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Membrane Interactions of Natural Cyclic Lipodepsipeptides of the Viscosin Group

Niels Geudens [a,1], Mehmet Nail Nasir [b,1], Jean-Marc Crowet [b], Jos M. Raaijmakers [c], Krisztina Fehér [a], Tom Coenye [d], José C. Martins [a], Laurence Lins [b], Davy Sinnaeve [a,*], Magali Deleu [b,**]

[a] NMR and Structural Analysis Unit, Ghent University;, Belgium
[b] Laboratory of Molecular Biophysics at Interfaces, University of Liège, Gembloux, Belgium
[c] Department of Microbial Ecology, Netherlands Institute of Ecology, Wageningen, Netherlands
[d] Laboratory of Pharmaceutical Microbiology, Ghent University, Belgium

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ABSTRACT

Many *Pseudomonas* spp. produce cyclic lipodepsipeptides (CLPs), which, besides their role in biological functions such as motility, biofilm formation and interspecies interactions, are antimicrobial. It has been established that interaction with the cellular membrane is central to the mode of action of CLPs. In this work, we focus on the CLPs of the so-called viscosin group, aiming to assess the impact of the main structural variations observed within this group on both the antimicrobial activity and the interaction with model membranes. The antimicrobial activity of viscosin, viscosinamide A, WLIP and pseudodesmin A were all tested on a broad panel of mainly Gram-positive bacteria. Their capacity to permeabilize or fuse PG/PE/cardiolipin model membrane vesicles is assessed using fluorescent probes. We find that the Glu2/Gln2 structural variation within the viscosin group is the main factor that influences both the membrane permeabilization properties and the minimum inhibitory concentration of bacterial growth, while the configuration of the Leu5 residue has no apparent effect. The CLP-membrane interactions were further evaluated using CD and FT-IR spectroscopy on model membranes consisting of PG/PE/cardiolipin or POPC with or without cholesterol. In contrast to previous studies, we observe no conformational change upon membrane insertion. The CLPs interact both with the polar heads and aliphatic tails of model membrane systems, altering bilayer fluidity, while cholesterol reduces CLP insertion depth.
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1. Introduction

Cyclic lipodepsipeptides (CLPs) are bacterial non-ribosomal peptides, especially reported for *Pseudomonas* and *Bacillus* spp., and are composed of an oligopeptide, cyclized through a lactone (depsi) bond, and a fatty acid moiety [1–3]. The wide variety of CLPs that have been described so far were categorized into distinct groups based on oligopeptide length, amino acid sequence and macrocycle size. For *Pseudomonas* CLPs, these groups include the viscosin, orfamide, amphisin, tolaasin, syringopeptin, syringomycin, entolysin and xantholysin groups [2,4,5]. *Pseudomonas* CLPs are involved in several secondary functions, such as cell motility, adhesion and biofilm formation [6–8], or ecological functions, such as promoting plant-growth [6,9] or triggering a defense response in plants [8,10]. Finally, CLPs have been reported to display a range of antagonistic properties, such as antifungal [11], antibiotic [12,13], insecticidal [14], antiviral [15] and antioomycete activity [16].

The viscosin group is currently one of the largest groups of CLPs, its members including viscosin [15,17], the viscosinamides [11,18], WLIP [19], the pseudodesmins [13], the massetolides [12] and the pseudophomins [20]. Some variations exist in terms of identity of the hydrophobic amino acid (Leu, Ile or Val) positions at 4 and 9 and the fatty acid tail length (10 to 12 carbons). The remaining structural variations are at positions 5, where either an L- or D-Leu residue is found, and at position 2, where a D-Glu or D-Gln residue is found (Fig. 1). This has led us to propose two ways to divide the viscosin group into subgroups: respectively into the L- and D-subgroups, and the E- and Q-subgroups. The E/Q subgroup division represents a notable structural variation, as the Glu residue in the E-subgroup represents the only ionisable group under physiological conditions, while CLPs of the Q-subgroup are always uncharged. Thus each CLP of the viscosin group can be characterized with a double label according to both types of subgroups, being EL (for instance, viscosin), ED (WLIP), QL (viscosinamide A) or QD (pseudodesmin A). (Fig. 1)

* Corresponding author at: NMR and Structural Analysis Unit, Ghent University; Campus Sterre, S4, Krijgslaan 281, B-9000 Gent, Belgium.
** Correspondence to: M. Deleu, Laboratory of Molecular Biophysics at Interfaces, University of Liège, 2 Passage des déportés, B-5030 Gembloux, Belgium.
E-mail addresses: davy.sinnaeve@ugent.be (D. Sinnaeve), magali.deleu@ulg.ac.be (M. Deleu).
[1] These authors contributed equally to this work.

