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Chromatographic purification and characterization of synthetic tryptophan-substituted gramicidin A analogues[1]

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Abstract

We report here experimental conditions for the one-step preparative purification of synthetic gramicidin A and 19 tryptophan-substituted analogues (with either phenylalanine or tyrosine residues) using reversed-phase chromatography. Methanol–water based mobile phases have been shown suitable for the elution of gramicidin analogues with one and two Trp→Phe substitutions, while addition of isopropanol was required for the purification of peptides with more than two Trp→Phe replacements. Using analytical RP-HPLC it is demonstrated that peptide retention increases as a monotonic function of the number of Trp→Phe substitutions, and that the position of a given amino acid substitution has little influence on peptide retention. All purified synthetic peptides give a clean, sharp characteristic peak which indicates the effectiveness of the purification protocol used. Size-exclusion HPLC reveals that purified Trp-substituted gramicidins exhibit a dimer/monomer conformational equilibrium in organic solvents similar to that observed for the naturally occurring gramicidin mixture. The usefulness of this series of Trp-substituted gramicidins in conformational studies of gramicidin incorporated in model membranes is also considered.

Keywords: Preparative chromatography; Gramicidin A analogues; Peptides; Antibiotics

1. Introduction

The linear gramicidins are pentadecapeptide antibiotics composed of alternating L- and D-amino acids. The sequence of gramicidin A (gA) is: formyl-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-ethanolamine. It has one of the most hydrophobic sequences known which span the lipid bilayer to form a monovalent cation-selective channel organized as a dimer of $\beta^{6.3}$ right-handed helices linked at the N-terminal ends [1–3]. The interest of the study of gramicidin is not only related to its function as an ion channel but also as a model for membrane-spanning regions of intrinsic membrane proteins and for protein/lipid interactions in general. Another motivation for the study of gramicidin as a model compound concerns protein folding, in particular the importance of the environment in determining the secondary structure of peptides and proteins [4]. It is well known that gA can adopt a variety of different monomeric and dimeric structures depending on its local environ-

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ment (organic solvents, lipid media, normal micelles, etc [1–3]). A time-dependent gramicidin dimer/monomer conformational equilibrium has been quantitatively characterized by size-exclusion HPLC in organic solvents [5–8], the predominant conformer at equilibrium in nonpolar solvents such as tetrahydrofuran (THF) being an intertwined helical dimer [5,6]. In contrast, in more polar solvents, such as dimethylsulphoxide or trifluoroethanol, monomeric forms predominate at equilibrium [5], the secondary structures of which being a function of the nature of the organic medium [9].

Upon incorporation into lipid bilayers from nonpolar solvents gA double-helices refold into $\beta^{6.3}$ helical dimers (channel state) [10–13]. However, it is still unclear why the intertwined helix is unstable in membranes while the channel state is very stable [14,15]. From spectroscopic investigations [16,17] it has been suggested that the interaction of gramicidin tryptophan residues with the bilayer surface, through Trp indol-NH hydrogen bonding, can be one of the driving forces responsible of this lipid-induced conversion [15]. Interestingly, using size-exclusion HPLC we have recently demonstrated that an all Trp→Phe substitution in gA dramatically changes its conformation in model membranes, resulting in a conformational rearrangement from β-helical monomers to double-stranded (ds) dimers [18]. This emphasizes the crucial role of the tryptophan residues in the stabilization of peptide's β-helical conformation in a lipid environment and underlines to which extent the interplay between amino acid sequence and environment may ultimately dictate the final folded state of a membrane peptide. In this context the spectroscopic observations of Sawyer et al. [19] with gramicidin B (in which Trp^{11} is replaced by Phe) also point to the importance of even single tryptophans for the unwinding of ds-dimers in a lipid environment.

In order to assess the specific role of each individual Trp side chain in the conversion of intertwined dimers to monomers in lipid media, the synthesis of a series of Trp-substituted gramicidins with systematic changes of one, two, three and four aromatic residues was planned. Recent developments in multiple synthesis have made it feasible to synthesize large numbers of peptide analogues simultaneously [20]. In this preliminary paper, we report on the purification and chromatographic behaviour of these extremely hydrophobic peptides in reversed-phase (RP) supports in preparative and analytical modes. Conditions for purification of linear gramicidins from a natural occurring mixture [21–24] and of some analogues with different amino acid substitutions obtained by more standard synthetic procedures have been reported by several laboratories [23,25,26]. Further characterization of the dimer/monomer conformational equilibrium of purified synthetic gramicidin analogues in organic solvent has been carried out using size-exclusion HPLC as previously described for the native peptide [27].

