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Synthesis and preliminary conformational analysis of TOAC spin-labeled analogues of the medium-length peptaibiotic tylopeptin B

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A set of analogues of the 14-residue peptaibol tylopeptin B, containing the stable free-radical 4-amino-1-oxyl-2,2,6,6,-tetramethylpiperidine-4-carboxylic acid (TOAC) at one or two selected positions, was synthesized by the solid-phase methodology. A solution conformational analysis performed by FTIR absorption and CD suggests that, in membrane-mimicking solvents, the labeled tylopeptin B analogues preserve the helical propensity of the parent peptide, with a preference for the α-helix or the 3_{10}-helix type depending upon the nature of the solvent. In aqueous environment, the spin-labeled analogues present a higher content of helical conformation as a consequence of the strong helix promoter effect of the conformationally constrained TOAC residue. We observed a progressive increase of the quenching effect of the nitroxyl radical on the fluorescence of the *N*-terminal tryptophan as TOAC replaces the Aib residue at positions 13, 8, and 4, respectively. A membrane permeabilization assay performed on two selected analogues, TOAC[8]- and TOAC[13]-tylopeptin B, showed that the labeled peptides exhibit membrane-modifying properties comparable with those of the natural peptaibiotic. We conclude that our TOAC paramagnetic analogues of tylopeptin B are good models for a detailed ESR investigation of the mechanism of membrane permeabilization induced by medium-length peptaibiotics.

Supporting information may be found in the online version of this article.

Keywords: antibiotics; conformation analysis; membrane activity; peptide synthesis; spin label; TOAC; tryptophan quenching

Introduction

Peptaibiotics are linear peptides of fungal origin with antibiotic activity, characterized by a high content of nonproteinogenic C^{α}-tetrasubstituted α-amino acids, an acylated *N*-terminus and a *C*-terminal 1,2-amino alcohol such as phenylalaninol or leucinol [1]. According to the number of residues in the sequence, they are classified in three groups: (i) short (from 4- to 10-mers), of which trichogin GA IV represents the prototype; (ii) medium length (from 14- to 16-mers), a less investigated subclass; and (iii) long (from 17- to 21-mers), with alamethicin as the most extensively studied. It is generally accepted that they are membranolytic antibiotics. However, although alamethicin was shown to form voltage-dependent pores, the detailed mechanism of short-length and medium-length peptaibiotics is largerly unknown.

Recently, we reported that tylopeptin B, a 14-residue peptaibiotic originally extracted from the fruiting body of the mushroom *Tylopilus neofelleus*, is endowed with membrane-modifying properties, which can be related to the ability of the peptide to adopt an amphipathic helical structure in membrane-mimicking enviroments [2]. To obtain more detailed information about the mechanism of membrane permeabilization of this medium-length peptaibiotic, we planned to synthesize a series of analogues in which the achiral tetrasubstituted α-amino acid 4-amino-1-oxyl-2,2,6,6,-tetramethylpiperidine-4-carboxylic acid (TOAC) replaces the Aib residue at selected positions in the tylopeptin B sequence. The TOAC amino acid, in which a stable nitroxyl radical is fixed on a rigid heterocyclic structure, was used as a paramagnetic probe to study by electron spin resonance (ESR) the mode by which peptides insert into a membrane. In particular, detailed information about the location, orientation, and aggregation of membrane-active peptides in the phospholipid bilayer was obtained for the TOAC-labeled peptaibiotics alamethicin F50/5 [3,4] and trichogin GA IV [5], in which one or two Aib residues in the sequence are replaced by the free-radical-containing amino acid.

Here, we present the solid-phase synthesis of the mono-labeled and bis-labeled tylopeptin B analogues shown in Table 1, which were prepared by avoiding any harsh acid treatment of the peptide-resin to preserve the free-radical character of the TOAC amino acid [6]. In addition, to obtain larger amounts of labeled peptides required to perform further detailed ESR studies, we

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Table 1. Amino acid sequences of tylopeptin B (**T**) and its TOAC-containing analogues **1–5**

T	Ac-Trp-Val-Aib[3]-Aib[4]-Ala-Gln-Ala-Aib[8]-Ser-Aib[10]-Ala-Leu-Aib[13]-Gln-Lol
1	Ac-Trp-Val-Aib-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-**TOAC[13]**-Gln-Lol
2	Ac-Trp-Val-Aib-Aib-Ala-Gln-Ala-**TOAC[8]**-Ser-Aib-Ala-Leu-Aib-Gln-Lol
3	Ac-Trp-Val-Aib-**TOAC[4]**-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
4	Ac-Trp-Val-**TOAC[3]**-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
5	Ac-Trp-Val-**TOAC[3]**-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-**TOAC[13]**-Gln-Lol

also synthesized the series of analogues shown in Table 2, which present a less sterically crowded *N*-terminal sequence.

By FTIR and CD, the conformational preferences of tylopeptin B analogues were compared with those of the parent peptide, and the quenching effect of the TOAC aminoxyl radical on the fluorescence emission of the *N*-terminal Trp residue was investigated. The membrane modifying properties of a few selected TOAC analogues were compared with those of tylopeptin B by the ability to induce permeabilization of phospolipid bilayers.

A preliminary communication of a limited part of this work has been reported [7].