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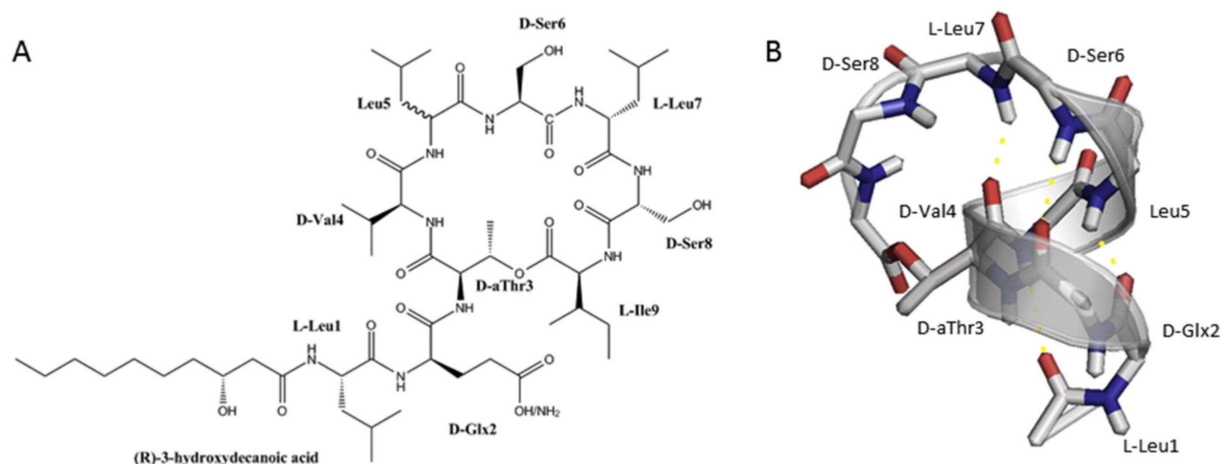

Fig. 1. A) Molecular structure of the principal viscosin group CLPs, which possess either a D-Gln (Q-subgroup) or a D-Glu (E-subgroup) at position 2, and an L-Leu (L-subgroup) or a D-Leu (D-subgroup) at position 5, B) Solution conformation of viscosinamide A, which is conserved for all viscosin group members [18]. The fatty acid tail was omitted for clarity.

The solution conformation of viscosin group CLPs has been established as a left-handed α-helix ranging from Leu1 to Ser6, followed by a short 'loop' that ends in the depsi bond between the oligopeptide C-terminus and the Thr3 side-chain (Fig. 1) [18,21]. The conformation is stabilized by the backbone hydrogen bonds Val4 NH → HDA CO, Leu5 NH → Leu1 CO, Ser6 NH → Gln2 CO, Leu7 NH → Val4 CO and Ser8 NH → Thr3 CO. The D/L stereo inversion at Leu5 that divides the viscosin group was found to have no impact on the backbone conformation, only changing the orientation of the Leu5 side-chain and slightly modifying the amphipathicity of the molecule [18].

Although antimicrobial activity has been reported for all viscosin group CLPs, the collections of microorganisms used in the individual studies are mostly non-overlapping, thus impeding a general comparison between the CLPs. For those few CLPs that were tested on the same microorganisms, some significant differences were found [12,22]. The molecular mechanism underlying these activities is still poorly understood, although it has been recognized that interactions with the cytoplasmic membrane of target cells is a key factor. *Pseudomonas* CLPs generally possess a high binding affinity towards lipid model membranes [23]. Of all CLPs within the viscosin group, only for WLIP (ED) an investigation of the membrane interaction study has been performed. It was found to insert into in large unilamellar vesicles (LUVs) composed of varying amounts of phosphatidylcholine (PC), sphingomyelin and sterols [24]. Concentration-dependent calcein release without vesicle destruction was subsequently observed. A separate study also showed that WLIP lyses red blood cells [25], with a colloid-osmotic shock caused by transmembrane pores as the proposed mechanism. For pseudodesmin A (QD), it was observed that its synthetic enantiomer has unaltered antibiotic activity, arguing against receptor-based interactions playing a role. This underpins the paradigm that interactions with the cellular membrane are the principle determinant for activity [26].