2. Experimental

2.1. Materials

Gramicidin (natural mixture) (gA′) was purchased from Sigma (St. Louis, MO, USA) and was used without further purification. All organic solvents were either HPLC or spectroscopic grade from Merck (Darmstadt, Germany). Doubly-distilled water was purified by passing it through a Milli-Q system (Millipore, Bedford, MA, USA). The eluents were always filtered and degassed through a 0.45-μm nylon filter (Micron Separation, Westboro, MA, USA) except for THF which was passed through a 0.50-μm regenerated cellulose filter (Micro Filtration Systems, Dublin, CA, USA). All other chemicals were of analytical grade.

2.2. Apparatus

The instrumentation for analytical HPLC (from Waters Chromatography Division, Milford, MA, USA) consisted of M-510 solvent-delivery systems, an automated gradient controller, a U6K universal injector and a photodiode array detector Model 996 (in some cases an M-481 or 490 multiwavelength detector was used). Chromatograms were stored and processed in a computer using the Millenium Chromatography Manager Software (Waters).

Preparative RP-HPLC was carried out using a Waters Delta Prep 3000 system. Absorbance was monitored with a Waters M-481 detector coupled to

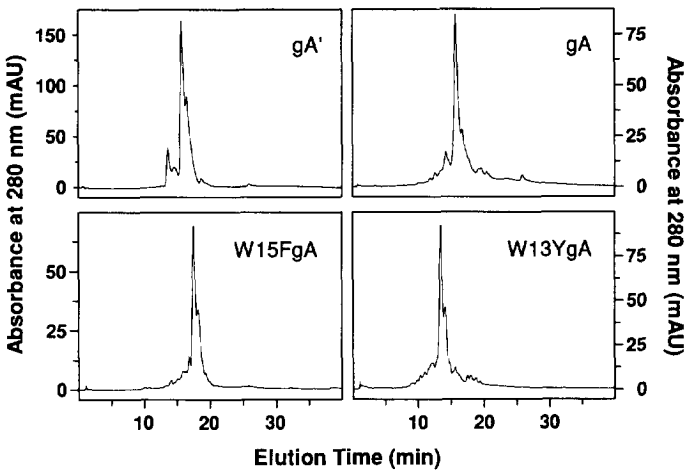

Fig. 1. Analytical RP-HPLC profiles of gA' and crude synthetic peptides gA, W15FgA and W13YgA under gradient conditions. Chromatography was performed with a linear gradient from methanol–water 70:30 to 100% methanol in 30 min followed by an isocratic period of 10 min. Flow-rate, 1.0 ml/min. In all cases 25 μl of a 1 mg/ml peptide sample was injected.

an SP-4290 integrator (Spectra-Physics, San Jose, CA, USA).

2.3. Peptide synthesis

Peptides were synthesized by simultaneous multiple peptide synthesis methodology [20] using a combination of t-Boc and Fmoc chemistry on co-poly(styrene–1% divinylbenzene) phenylacetamidomethyl (Pam) resin (0.55 mequiv./g resin). The peptides were cleaved from the resin by treatment with ethanolamine. The identities of the peptides were confirmed by time-of-flight mass spectroscopy using a BIOION 20 spectrometer. Details of the peptide synthesis will be published elsewhere (manuscript in preparation). The synthetic gramicidins were the following: gA, four analogues with one Trp→Phe substitution (W9FgA, W11FgA, W13FgA and W15FgA), six with two substitutions [W(9,11)FgA, W(9,13)FgA, W(9,15)FgA, W(11,13)FgA, W(11,15)FgA and W(13,15)FgA], four with three substitutions [W(9,11,13)FgA, W(9,11,15)FgA, W(9,13,15)FgA and W(11,13,15)FgA], one with four substitutions [W(9,11,13,15)FgA], and four with one Trp→Tyr substitution (W9YgA, W11YgA, W13YgA and W15YgA). These substituted gramicidin analogues have been named with the abbreviation of the native peptide, gA, preceded by: first, the one-letter code of the original amino acid; second, its sequence position(s); and third, the one-letter code of the substituting amino acid.