Materials and Methods

General

Fmoc-TOAC-OH was synthesized as reported previously [8,9]. H-*L*-Lol-2-chlorotrityl resin (200–400 mesh, loading 0.45 mmol/g resin) was purchased from Iris Biotech (Marktredwitz, Germany). Fmoc-amino acids were supplied from Novabiochem (Merck Biosciences, La Jolla, CA, USA), and all other amino acid derivatives and reagents for peptide synthesis were purchased from Sigma-Aldrich (St. Louis, MO, USA). HATU was purchased from GLS (Shanghai, China). 5,6-Carboxyfluorescein (CF), cholesterol, and *L*-α-phosphatidylcholine (from egg yolk, type XVI-E) were Sigma-Aldrich products.

Table 2. Amino acid sequences of analogues **6** and **9** of tylopeptin B and their corresponding TOAC-labeled peptides **7**, **8**, **10**, and **11**

6	Ac-Trp-Val-Aib-**Ala[4]-Aib[5]**-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
7	Ac-Trp-Val-Aib-**Ala[4]-TOAC[5]**-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
8	Ac-Trp-Val- **TOAC[3]-Ala[4]-Aib[5]**-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
9	Ac-Trp-**Ala[2]**-Aib-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
10	Ac-Trp-**Ala[2]-TOAC[3]**-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-Aib-Gln-Lol
11	Ac-Trp-**Ala[2]-TOAC[3]**-Aib-Ala-Gln-Ala-Aib-Ser-Aib-Ala-Leu-**TOAC[13]**-Gln-Lol

Analytical HPLC separations were carried out on a Dionex (Sunnyvale, CA, USA) Summit dual-gradient HPLC apparatus, equipped with a four-channel UV-Vis detector, using a Vydac C_{18} (250 × 4.6 mm, 5 μm, flow rate at 1.5 ml/min) from W. R. Grace (Columbia, MD, USA). In preparing the mobile phase, deionized water was further purified using a milliQ reagent grade water system from Millipore (Bedford, MA, USA). The eluants A (aqueous 0.1% TFA) and B (90% aqueous acetonitrile containing 0.1% TFA) were used for the preparation of binary gradients. Analyses were carried out under the following elution conditions: (i) 3 min isocratic 35% B; linear gradient 35%–90% B in 30 min; and (ii) 3 min isocratic 50% B; linear gradient 50%–90% B in 30 min. Semi-preparative HPLC was carried out on a Shimadzu (Kyoto, Japan) series LC-6A chromatographic apparatus, equipped with two independent pump units, a UV-Vis detector, and a Vydac C_{18} column (250 × 22 mm, 10 μm, flow rate at 15 ml/min). Elutions were carried out with the same mobile phases described previously, using a linear gradient from 35% to 90% B in 20 min. MS spectral analyses were carried out on a Mariner API-ToF workstation (PerSeptive Biosystems, Framingham, MA, USA), operating in the positive mode.

Solid-Phase Peptide Synthesis

Tylopeptin B and its TOAC-containing analogues were synthesized in a 0.05 mmol scale using an Advanced Chemtech (Louisville, KY, USA) 348-Ω peptide synthesizer, starting from *H*-Lol-2-chlorotrityl resin. The TBDMS group was used to mask the Ser side chain. Fmoc deprotection was achieved with 20% piperidine in DMF (5 + 15 min). Amino acid couplings were performed in the presence of HATU/DIPEA (4 equivalents of the carboxyl component, reaction time 45–60 min), except in the case of Fmoc-Trp-OH and Fmoc-Gln-OH, which were introduced on the peptide-resin as preformed 1-hydroxybenzotriazolyl active esters. A single coupling protocol was used to acylate Lol, Leu[12], Ala[7], Gln[6], and Val[2]; in all other cases, the coupling step was repeated. Attempts to improve the coupling yields of selected sterically hindered residues by microwave irradiation was performed in a CEM Discover (Matthews, NC, USA) apparatus, heating for 10 min (25 W) giving an end temperature of 70 °C. *N*-Terminal acetylation of the peptide resin was performed by a repeated treatment with acetic anhydride (0.75 mmol) and DIPEA (0.2 mmol) in DMF for 30 min. After removal of the TBDMS group by a 5-min treatment with 0.1 M TBAF in DMF, the 1,2-aminoalcohol peptide was cleaved from the resin upon three to four treatments with 30% HFIP in DCM for 30 min or with a mixture of acetic acid–TFE–DCM (2 : 2 : 6 by volume) [10]. The filtrates were combined and evaporated to dryness providing the crude peptide, which in the case of TOAC-containing tylopeptin B analogues presents an intact nitroxyl radical. The crude peptides were purified by semi-preparative HPLC to afford the desired product, which was characterized by analytical HPLC and ESI-MS (Table SI1 in the Supporting Information).

Alternatively, the synthesis of the TOAC-containing tylopeptin B analogues were performed according a previously published protocol [2] using the trityl group to mask the Gln side chain and the Boc and *tert*-butyl groups to protect the Trp and Ser side chains, respectively. Assembly of peptides was performed by the Fmoc/HATU method, as described earlier. After cleavage of the peptide-alcohol from the resin, the side-chain-protecting groups were removed by a 90-min treatment with a TFA/phenol/thioanisole/water/1,2-ethanedithiol mixture (82 : 5 : 5 : 5 : 2.5 by

Journal of PeptideScience

volume). After this harsh acid treatment, the regeneration of the nitroxyl radical in the TOAC-containing peptides was performed by an alkaline treatment with 0.02 M ammonium acetate (pH 10) for 4–5 h [6].