The goal of this work is to perform a comparative study in terms of antimicrobial activity and model membrane interaction of the four principal CLPs of the viscosin group (viscosin, viscosinamide A, pseudodesmin A and WLIP), which only feature structural variations according to the D/L and Q/E subgroup dichotomies (Fig. 1). The impact of these specific structural variations on the antimicrobial activity and the capacity to permeabilize Gram-positive mimicking model membrane vesicles (consisting of a PG/PE/cardiolipin mixture) are evaluated. Using model membranes with the same composition, it is tested whether the CLPs can induce membrane fusion, and whether the CLPs undergo conformational changes upon membrane insertion using circular dichroism (CD) spectroscopy. Finally, inspired by previous membrane interaction studies of WLIP (ED) with model membranes consisting out of PC, sphingomyelin and sterols [24], the effects of the interaction between viscosinamide A (QL) on eukaryotic mimicking POPC model membrane structure with or without cholesterol and the CLP conformation are investigated using Fourier-transform infrared (FT-IR) and CD spectroscopy [27].

2. Experimental procedures

2.1. Materials

Viscosinamide A was obtained from *Pseudomonas fluorescens* DR54 as described before [18]. Pseudodesmin A was synthesized using the procedure described by De Vleeschouwer *et al.* [26]. WLIP was a kind gift from Prof. M. Höfte (Ghent, Belgium). Viscosin was extracted from *Pseudomonas* sp. SBW25 which was grown on Kings B medium for 2–3 days at 28 °C. The CLP was extracted and partially purified as described by De Souza *et al.* [28]. In short, bacterial cells were collected from the medium and suspended in milliQ water. After centrifugation, the supernatant was acidified to pH 2, upon which the CLP precipitates. The precipitate was collected by centrifugation and washed twice with acidic milliQ water. Finally, the resulting pellet is resuspended by adjusting the pH to 8. After centrifugation, the supernatant is again acidified to pH 2. For final purification, the precipitate is dissolved in methanol and injected into a Prostar HPLC device (Agilent Technologies) equipped with a Luna C-18(2) preparative reversed phase column (250 × 21.2 mm, 5 μm particle size). For both CLPs, an elution gradient of CH_3CN/H_2O (75:25 to 100:0) was applied over a 30 min period at a flow rate of 17.5 mL min^{-1}, while the column was kept at 35 °C.

Trifluoroethanol (TFE), DMSO, Trizma base and deuterium oxide (D_2O) were purchased from Sigma Aldrich (St. Louis, MO) and chloroform and methanol from Merck (Darmstadt, Germany). The purity of all solvents was at least 99% and the solvents were used without further purification. The ultrapure water was provided from Millipore system (Bedford, MA) and had a resistivity of 18.2 MΩ. Triton-X-100 was provided from Sigma Aldrich (Saint Louis, MO). 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), p-xylylenebis[pyridinium] bromide (DPX) and octadecyl rhodamine chloride (R18) were purchased from Molecular Probes (Eugene, OR). Egg phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and cardiolipin procured from Sigma Aldrich (St. Louis, MO) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). The SIV peptide (NH_2-GVFVLGFLGFLA-$CONH_2$) was obtained from Neosystem (Strasbourg, France) and was 90% pure.

2.2. Antimicrobial activity testing

Antimicrobial activity against a reference set of microorganisms (Table 1) was assessed by a broth microdilution method [29]. Strains

Table 1
CLP minimal inhibitory concentrations (MIC½; μg.mL^{-1}). n.d. = not determined.