2.4. High-performance liquid chromatography

Preparative separations for each peptide were conducted at room temperature on a Waters Delta-Pak C_{18} cartridge (d_p 15 μm, pore size 300 Å, 100×25 mm I.D.), at a flow-rate of 20.0 ml/min. Crude peptide (25–50 mg) was dissolved in the mobile phase mixed solvent, filtered through a 0.45-μm syringe filter Acrodisc CR PTFE (Gelman Sciences, Ann Arbor, MI, USA) and injected. The detector was set at 222 or 280 nm. Fractions of 25 ml were collected and their purity was controlled by analytical RP-HPLC. Fractions of the desired purity (>95%) were combined and, after removal of the solvent, the peptide was lyophilized from glacial acetic acid. Gramicidin concentrations were determined by vacuum drying to constant weight and verified by UV spectroscopy [23] and/or by size-exclusion HPLC.

Analytical RP-HPLC was carried out using a LiChrospher 100 RP-18 (d_p 5 μm, 125×4 mm I.D.) cartridge from Merck. For crude peptide analysis, injections between 15–50 μl of 1 mg/ml peptide concentration were eluted at room temperature at a flow-rate of 1.0 ml/min. Separations were achieved

using either a linear gradient of 70 to 100% methanol in water in 30 min, or isocratically using a mobile phase of methanol–water or methanol–isopropanol–water at different compositions depending on the number of Trp substitutions in the peptide (see below).

Size-exclusion chromatography was performed at room temperature using a Waters 100-nm pore-size Ultrastyragel 1000 Å column (300×7.8 mm I.D.) eluted with THF [5–8,27]. The flow-rate was 1.0 ml/min and the eluting peptides were detected at 280 nm except for the analogue with four Trp→Phe substitutions, which was detected at 260 nm.

3. Results and discussion

We first examine peptide crude preparations using analytical RP-HPLC in gradient conditions. Fig. 1 shows, as an example, the elution profiles corresponding to the commercially available gA′, synthetic gA, and analogues W15FgA and W13YgA. The reference run of gA′ (a mixture of gramicidin A, B and C) illustrates the resolution of the column and resolves the gA dominant species (elution time 15.7 min), gramicidin C (elution time 13.6 min) and gramicidin B (elution time 18.6 min). Gramicidin B and C differ in having Phe and Tyr, respectively, in place of Trp in position 11. The peak eluted after the main gA and gramicidin C ones corresponds to the species present in the natural mixture with Ile instead of Val in position 1. The resolution observed for gA′ in Fig. 1 is similar to that reported by Koeppe and Weiss [21] in the purification of gramicidins A, B and C from the naturally occurring mixture from *Bacillus brevis* using a phenyl-silica column, and to that reported by Fields et al. [23] using a RP-C_{18} support under isocratic conditions. In the chromatograms corresponding to synthetic gramicidins shown in Fig. 1, a main peak appears at different retention times, i.e. 15.7 min for gA, 17.5 min for W15FgA and 13.4 min for W13YgA, which represents the major component of the crude product. It should be noted that the retention times for the analogues W11FgA and W11YgA were identical to those obtained for gramicidin B and C, respectively, from the natural mixture. The presence of one Phe residue in the peptide sequence with a more hydrophobic phenyl side chain relative to the indol group of tryptophan causes an increase in the peptide retention time. This can be ascribed to favoured peptide interactions with the alkyl chains of the support. In fact, this change in retention characteristics was similar to that observed for gramicidin B relative to gA in the natural mixture (see Fig. 1). Furthermore, this chromatographic behaviour is in agreement with recent HPLC studies on peptides related to gramicidin A [28] and gramicidin S (a hydrophobic cyclodecapeptidic antibiotic) [29] which shows that the antibiotics with a higher effective hydrophobicity are eluted more slowly from an RP-octadecanoyl column with methanol–water based mobile phases. As expected, the retention time for the synthetic analogue with a Trp→Tyr replacement was smaller than gA. In this respect, a similar change on protein retention in hydrophobic interaction chromatography has been recently reported for a small recombinant protein [30] when examining the effect of a single Tyr→Trp amino acid substitution.