Antibacterial Activity Assays

Peptide antibacterial activity was tested against Gram-positive and Gram-negative bacteria by the standardized disk diffusion Kirby–Bauer method [11] using the Mueller–Hinton culture medium pH 7.2–7.4 [12] recommended by the National Committee for Clinical Laboratory Standards and 6-mm diameter disks. In the case of streptococci, a sheep-blood agar medium was exploited. Disks were prepared by using blank paper disks (Whatman) previously autoclaved and saturated with the peptide solution. The peptide samples were dissolved in DMSO such as to give a 10 mg/ml solution. An inoculum size of 10^5 CFU/ml (10^9 CFU/ml in case of *Pseudomonas aeruginosa*) was swabbed onto the test medium and left to dry at room temperature for about 15 min. The peptide-impregnated disks (100 μg/disk) were placed aseptically onto inoculated plates and incubated at 37 °C. The results were read after 24 h of incubation by measuring the inhibition zones (in millimeters). The antibacterial activity of peptides was tested against clinical isolates of bacteria and reference bacterial strains: *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 19615, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Salmonella entereditis* 13076, and *Proteus mirabilis* ATCC 10975, where ATCC stands for American Type Culture Collection strains. The well-known antibiotics bacitracin and tetracyclin (10 μg/disk) were used as control test (Table 3).

Spectroscopic Techniques

Conventional cw-ESR measurements were performed using a Bruker ER 200D (9–10 GHz) spectrometer with a standard TE_{102} cavity. Peptide samples were 0.5 mM in $CHCl_3$, and dissolved oxygen was removed by several freeze–pump–thaw cycles.

The solution FTIR absorption spectra were recorded at 293 K using a Perkin–Elmer model 1720X FTIR spectrophotometer, nitrogen flushed, equipped with a sample-shuttle device, at 2 cm^{-1} nominal resolution, averaging 100 scans. Solvent (baseline) spectra were recorded under the same conditions. For data processing, the software SpectraCalc provided by Galactic (Salem, MA, USA) was employed. Cells with path lengths of 1.0 and 10 mm (with CaF_2 windows) were used. Spectrograde deuterated chloroform (99.8%) was purchased from Merck.

Fluorescence measurements were performed in quartz cuvettes with 1-cm path length (Hellma, Müllheim, Germany) using a Perkin–Elmer LS 50B spectrofluorimeter with both emission and excitation band passes set at 10 nm. The Trp residue of tylopeptin B analogues was excited at 280 nm, and emission spectra were recorded from 290 to 450 nm, averaging 32 scans. Peptide solutions were 1 μM in methanol, as determined by quantitative UV absorbtion measurements ($\varepsilon_{Trp} = 6170\ M^{-1}\ cm^{-1}$ at 282 nm).

CD spectra were recorded at 293 K (bandwidth 2 nm, scanning speed 20 nm/min, response 2 s), using a Jasco (Tokyo, Japan) model J-715 spectropolarimeter equipped with a Haake thermostat (Thermo Fisher Scientific, Waltham, MA, USA), averaging eight scans. Baselines were corrected by subtracting the solvent contribution, and CD curves were smoothed by the binomial method provided by the Spectra Analysis program (Jasco). Cylindrical, fused quartz cells of 0.5 and 0.2 mm path length (Hellma, Müllheim, Germany) were employed. The data are expressed in terms of $[\theta]_R$, the mean residue ellipticity (degree·cm^2·$dmol^{-1}$). CH_3OH 99.9% and TFE 99.5%, spectrophotometric grade, were purchased from Sigma. SDS, 99% purity, was a Pierce Chemical Co. (Rockford, IL, USA) product and was used without recrystallization. Deionized water was further purified using a milliQ reagent grade water system from Millipore.

Liposome Leakage Assay

Peptide-induced leakage from egg phosphatidylcholine vesicles was measured at 293 K using the CF-entrapped vesicle technique [13] and a Perkin–Elmer model MPF-66 spectrofluorimeter. CF-encapsulated small unilamellar vesicles (egg PC/cholesterol, 7 : 3) were prepared by sonication in Hepes buffer, pH 7.4. The phospholipid concentration was kept constant (0.06 mM), and increasing peptide/lipid molar ratios (R^{-1}) were obtained by adding aliquots of methanol solutions of peptides, keeping the final methanol concentration below 5% by volume. After rapid and vigorous stirring, the time course of fluorescence change corresponding to CF escape was recorded at 520 nm (6 nm band pass) with λ_{exc} 488 nm (3 nm band pass). The percentage of released CF at time t was determined as $(F_t - F_0)/(F_T - F_0) \times 100$, with F_0 = fluorescence intensity of vesicles in the absence of peptide, F_t = fluorescence intensity at time t in the presence of peptide, and F_T = total fluorescence intensity determined by disrupting the vesicles by addition of 50 μl

Table 3. Antibacterial activity of tylopeptin B (**T**) and its analogues **6** and **9**[a]

Bacterial strain	Diameter inhibition zone (millimeter)				
	Bacitracin	Tetracyclin	Tylopeptin B	**6**	**9**
Staphylococcus aureus ATCC 25923	20	31	12	11	10
Streptococcus pyogenes ATCC 19615	nd[b]	32	19	18	19
Escherichia coli ATCC 25922	25	28	8	7	7
Pseudomonas aeruginosa ATCC 27853	26	nd	—[c]	—	—
Klebsiella pneumoniae ATCC 13883	nd	26	7	—	—
Salmonella enteritidis 13076	nd	26	—	—	—
Proteus mirabilis ATCC 10975	nd	32	—	—	—

[a]Peptide concentration: 100 μg/disk. Bacitracin and tetracyclin were used as control (concentration: 10 μg/disk).
[b]nd, not determined.
[c]no inhibition zone.

of a 10% Triton X-100 solution. The kinetics experiments were stopped at 20 min.

