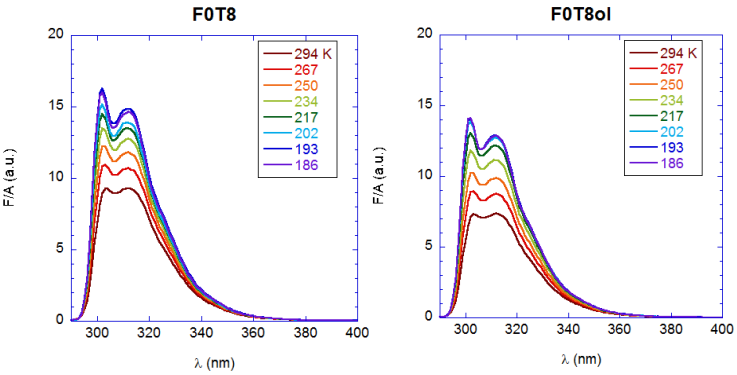

Figure 6. vT Fluorescence spectra of **F0T8-OMe** (left) and **F0T8-ol** (right).

From the integrals of the absorbance-normalized spectra it is possible to calculate the quenching efficiency (Figure 7). Since, whatever the quenching mechanism, the quenching efficiency decreases with increasing distance between fluorophore (fluorene) and quencher (TOAC), these data clearly indicate that this distance is slightly lower in **F0T8-ol** than in **F0T8-OMe**, in agreement with the EPR results. Assuming a Förster energy transfer mechanism of quenching, and correcting for the

temperature variations of the unperturbed quantum yield and of the methanol refractive index,[29] an estimate for the relative variations in interprobe distance can be obtained, allowing more quantitative considerations. (Figure 8). Both the difference between the two analogs and the temperature-induced distance variation are relatively small.

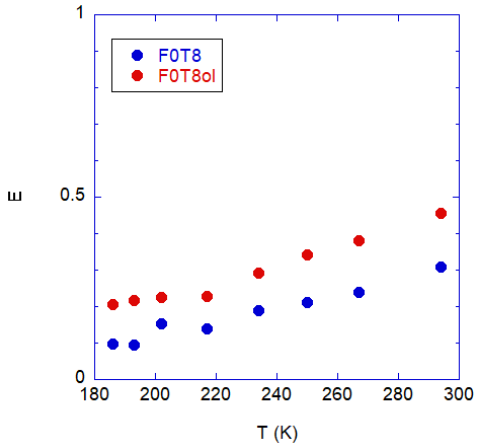

Figure 7. Quenching efficiency of **F0T8-OMe** and **F0T8-ol** as a function of increasing temperature.

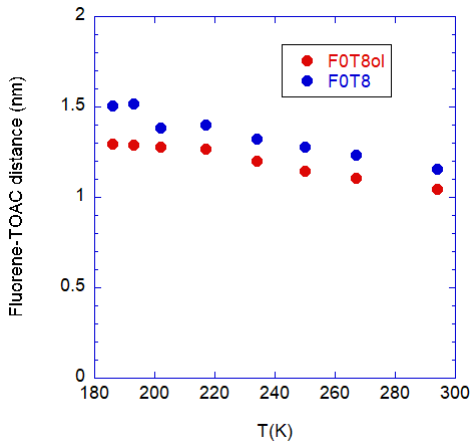

Figure 8. Interprobe distances for **F0T8-OMe** and **F0T8-ol** as a function of increasing temperature.

The results of our vT fluorescence analysis prove that peptide length of both **F0T8-OMe** and **F0T8-ol** does not change dramatically in response to temperature variation. More to the point, no sizeable differences could be detected between the two peptides, despite the dramatically different response of the corresponding analogs **T8-OMe** and **T8-ol** to temperature variations, highlighted by vT SRCD (see Figure S15, SI). In summary, both fluorescence and EPR results point to the onset of a screw-sense interconversion, associated to a modest length variation.