Similar elution profiles were obtained for the remaining synthetic gramicidins studied in this work (not shown). As a general rule, it was evidenced that the retention time of the main peak varies as a function of the number of Trp→Phe substitutions. Thus, the presence of an additional Phe residue results in a retention in peptide elution of 2–3 min. Curiously, this effect seems additive as demonstrated in the retention time values obtained for analogues with two, three and four Trp→Phe replacements (Table 1). The monotonic increase in the retention time suggests that the interactions of the phenyl groups with the support are largely important in the peptide elution behaviour. On the other hand, the results seem to rule out possible intramolecular stacking interactions between aromatic rings of peptide side chains which are clustered at the C-terminal end during the elution of the peptides. It should be noted that for all these series of gramicidin analogues the relative position of the Phe residue in the sequence has little influence on the chromatographic retention of the peptides, which is mainly governed by the number of phenylalanine residues (see Table 1).

It is well known that gA′ can be present in organic solvents in dimeric and monomeric forms [5–8], and that the ratio between the two forms varies as a

Table 1
Retention times corresponding to synthetic gA and tryptophan-substituted gramicidins in analytical RP-HPLC under gradient conditions

Peptide	Retention time (min)
gA	15.7±0.1
W9FgA, W11FgA, W13FgA, W15FgA	18.0±0.6
W(9,11)FgA, W(9,13)FgA, W(9,15)FgA W(11,13)FgA, W(11,15)FgA, W(13,15)FgA	21.0±0.8
W(9,11,13)FgA, W(9,11,15)FgA W(9,13,15)FgA, W(11,13,15)FgA	23.5±0.8
W(9,11,13,15)FgA	26.0±0.2
W9YgA, W11YgA, W13YgA, W15YgA	13.6±0.2

function of solvent polarity and peptide concentration, among other factors [5,6,27]. In fact, the occurrence of dimeric species, such as double-stranded antiparallel helices, eluting after the main peak has been suggested in the purification of synthetic gramicidin C [23]. Thus, by injection of several diluted aliquots prepared from each corresponding stock 1 mg/ml sample it was verified that for the peptide analogues studied here the peak eluting after the main gramicidin peak did not correspond to dimeric forms. On the other hand, the crude peptide preparations eluted from a size-exclusion column, as described previously for the natural gramicidin mixture [5,27], give rise to one main peak corresponding to the monomeric species. Additional evidence supporting the above results was obtained when a synthetic gA sample was prepared containing a high proportion of ds-dimers (about 50% as deduced from size-exclusion HPLC): the elution profile in RP-HPLC was identical to that observed from a sample with the same peptide concentration but containing more than 98% of monomeric forms.

Next, crude peptide samples were purified in one-step over a reversed-phase preparative column. Originally, a mobile phase consisting of methanol–water was used for gA and single amino acid substituted analogues [21,23,28]. A linear gradient from methanol–water 70:30 (v/v) to 100% methanol in 30 min allows the recovery of gramicidins with high purity (see below). However, these chromatographic conditions were not optimal for preparative separations of analogues with two, three or four Trp→Phe substitutions due to higher hydrophobicities of the peptides (which are retained into the column) and poorer solubilities in the initial mobile phase at the concentrations used in preparative conditions. In fact, it was necessary to fit the eluent composition for either to improve peptide dissolution or to minimize adsorptive interactions with the alkyl chains of the solid support. Good resolutions and recoveries were achieved for analogues with two Trp→Phe substitutions using linear gradients from methanol–water 80:20 (v/v) to 100% methanol in 30 min. Linear gradients from methanol–isopropanol–water 80:0:20 (v/v) to 75:25:0 (v/v) and from 60:20:20 (v/v) to 50:50:0 (v/v) in 30 min were used for analogues with three and four Trp→Phe substitutions, respectively. These non-buffered methanol–water mobile phases can be successfully used without irreversible binding of the peptide to the reversed-phase support. Buffered methanol–water eluents as described by Fields et al. [23] also proved to be adequate in purification of gA analogues with higher hydrophobicities.