Results and Discussion

Solid-Phase Synthesis of Mono-Substituted and Bis-Substituted TOAC Tylopeptin B Analogues

The high proportion of sterically hindered $C^{\alpha,\alpha}$-dialkylated α-amino acids and the presence of acid-sensitive sequences (e.g., Aib-Pro/Hyp) in peptaibiotics have discouraged the synthesis of these peptides by the solid-phase methodology for a long time. With the advent of very acid-labile resin, effective coupling reagents and, more recently, microwave irradiation, the number of peptaibiotics synthesized automatically on solid-phase has constantly increased [14,15]. We too have recently reported the solid-phase synthesis of tylopeptin B using the Fmoc/*t*But methodology, starting from the acid-sensitive *H*-Lol-2-chlorotrityl resin [2]. To extend this methodology to the synthesis of TOAC-containing tylopeptin B analogues, two additional difficulties have to be taken into account: (i) the low reactivity of the amino function of the TOAC residue [8], which hampers down the incorporation of the next amino acid residue in the sequence; and (ii) the sensitivity of the nitroxide moiety to strongly acidic conditions [8,16], commonly used for peptide cleavage from the resin and/or side-chain deprotections, which can destroy the free-radical character of the TOAC residue. Despite the possibility to regenerate the nitroxyl radical by submitting the crude peptide to a basic treatment [8,16], we took the opportunity of the presence of a resin linker cleavable under very mild acid conditions [10] to change our synthetic strategy. By adopting a minimum protection strategy, Gln and Trp residues were introduced without side-chain protecting groups, as preformed active esters, and only the alcoholic function of Ser was protected by the fluoride sensitive TBDMS group. After the assembly of peptides on the solid support, the TBDMS group was easily removed by a short treatment with 0.1 M TBAF in DMF, and the peptide alcohols were cleaved from the resin by a mild acid treatment, which does not destroy the free-radical character of the TOAC residue.

Analysis by RP-HPLC of the tylopeptin B analogues revealed marked differences in the homogeneity of the eluted materials (Figure 1). Mono-substituted analogues with TOAC at position 8 or 13 were the most homogeneous compounds, presenting a major HPLC peak corresponding to the expected peptide. On the contrary, several by-products were formed during the synthesis of analogues containing a TOAC residue at position 3 or 4. Here, the desired compound was obtained only as a minor component (Figure 1(C) and (D)), even by extending coupling times and using microwave irradiation. MS analysis revealed that most of the by-products corresponded to sequences missing one or more residues *after* the TOAC amino acid. This finding suggested that a higher structuration of the growing 10-mer peptide occurred [16], possibly favored by aggregation phenomena involving the unprotected side chains of the two Gln residues [17].

For the synthesis of [$TOAC^{3,13}$]tylopeptin B, we decided to protect the Gln and Trp side chains, in addition to the Ser alcoholic function. The efficacy of this strategy was tested first by repeating the synthesis of the mono-substituted analogue [$TOAC^{8}$]tylopeptin B. The resulting peptide, protonated at the nitroxide group after the acidic treatment (95% TFA) required to remove the side-chain protecting groups [18], was eluted about 6 min before the corresponding free radical. Under alkaline conditions (20 mM ammonium acetate, pH 10) [6], the recovery of the nitroxyl radical character was completed in 5 h (Figure 2(A)). The ESR spectrum confirmed the regeneration of the free radical of the TOAC