Conclusions

The sequence of peptaibols - naturally-occurring, membrane-active peptides - is characterized by the presence of a C-terminal aminoalcohol. In previous studies, we found that by replacing the C-terminal 1,2-aminoalcohol Leucinol of the peptaibol trichogin with the corresponding methyl ester (-Leu-OMe) or free carboxylic acid (-Leu-COOH) the anticancer activity of the native peptide was deeply affected.[6] Aiming at shedding light on that behavior, we acquired both synchrotron radiation circular dichroism (SRCD) and fluorescence spectra in a wide temperature interval for the naturally-occurring peptaibol trichogin and several analogs. Our vT-SRCD study revealed the occurrence of a reversible, temperature-driven conformational switch from right-handed to left-handed helix triggered by the presence of a C-terminal methyl ester or free carboxylic acid. Several other **Tric-ol** analogs were analyzed by vT-SRCD, revealing further details about the screw-sense inversion stabilized at cryogenic temperatures. The vT-NMR study enabled the identification of the key residues involved in the transition (namely Aib^{8} and Leu^{11}-OMe).

By combining the vT-fluorescence data with those obtained by CW EPR at 80K and PELDOR on double spin-labeled trichogin analogs, we were able to rule out any significant peptide-length modulation associated with the temperature-driven structural change, assigning the conformational changes occurring at very low temperatures exclusively to the screw-sense inversion. A significant population of more elongated peptide molecules in the presence of a C-terminal methyl ester was also identified, highlighting the role of this C-terminal moiety in increasing structural flexibility.

Several publications were reported describing screw-sense inversion of synthetic helical polymers, induced by a variety of external stimuli such as metal complex,[30] light,[31] pH or solvent,[32] small-molecule interactions[33] aiming at building stimuli-responsive materials. The exploitation of biopolymers is even more of interest, as it gives the material a biocompatible essence.[3b, 34] Several literature studies reported the development and exciting applications of *bioinspired* systems undergoing controlled interconvertion between left- and right-handed helices.[3b,35] Helix-inducer, achiral, C$^{\alpha}$-tetrasubstituted residues such as Aib can play a significant role in promoting such helix handedness interconversion.[36] The ability of Aib-containing peptides to adopt a left-handed screw sense in the presence of several L-amino acids has been linked to the position of the L-amino acid(s) in the sequence[37] or to the sequence propensity to adopt type-II β-turns.[38] Also some *chiral*, C$^{\alpha}$-tetrasubstituted residues, among which the naturally-occurring Isovaline, could be accommodated in mismatched helix.[39]

None of the papers reporting thermo-directed screw-sense inversion on peptides - or other organic substrates - so far connected the molecule propensity to switch with its C-terminal capping moiety. We herein demonstrated the ability of a C-terminal methyl ester or carboxylic acid to actively promote the onset of a temperature-driven screw-sense inversion. This information will help developing smart materials with stimuli-responsive properties or studying the biological role of post-translational modifications affording esters on membrane proteins.[40]

Naturally-occurring trichogin (**Tric-ol**) possess a strong and non-selective cytotoxic activity. We note that all trichogin analogs promptly undergoing the conformational switch (such as C-terminal methyl esters or free -COOH, and the Leu[4]-containing analog) are also the least active against both cancer cells and bacteria.[6] On the other hand, those displaying the smallest propensity to change - or even a stabilization of - their 3D-structure at low temperatures, such as **K56-ol**, were found to be selectively active against cancer cells, leaving healthy cells unaffected.[6, 41] Several parameters (such as net charges, hydrophobicity, ...) influence potency and selectivity of bioactive peptides. Perhaps a more rigid 3D-structure helps in achieving selective recognition of target cells, too. Further vT-SRCD studies on bioactive peptides might contribute in shedding light on such an intriguing structure-activity relationship.

Experimental Section

Synthesis. Synthetic procedure, HPLC profiles and ESI-MS spectra for unpublished sequences, namely **F0T8-ol**, **L4T8-ol**, and **T18-ol** are reported in the *Supporting Information (SI)*.

SRCD experiments were performed using a nitrogen-flushed Module A end-station spectrophotometer at B23 Synchrotron Radiation CD Beamline at the Diamond Light Source, Oxfordshire, UK.[42] Peptide concentration in spectrophotometric-grade methanol (or isopropanol) solutions has been corrected taking into account the temperature-dependent variation of solvent density. The corrected values are reported in Table S1 (*SI*).