Organism	Strain	Pseudodesmin A	Viscosinamide A	WLIP	Viscosin
Enterococcus faecalis	LMG 8222	4	16	>32	16
Streptococcus pyogenes	LMG 15868	1	2	16	4
Streptococcus pneumoniae	LMG21598	1	16	32	32
Enterococcus faecium	LMG 9431	4	8	32	16
Micrococcus luteus	ET067	2	8	>32	>32
Mycobacterium smegmatis	LMG10267	8	8	8	16
Propionibacterium acnes	LMG 16711	1	4	4	4
Clostridium perfringens	NCTC 8798	2	8	32	16
Bacillus cereus	ATCC11178	n.d.	8	32	>32
Bacillus subtilis	ATCC6633	n.d.	8	>32	32
Staphylococcus aureus	LMG 10147	4	8	>32	32
Staphylococcus aureus	Mu50	16	16	>32	>32
Staphylococcus aureus	ET197	32	>32	>32	>32
Staphylococcus aureus	ET199	32	>32	>32	>32
Staphylococcus epidermidis	ET086	>32	>32	>32	>32
Aspergillus fumigatus	IHEM 17907	>32	>32	>32	>32
Candida albicans	SC5314	n.d.	>32	>32	>32

with LMG designation were obtained from the BCCM/LMG Bacteria Collection (Ghent, Belgium) while strain NCTC 8798 was obtained from the National Collection of Type Cultures (HPA, London, UK). Strains with ET designation (Table 1) were selected from a collection of isolates recovered from the endotracheal tube of mechanically ventilated patients [30]. *S. aureus* strain Mu50 was a kind gift of P. Vandamme (Ghent, Belgium). All strains were grown aerobically at 37 °C on Brain Heart Infusion (Oxoid, Erembodegem, Belgium), with the exception of *C. perfringens* NCTC 9789 which was grown aerobically at 37 °C on Reinforced Clostridial Medium (Oxoid). Vancomycin (Sigma-Aldrich, St. Louis, MO) and daptomycin (SelleckChem, Boston, MA) were included as reference antibiotics to monitor performance of the assay. The minimal concentration that inhibited growth by at least 50% compared to the untreated control (MIC½) was used as a measure of activity. MIC½ values were determined using flat-bottomed 96-well microtiter plates (TPP, Trasadingen, Switzerland). Concentrations of CLPs and antibiotics tested ranged from 0.016 to 32 μg/mL. The inoculum was standardized at approx. 5×10^5 colony forming units mL^{-1}. The plates were incubated at 37 °C for 24 h and the optical density was determined at 590 nm using a multilabel microtitre plate reader (Envision Xcite, Perkin Elmer LAS, Waltham, MA). In accordance with CLSI guidelines, MIC½ values were considered identical if they did not differ more than one two-fold dilution factor.

2.3. Membrane permeabilization and fusion induced by CLPs

All fluorescence experiments were performed on a LS-50 B Perkin Elmer fluorimeter. Two complementary types of fluorescence experiment were performed to investigate the permeabilizing and fusogenic properties of the CLPs towards membranes, as previously described [31,32].

2.3.1. Permeability

The HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid)/DPX (p-xylylenebis[pyridinium] bromide) assay of Ellens *et al.* [33] was used to monitor vesicle leakage. SUVs consisting of PG, PE and cardiolipin (7:2:1) were prepared by dissolving the lipids in chloroform:methanol (2:1) and evaporating the solvent, thereby forming a film of lipids. Rehydrating this film with 1 mL buffer solution (Tris 10 mM, NaCl 150 mM at pH 7.4), 1 mL 37.5 mM HPTS (fluorescent probe)/45 mM NaCl solution and 1 mL 135 mM DPX (quencher)/20 mM NaCl solution, vortexing and sonication (8 × 2 min) provided SUVs with a mean size of 73.2 ± 3.5 nm. The non-encapsulated probe and quencher were removed by eluting the vesicles through a freshly prepared Sephadex G-75 column. The expected HPTS and DPX concentrations encapsulated by the vesicles are 12.5 mM and 45 mM respectively. An excitation wavelength of 450 nm was used, while the emission wavelength was recorded at 512 nm.

Leakage of the dye and quencher, occurring due to the onset of permeability, leads to their dilution and thus restoration of the fluorescence. The peptides were dissolved in DMSO given their low solubility in water. The peptide was added in different concentrations as 10 μL solutions in DMSO to 750 μL of 25 μM SUV solution. The fluorescence response is expressed as the release percentage %R:

$$\%R = \frac{(F_{sample} - F_{blank})}{(F_{max} - F_{buffer})} \times 100\%$$

Where F_{sample} is the measured fluorescence of the sample after 15 min, F_{blank} and F_{buffer} are the measured fluorescence during a blank experiment adding only solvent or buffer, respectively. Finally, F_{max} is the maximum release obtained through the addition of 0.5% Triton-X100. Each fluorescence measurement was repeated 2 times on two different liposome batches. The data points were then fitted to a four-parameter Hill equation.

$$y = D + \frac{A - D}{\left[1 + \left(\frac{x}{C_{½}}\right)^{B}\right]}$$

where A is the estimated response at zero concentration, B is the slope factor, $C_{½}$ is the mid-range concentration (inflection point) and D is the estimated response at infinite concentration. During the fitting, confidence intervals on each parameter were determined using a Monte Carlo procedure using 2000 repetitions to obtain 95% confidence intervals.