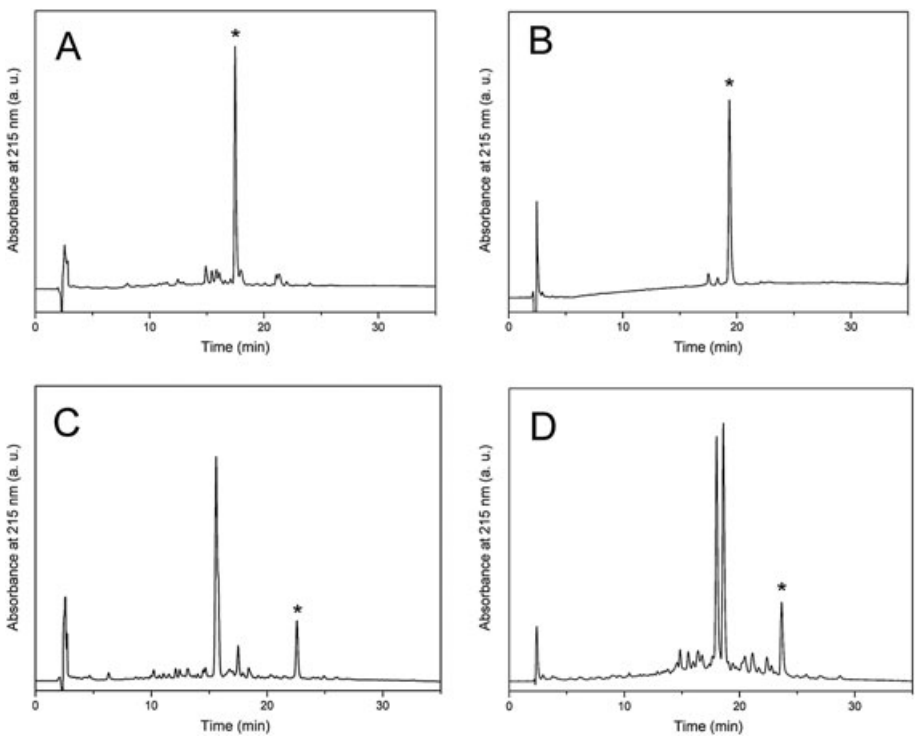

Figure 1. HPLC profiles of the crude synthetized analogues: (A) [$TOAC^{8}$]tylopeptin B (**2**); (B) [$TOAC^{13}$]tylopeptin B (**1**); (C) [$TOAC^{3}$]tylopeptin B (**4**); (D) [$TOAC^{3,13}$]tylopeptin B (**5**). Starred peaks refer to the expected products.

Journal of PeptideScience

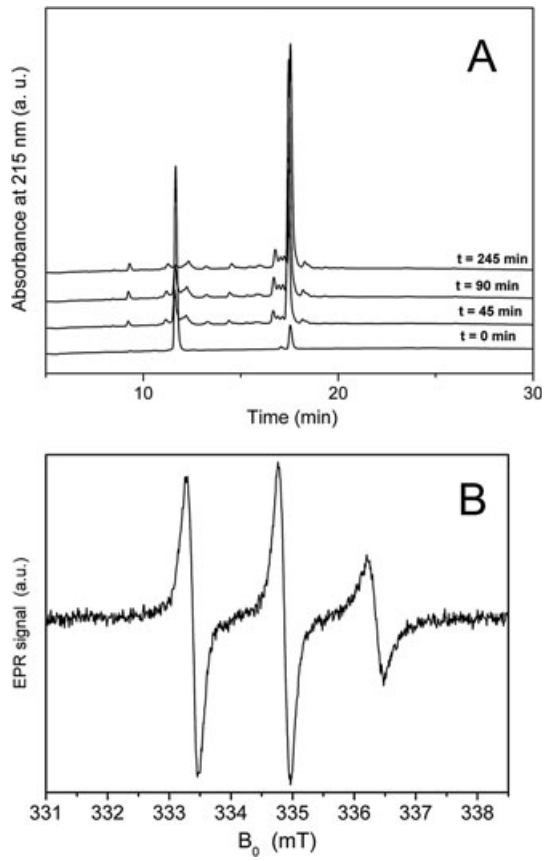

Figure 2. Regeneration of the free-radical character in the [TOAC[8]]tylopeptin B analogue (**2**) after side-chain deprotection with 95% aqueous TFA. (A) HPLC profiles at different times of the reaction mixture during the alkaline treatment with 20 mM ammonium acetate (pH 10); (B) ESR spectrum of the peptide in $CHCl_3$ after reconstitution of the radical (a_N = 1.485 mT; *g* factor = 2.0044).

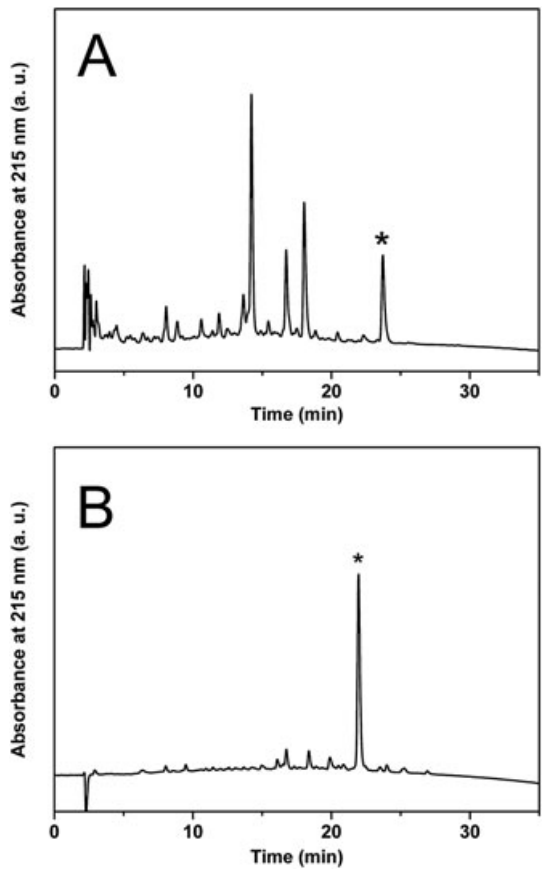

Figure 3. (A) HPLC profile of the crude [TOAC[3, 13]]tylopeptin B analogue (**5**), after the alkaline step required to regenerate the nitroxyl moieties; (B) HPLC profile of the crude [Ala[2], TOAC[3, 13]]tylopeptin B analogue (**11**). Starred peaks refer to the expected products.

residue (Figure 2(B)). Despite the promising results obtained for the mono-substituted analogue, the same strategy applied to the [TOAC[3, 13]] tylopeptin B analogue did not improve the outcome of the synthesis. Again, we obtained several by-products with the TOAC bis-substituted peptide as a minor component (Figure 3(A)). In summary, the synthesis of tylopeptin B analogues with TOAC replacing the Aib residue at position 3 or 4 proved to be particularly difficult, in fact not affording the corresponding bis-substituted analogues.