Continuous-wave (CW) and Pulse Electron-Electron Double Resonance (PELDOR) EPR measurements. *Sample preparation.* The double spin-labeled analogs of trichogin nOct-TOAC[1]-Gly-Leu-Aib-Gly-Gly-Leu-TOAC[8]-Gly-Ile-Leu-Lol (**T18-ol**) and nOct-TOAC[1]-Gly-Leu-Aib-Gly-Gly-Leu-TOAC[8]-Gly-Ile-Leu-OMe (**T18-OMe**) were dissolved in a 95:5 (*v*/*v*) methanol (MeOH)/ethanol(EtOH) mixture (both solvents from Ekros-Analytics, St. Petersburg, Russian Federation). The peptide concentrations varied in the range 0.54-8 mM. The solutions were placed in 2.9-mm o.d. EPR tubes, degassed, and sealed. The sample formed a transparent glass after shock-freezing in liquid nitrogen. *CW EPR experiments* were carried out on an X-band Bruker E380 EPR spectrometer using a dielectric Bruker ER 4118 X-MD-5 cavity and an Oxford Instruments CF-935 cryostat for cooling. The cavity was cooled down to 90 K through a nitrogen flow. The modulation amplitude was 0.05 mT, the modulation frequency 100 kHz, and the microwave power set to a level low enough to avoid spectra saturation. *The PELDOR experiments* were performed on an X-band Bruker ELEXSYS E580 EPR spectrometer using a split-ring Bruker ER 4118 X-MS-3 cavity. The cavity was cooled to 78 K. The 3-pulse PELDOR experiments were done with a microwave pulse sequence $\pi/2_{(\nu A)}$-T-$\pi_{(\nu B)}$-(τ-T)-$\pi_{(\nu A)}$-τ-$echo_{(\nu A)}$ where the ν_A frequency refers to the echo-forming pulses and the ν_B frequency to the additional pumping pulse. The pumping pulse was initially set with a negative T delay of -188 ns, and then it was scanned forward with a time step of 4 ns. The frequency offset ν_A-ν_B was 70 MHz. The pumping and observation frequencies were set symmetrically around the center of the resonator dip, with ν_B applied at the maximum of the EP EPR. The length of all pulses was set to 32 ns. The amplitudes of π/2- and π-pulses at ν_A were tuned independently to provide maximum of the Hahn echo. A two-step phase-cycling (+,+,+), (-,+,-) was performed, where the first two signs refer to the phases of echo-forming pulses and the third to the phase of detection. The amplitude of π-pulse at ν_B was set as to invert the echo at ν_A=ν_B. The time τ was about 0.8 μs. The PELDOR signal distortions occurring with the passage of pumping pulse through the detection pulse were eliminated as previously described.[43] The normalized pair $V_n(T)$ time traces were obtained from eq. 2[44] and Fourier transformation was performed. The multi-Gaussian Monte Carlo fitting was performed using homemade program in Pascal.NET software.[23] Three Gaussians were enough to attain good agreement with experimental PELDOR data (see Fig. 10S, *Supporting Information*).

Fluorescence and Absorbance Spectroscopy. B23 beamline is also equipped with a fluorimeter that allows fluorescence measurements at very low temperatures. Using that spectrophotometer we measured the effect of temperature variations on the fluorescence of trichogin analogs bearing a fluorophore (fluorenylmethyloxycarbonyl, Fmoc) and a quencher (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid, TOAC) at their N- and C-terminus, respectively. All samples were prepared in spectrophotometric grade methanol (ACS, >99.9%). For reference, L-Trp, 20 μM in H_2O (pH adjusted to 6.1) at 24.5 °C was also measured. The real temperature (not the set one) is always indicated in the text. Set and real temperatures are reported in table S2, *SI*. Fluorescence spectra were measured under the following conditions: scanning speed 2 nm/s, PMT HV 600 V, bandwidths 6 nm in excitation, 1.5 nm in emission, excitation wavelength 265 nm. For a proper comparison of the acquired spectra with those of F0 (reference compound), the spectra had to be normalized by the absorbance at the excitation wavelength (265 nm) of the fluorene moiety alone (while also TOAC contributes to the total absorbance). This was estimated by using the absorbance at 299 nm, and the ratio between the absorbances at the two wavelengths obtained for F0 (see *SI*).

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Keywords: antimicrobial peptide • helix handedness • conformational transition • screwsense switch • synchrotron radiation circular dichroism

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