2.3.2. Membrane fusion

Similar as for the permeability assay, model membrane vesicles consisting of PG, PE and cardiolipin (7:2:1) were used. Dilution of the octadecylrhodamine (R18) probe due to fusion/aggregation induces an increase of the dye fluorescence. For this fusion assay [34,35], two different SUV fractions were prepared, one with a fluorescent marker present, and one without. 'Marked' liposomes were prepared by co-dissolving R18 at a self-quenching concentration together with the lipids (R18/lipids 5.7w%) when preparing the liposomes. Rehydrating the lipid film with buffer solution, vortexing and sonication (5 × 2 min) provided the marked and unmarked SUVs. For the assay, marked and unmarked SUVs are mixed in a 1:5 ratio, with a total lipid concentration of 12.8 μM. An excitation wavelength of 560 nm was used, while the emission wavelength was set to 590 nm. The peptide was added in different concentrations as 25 μL solutions in TFE to 750 μL solution. Maximum fluorescence (E_{max}) response was determined by adding a

600 μM Simian Immunodeficiency Virus (SIV) peptide solution, used as positive control. The response of the mixing of marked and unmarked liposomes and peptide is compared to that of the mixing of marked liposomes, buffer and peptide.

2.4. Preparation of multilamellar vesicles (MLVs)

Multilamellar vesicles (MLVs) in the presence or in the absence of CLPs were prepared as described in Nasir *et al.* [36], for CD and IR analysis. The MLVs either consisted of pure POPC, POPC and cholesterol (70/30, molar ratio) or PG, PE and cardiolipin (7:2:1), as indicated for each experiment. Briefly, molecules were co-dissolved in the minimal volume of methanol-chloroform mixture. The solvent was evaporated under nitrogen in order to obtain a lipidic film, which was dried under vacuum for at least 3 h. The resulting films were hydrated by deuterium oxide for IR experiments or by ultrapure water for CD experiments and the hydrated films were dispersed by vortexing. The lipid/peptide molar ratios were 5 for CD experiments and 20 for IR experiments.

2.5. CD spectroscopy

Far-UV CD spectra were collected between 190 nm and 260 nm using a Jasco Circular Dichroism Spectrometer model J-810 (Easton, MD) at room temperature with a 1 mm optical path length quartz cell. CD spectra of the peptides in TFE were all recorded with a concentration of 0.22 mM. Measurement of vesicles consisting of PG:PE:cardiolipin, POPC pure and POPC and chol were performed with a total lipid concentration of 5 mM in the presence of 0.99 mM CLPs. The solvent (or lipids) contribution was removed by subtracting the spectrum of the pure solvent (or lipids) taken under the same conditions. None of the blanks showed dichroic activity. Each spectrum was smoothed by a Savitzky-Golay filter [37] using the Jasco Spectra Manager software provided with the spectrometer.

2.6. FT-IR spectroscopy

Infrared spectra were recorded by means of a Bruker Equinox 55 spectrometer equipped with a liquid nitrogen-cooled mercury–cadmium-telluride detector using 128 scans at 4 cm^{-1} resolution. During the data acquisition, the spectrometer was continuously purged with filtered dried nitrogen. All experiments were performed using a demountable cell equipped with CaF_2 windows. The sample compositions were D_2O (as a hydrated film), POPC and POPC:cholesterol (70:30) and were measured with and without the presence of CLPs. The solvent spectra were subtracted from the sample spectra taken under the same conditions. Each spectrum is the representative of at least three independent measurements.