To achieve a higher amount of the tylopeptin B analogue doubly spin labeled at the *N*-terminal and *C*-terminal segments, we considered the possibility to modify the crowded *N*-terminal sequence by replacing Val[2] by Ala or by exchanging residues 4 and 5 so as to separate the two consecutive $C^{\alpha,\alpha}$-dialkylated amino acids -Aib[4]-Aib[5]-. As the antibacterial activity of [Ala[2]]tylopeptin B and [Ala[4], Aib[5]]tylopeptin B (respectively **9** and **6** in Table 2) resulted comparable with that of the parent peptide **T** (Table 3), we planned the synthesis of their spin-labeled analogues according to the minimum protection strategy. Using this strategy, [Ala[4], TOAC[5]]tylopeptin B (**7**) was obtained in very good yield. On the contrary, the synthesis of [TOAC[3], Ala[4], Aib[5]] tylopeptin B (**8**) failed, showing that the α-amino function of TOAC[3] was reluctant to react with the next β-branched amino acid (Val). Consequently, by replacing Val[2] by Ala in the tylopeptin B sequence, we could obtain [Ala[2], TOAC[3]]tylopeptin B (**10**) in acceptable yield. At this point, we moved on with the synthesis of the bis-substituted analogue [Ala[2],TOAC[3, 13]]tylopeptin B (**11**). The HPLC profile of the crude product revealed only a major peak, corresponding to the desired compound (Figure 3(B)).

Conformational Studies

By a combination of different spectroscopic techniques and molecular dynamics calculations, we have previously established that tylopeptin B is largely helical in solution with a preference for the α-helix or 3_{10}-helix type depending upon the nature of the solvent [2]. For the TOAC spin-labeled analogues of tylopeptin B, the conformational analysis was performed by FTIR absorption and CD spectroscopy because the presence of the paramagnetic probe dramatically reduces the efficacy of the NMR technique.

The FTIR absorption spectra in $CDCl_3$ solution of tylopeptin B and its analogues **1–4** are dominated by a very intense absorption near 3305 cm^{-1} assigned to the N–H stretching mode of strongly *H*-bonded amide groups [19–21] (as shown in Figure 4 (A) for analogues **2** and **4**). Additional but very weak bands are seen at about 3410, 3480, and 3530 cm^{-1}. The 3530 cm^{-1} band is attributed to the asymmetrical N–H stretching mode of free (solvated) primary amide groups of the Gln side chains, whereas the corresponding symmetrical stretching mode falls at 3410 cm^{-1}, overlapping the N–H stretching mode of the free (solvated) secondary amide bonds of the backbone [2]. The

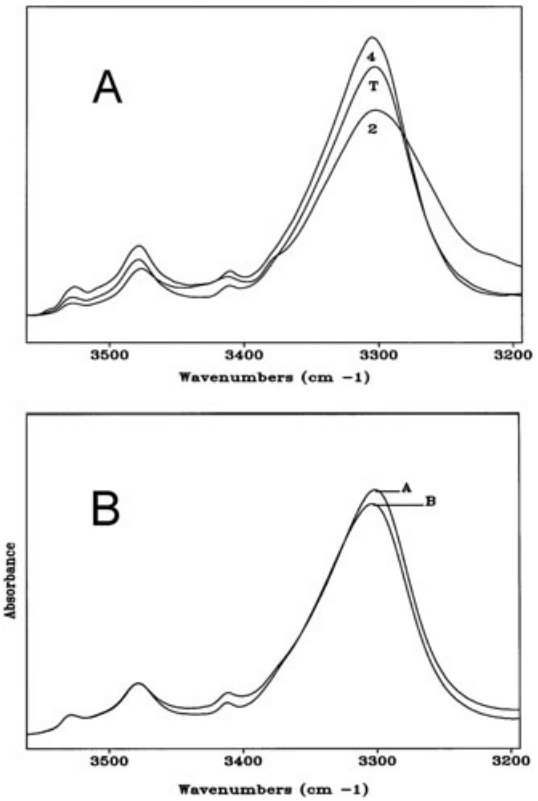

Figure 4. FTIR absorption spectra in $CDCl_3$ of the N–H stretching (amide A) region of (A) tylopeptin B (**T**) and [TOAC^{8}] (**2**) and [TOAC^{3}] (**4**) tylopeptin B analogues (peptide concentration: 1 mM); (B) [TOAC^{13}]tylopeptin B (**1**) at 1 mM (A) and 0.1 mM (B) peptide concentrations.

3480 cm^{-1} band is assigned to the indole N–H stretching mode of the Trp side chain [2]. At the lowest concentration examined (0.1 mM), the 3305 cm^{-1} band is still quite intense in the whole series of compounds, which supports the occurrence of an extensive set of intramolecular C=O···H–N hydrogen bonds, typical of an overwhelmingly helical conformation. Thus, the replacement of Aib residues in tylopeptin B by TOAC does not change the propensity of the peptide to adopt a helical structure in the apolar solvent $CDCl_3$. The spectra of the labeled tylopeptin B analogues do not change significantly upon increasing peptide concentration (as shown in Figure 4(B) for analogue **4**), which suggests that self-aggregation does not play a major role in the behavior of these analogues. The FTIR spectra of analogues **6** and **7** in the N–H (3600–3200 cm^{-1}) and C=O (1800–1600 cm^{-1}) stretching regions were characterized by similar absorption bands (Table SI2). The poor solubility in $CDCl_3$ of analogues **9–11** did not allow a structural investigation by FTIR at the tested concentrations.