All fractions derived from the preparative separations were collected and monitored by analytical RP-HPLC under gradient conditions in a routine way. Fractions of the desired purity (>95%) were combined and analyzed by RP-HPLC under isocratic conditions. For this purpose, the mobile phase basically consisting of a mixture of methanol–water was slightly modified for the analysis of each series of Trp-substituted gramicidins in order to achieve elution of all peptides with optimal resolution between 4.5–6 min. For single- and di-substituted Trp→Phe gramicidins a small increases in the methanol proportion was enough whereas for tri- and four-substituted analogues some additional amounts of iso-

Table 2
Mobile-phase composition for peptide elution in 4.5–6 min in analytical RP-HPLC under isocratic conditions

Peptide	Eluent composition
gA	Methanol–water 80:20 (v/v)
W9FgA, W11FgA, W13FgA, W15FgA	Methanol–water 82:18 (v/v)
W(9,11)FgA, W(9,13)FgA, W(9,15)FgA W(11,13)FgA, W(11,15)FgA, W(13,15)FgA	Methanol–water 85:15 (v/v)
W(9,11,13)FgA, W(9,11,15)FgA W(9,13,15)FgA, W(11,13,15)FgA	Methanol–isopropanol–water 60:20:20 (v/v)
W(9,11,13,15)FgA	Methanol–isopropanol–water 63:21:16 (v/v)
W9YgA, W11YgA, W13YgA, W15YgA	Methanol–water 78:22 (v/v)

propanol were required (Table 2). As an example, Fig. 2 compares the elution profiles of the purified synthetic peptides gA, W15FgA, W(9,13)FgA and W(9,11,15)FgA with those corresponding to the crude peptide preparations. As demonstrated in the chromatograms a high degree of purity was achieved in the one-step purification of these analogues, each peptide exhibiting a sharp, characteristic elution profile. Also, similar results were obtained for the remaining synthetic analogues. The amino acid analysis of each purified peptide was within experimental error of theoretical values (data not shown).

Since size-exclusion HPLC has proved to be an excellent technique to monitor changes in the dimer/monomer conformational equilibrium of the natural gA mixture in organic solvents [5–8] it seemed interesting to verify whether the synthetic gramicidin analogues exhibit a similar conformational behaviour in these media. For this objective single-, di- and tri-substituted gramicidin samples were independently dissolved in a given pure organic solvent and 2-μl aliquots of each one were taken at different times and directly injected onto the size-exclusion column eluted with THF.

As an example, the chromatographic results illustrated in Fig. 3A for W(13,15)FgA indicate the presence of two peaks with elution volumes identical to those obtained for gA′ ds-dimers (7.9 ml) and gA′ monomers (8.4 ml) [5,12,13,27]. They also show that the monomers present in the ethanol sample at zero time (about 50 s after solvent addition to peptide powder) refolds to intertwined dimers to reach a final equilibrium state where the ds-dimeric configuration is clearly favoured. More interestingly,

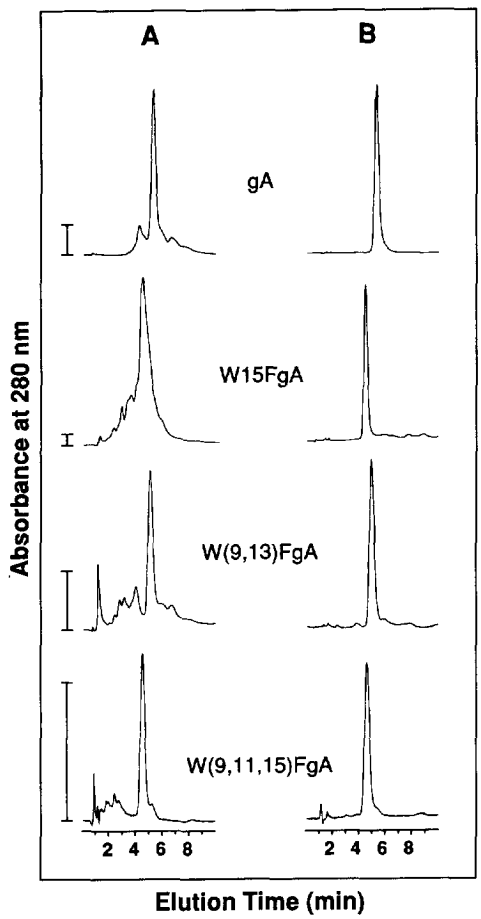

Fig. 2. Elution profiles corresponding to the synthetic gramicidins gA, W15FgA, W(9,13)FgA and W(9,11,15)FgA in analytical RP-HPLC under isocratic conditions. (A) Crude peptides; (B) purified peptides. For each peptide the mobile-phase composition is described in Table 2. Flow-rate, 1.0 ml/min. Bars on the left represent an absorbance of 4 mAU for each pair of chromatograms of a given analog (crude and purified).