In organic solvents or in micellar environment (30 mM SDS) (Figures 5(A) and SI1), the CD spectra of the labeled analogues exhibit two negative Cotton effects of moderate intensities, located near 222 nm (n–π^* transition of the peptide chromophore) and 205 nm (parallel component of the split peptide π–π^* transition) [22,23]. Any contribution by the TOAC nitroxyl side chain to the far-UV CD spectra can be neglected for such long, helical peptides [24]. This general pattern, which closely resembles that of the parent peptide [2], is reminiscent of the CD spectra of right-handed, predominantly helical peptides. Because the observed ratio between the ellipticities of the two negative bands $[\theta]_{222}/[\theta]_{205}$ is in the range 0.8–1.0 (Table SI3), it can be speculated that, in these solvents, the amount of the α-helix structure is largely prevailing over that of the 3_{10}-helix [25–28]. In aqueous environment, the spectra show a slightly decreased intensity with respect to those in organic solvents, and, most notably, the CD patterns of labeled peptides (**1**, **2**, **4**) change significantly from that of tylopeptin B (**T**) (Figure 5(B)). In particular, the spectra of the former compounds present a more intense positive band around 192–194 nm and two negative maxima at about 208 and 225 nm, which point to a progressive change in the population of the helical conformers, favoring the α-helical structure over the 3_{10}-helix, as the TOAC residue is moved from the peptide *C*-terminus to the *N*-terminus (from **1** to **4**). Thus, we obtained a direct spectroscopic demonstration that even a single TOAC residue can increase the α-helical content of peptides in aqueous environment. In Figure 6, the CD spectra of labeled and unlabeled peptides, modified at the *N*-terminal part of the tylopeptin B sequence, are presented. The CD curves of peptides **6** and **9**, both in organic (Figures 6(A) and SI2) and in aqueous (Figure 6(B)) solvents, closely resemble those of the parent peptide (**T**), showing that these analogues keep up the conformational preferences of tylopeptin B. Thus, as for tylopeptin B, the antimicrobial activity shown by **6** and **9** can be related to their ability to fold into an amphipathic helical structure in a membrane-mimicking environment. The CD spectra of the labeled analogues of [Ala^{2}]tylopeptin B, **10** and **11**, (Figures 6 and SI2) compare well with those of labeled tylopeptin B analogues **1–5** (Figures 5 and SI1), confirming that **10** and **11** can be considered good models to investigate the detailed mechanism of

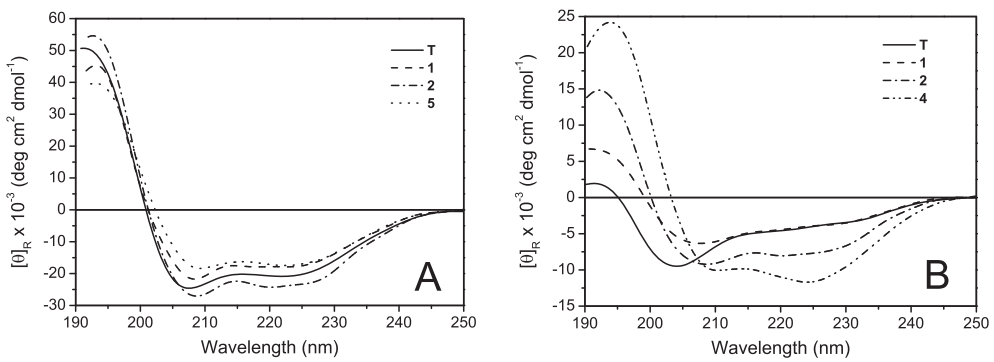

Figure 5. Far-UV CD spectra of tylopeptin B (**T**) and selected TOAC-labeled analogues (**1**, **2**, **4**, and **5**) in methanol (A) and in 10% aqueous methanol (B). Peptide concentration: 0.1 mM.

Journal of PeptideScience

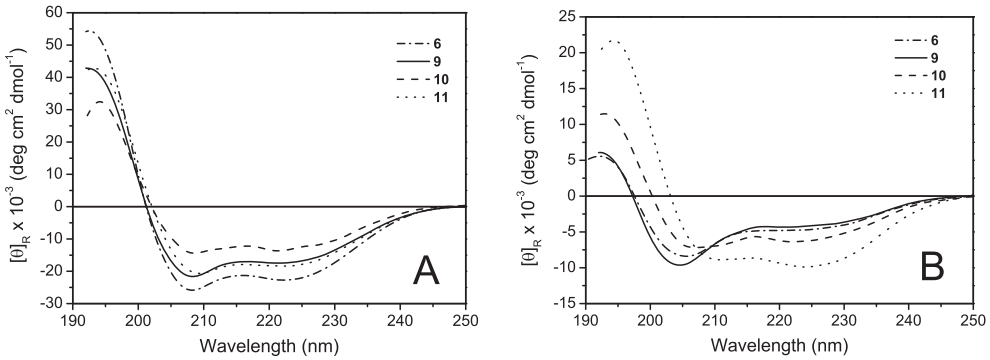

Figure 6. Far-UV CD spectra of [Ala^4, Aib^5] tylopeptin B (**6**), [Ala^2]tylopeptin B (**9**), and selected TOAC-labeled analogues (**10** and **11**) in methanol (A) and in 10% aqueous methanol (B). Peptide concentration: 0.1 mM.

membrane permeabilization by medium-length peptaibiotics by a combination of ESR techniques.

Quenching Effect of the Trp Fluorescence in TOAC-Containing Peptides

The presence of a Trp residue in the tylopeptin B sequence allowed us to test the quenching effect of the fluorescence of the indole moiety by the nitroxyl radical as a function of the position of TOAC along the peptide helix relative to the *N*-terminal Trp. Figure 7 shows the fluorescence spectra in methanol of tylopeptin B and its TOAC-containing analogues, which are characterized by a maximum near 347 nm typical for Trp in a polar environment [29]. The spectra show a progressive decrease in the fluorescence intensity as the TOAC residue moves along the peptide backbone from position 13 to 8 and to 4 of the tylopeptin B sequence, which roughly corresponds to a quenching of the Trp signal by 32%, 64%, and 94%, respectively. Considering that, in methanol, tylopeptin B and its TOAC-containing analogues adopt a prevalently helical structure, as shown by NMR [2], and by the CD spectra recorded in this work, it comes out that the ordered secondary structure keeps relatively apart the Trp/TOAC pair, which relaxes by a long-range, possibly electron transfer, mechanism [30].

Membrane Modifying Properties

A preliminary study of the membrane-modifying properties of the TOAC tylopeptin B analogues were performed by measuring the leakage of CF, entrapped in phospholipidic vesicles, induced by increasing amount of peptide. As shown in Figure 8, the tested labeled analogues [TOAC^{13}] (**1**) and [TOAC^8]-tylopeptin B (**2**) exhibit a very high activity in this membrane permeabilization assay, only slightly smaller than that of our reference peptaibiotic trichogin GA IV, but comparable with that of the parent peptide.

Conclusions

A series of analogues of the medium-length peptaibiotic tylopeptin B was synthesized in which one or two Aib residues in the sequence were replaced by the nitroxide spin-labeled C$^{\alpha,\alpha}$-disubstituted α-amino acid TOAC. The solid-phase synthesis of these peptides, containing a large amount of sterically hindered residues and the acid-labile nitroxide free radical, was performed either by the standard Fmoc/*t*But strategy, with regeneration of the free radical of the TOAC residue after the final deprotection step, or by the Fmoc/TBDMS strategy, which, avoiding any harsh acidic treatment of the peptide resin, preserves the spin label intact. Both strategies gave comparable results in the synthesis of the labeled tylopeptin B analogues, but the replacement of the Aib residue at position 3 or 4 by TOAC was particularly troublesome. As a consequence, to obtain the bis-substituted [TOAC^{3,13}] tylopeptin B analogue, we chose as parent peptide the less hindered [Ala^2]tylopeptin B, which presents an antibacterial activity comparable with that of the natural peptide. Conformational studies, carried out by FTIR absorption and CD spectroscopy,

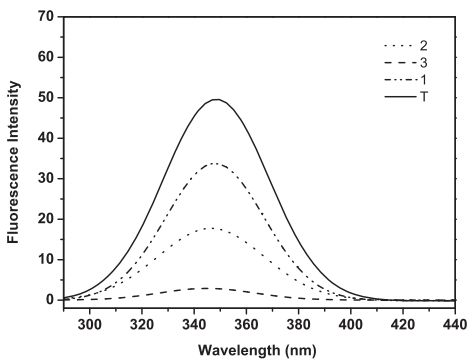

Figure 7. Trp fluorescence spectra of tylopeptin B (**T**) and selected TOAC-labeled analogues (**1–3**) in methanol. Peptide concentration: 5 μM.

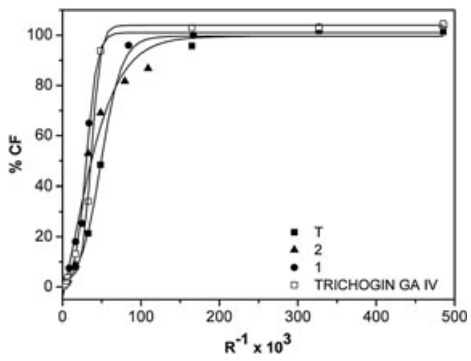

Figure 8. Peptide-induced carboxyfluorescein (CF) leakage, for different ratios peptide/lipid (R^{-1}) from egg PC/cholesterol unilamellar vesicles for tylopeptin B (**T**) and selected TOAC-labeled analogues (**1** and **2**) and for the reference peptaibiotic trichogin GA IV.

demonstrated that all of the TOAC-based tylopeptin B analogues adopt a helical (mostly α-helical) structure even in an aqueous environment. They also exhibit membrane-modifying properties comparable with those of the parent peptide.

In summary, our TOAC spin-labeled analogues of tylopeptin B can be considered good models to study in detail, by a combination of ESR techniques, the mechanism of membrane permeabilization by medium-length peptaibiotics [Milov AD, Gobbo M, Toniolo C, Tsvetkov Yu D, Pulsed electron-electron double resonance (PELDOR) of two doubly labelled peptides: tylopeptin B and heptaibin. Determination of distances between labels and effects of orientation. Submitted for publication].

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