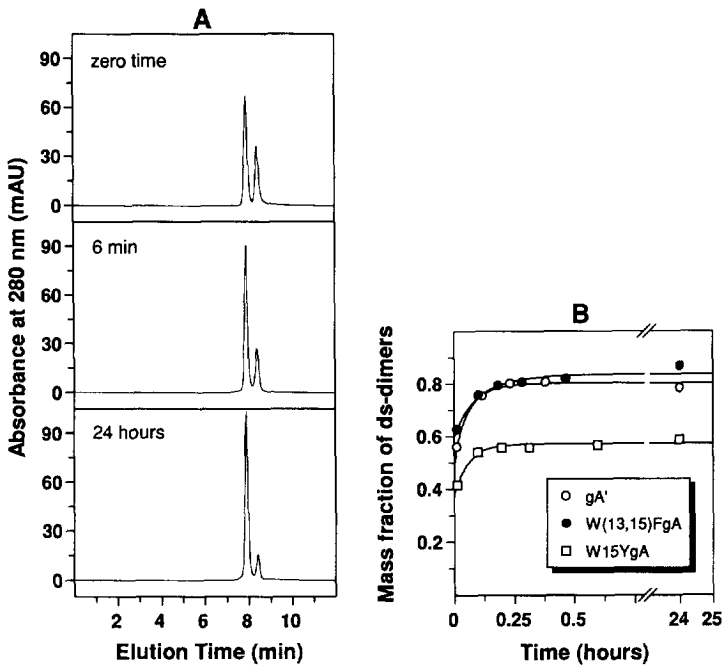

Fig. 3. (A) Chromatograms corresponding to a purified W(13,15)FgA sample at three different times of incubation at room temperature in ethanol. (B) Kinetic profiles of the dimerization process in ethanol for gA′ and purified W(13,15)FgA and W15YgA; the mass fraction of ds-dimers is plotted against the time at which each aliquot was taken. The lines correspond in all cases to the fitting of the experimental data to a simple model of reversible dimerization. Chromatography was carried out at a flow-rate of 1.0 ml/min with tetrahydrofuran as eluent on a size-exclusion Ultrastyragel 1000 Å column. In all cases the peptide concentration was 1 m*M* and the injection volume was 2 μl.

Fig. 3B shows that the dimer/monomer ratio in organic solution either at a given time or at equilibrium varies with both the number of substituted tryptophans and the nature of the side chain. Thus, the higher the number of phenyl groups the higher the extent of dimerization. In this context, it should be noted that infrared spectroscopic investigations in this solvent using gramicidin analogues where tryptophans are replaced by non-coded aromatic amino acids reveal that modification of all or some side chains can alter the dimer/monomer equilibrium as deduced from the analysis of the peptide Amide I band [25]. In addition, in our case, it is also remarkable that the analogues with one tyrosine depict a decrease in the mass fraction of ds-dimers (about 0.57) regardless of the position of the residue in the sequence when examined under identical conditions as gA′. In nonpolar solvents such as THF a more slow time-dependent dimerization was also observed for the analogues included in Fig. 3B but the proportion of ds-dimers at equilibrium was higher than in ethanol, reaching about 78% of dimeric forms for W15YgA (not shown).

The previous quantitative characterization of the dimer/monomer conformational equilibrium of Trp-substituted gramicidins in organic solvents is essential in order to select experimental conditions to prepare peptide samples with a given ratio of conformers for further incorporation in lipid environments as we have demonstrated in studies on the conformational transitions of gramicidin A [12,13,27] and their optically reversed analogue W(9,11,13,15)FgA$^-$ [18] in phospholipid model membranes. The use of size-exclusion HPLC in combination with spectroscopic techniques will be very useful in the elucidation of the role of each tryptophan residue of gramicidin due to its importance in the unwinding of ds-dimers in the bilayer and in the stabilization of the channel gramicidin configuration [13,15–19]. Studies in this direction are now in progress using these

series of Trp-substituted gramicidin analogues inserted in different phospholipid model systems.

Acknowledgments

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