3. Results

3.1. Antimicrobial activity testing

Minimal inhibitory concentrations (MIC½) of the four CLPs against a collection of bacteria and fungi were collected in the range 0.015–32 μg mL^{-1}, and are summarized in Table 1. All bacteria are Gram-positive, except *Mycobacterium smegmatis*, which is neither truly Gram negative nor positive due to its waxy coated cell wall. All CLPs displayed antibacterial activity against most tested bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and *Mycobacterium smegmatis*. None of the CLPs displayed inhibitory effects against the human-pathogenic fungi tested (*Aspergillus fumigatus, Candida albicans*). Overall, the antimicrobial activity of the tested CLPs appears to follow the trend: pseudodesmin A > viscosinamide A > > viscosin ≥ WLIP. There is a clear distinction between the CLPs of the Q-subgroup (pseudodesmin A, viscosinamide A) and those of the E-subgroup (WLIP, viscosin), whereby the latter generally display higher MIC½ values. It would thus appear that the presence or absence of the single ionisable functional group has a significant impact. In some particular cases, differences of more than a factor of 2 are observed between pseudodesmin A and viscosinamide A, and between WLIP and viscosin, but overall there is no systematic trend in the antimicrobial activity data that clearly reflects the D/L-subgroup division.

3.2. Membrane permeabilization and fusion induced by CLPs

The permeabilizing effect of the CLPs was tested using a fluorescence release assay [33]. The model membranes used in this study were small unilamellar vesicles (SUVs), consisting of PG, PE and cardiolipin (7:2:1), a composition commonly used to mimic Gram-positive bacterial cell membranes [38]. All CLPs were found to permeabilize the SUVs (Fig. 2), and the data were fitted to a four-parameter Hill equation (see materials and methods) (Table 2). For pseudodesmin A and viscosinamide A (Q-subgroup), there is a progressive increase of the release percentage in the range of ca. 2 to 8 μM, signifying part of the SUV population being permeabilized. For WLIP and viscosin (E-subgroup), a sharper increase in the range of ca. 3 to 7 μM is observed, also reflected by the significantly higher B parameters of the fitted Hill curve (Table 2). The exact reason for this difference in slope factor between Q- and E-subgroup CLPs, the latter possessing a single negative charge at pH 7.4, is unclear, but one could speculate that, after binding, the association of anionic CLPs to form a pore is less unfavourable in a membrane that already contains a high density of anionic molecules. At higher concentrations, the percentage of release of all CLPs reaches a plateau at ca. 100%. The concentration at the inflection point (parameter $C_{½}$), representing the concentration whereby the release percentage reaches half of its maximum value, allows ranking the CLPs in terms of permeabilizing capacity. The $C_{½}$ does not differ significantly between pseudodesmin A and viscosinamide A, and between WLIP and viscosin, indicating that the D/L stereo inversion overall has no significant impact on the CLP permeabilizing concentration capacity. There does appear to be a more pronounced difference between the uncharged CLPs of the Q-subgroup and the charged E-subgroup, the uncharged CLPs having at least 1 μM lower $C_{½}$ concentrations.

The capacity to induce membrane fusion was tested by observing the intermixing of lipid leaflets of different vesicles by mixing vesicles that are unlabeled or labeled with octadecylrhodamine (R18), a lipophilic fluorescent probe, at a self-quenching concentration [34,35]. Similar as for the permeability assay, model membranes used in this study consisted of PG, PE and cardiolipin (7:2:1). For all CLPs, no vesicle fusion or aggregation was detected at the concentrations where permeability occurs (see supplementary Fig. S1). However, at CLP concentrations above 20 μg mL^{-1}, there is a similar increase in fluorescence response for the samples containing only marked liposomes and for those containing both marked and unmarked liposomes. This indicates that, at these concentrations, the CLPs solubilize the vesicles due to the formation of mixed micelles, likely composed of lipopeptides and lipids.

3.3. Influence of lipid membranes on the CLP conformation

The conditions upon membrane insertion prohibit a liquid-state NMR conformational analysis similar to what took place previously for viscosinamide A and pseudodesmin A in free solution [18]. Therefore, CD spectroscopy was used instead to assess whether any significant changes in CLP conformation occur upon insertion into bacterial model membranes, consisting out of PG, PE and cardiolipin (7:2:1) lipid mixture. Given that viscosin group CLPs also have reported hemolytic [25] and antifungal [11,28,39] activity, we found it relevant to investigate the interaction with POPC eukaryotic model membranes, both with and without cholesterol. To assess both the impact on the CLP conformation and the membrane structure itself, CD spectroscopy was complemented with FT-IR spectroscopy. Both techniques are complementary, with CD being more sensitive to the presence of helices and

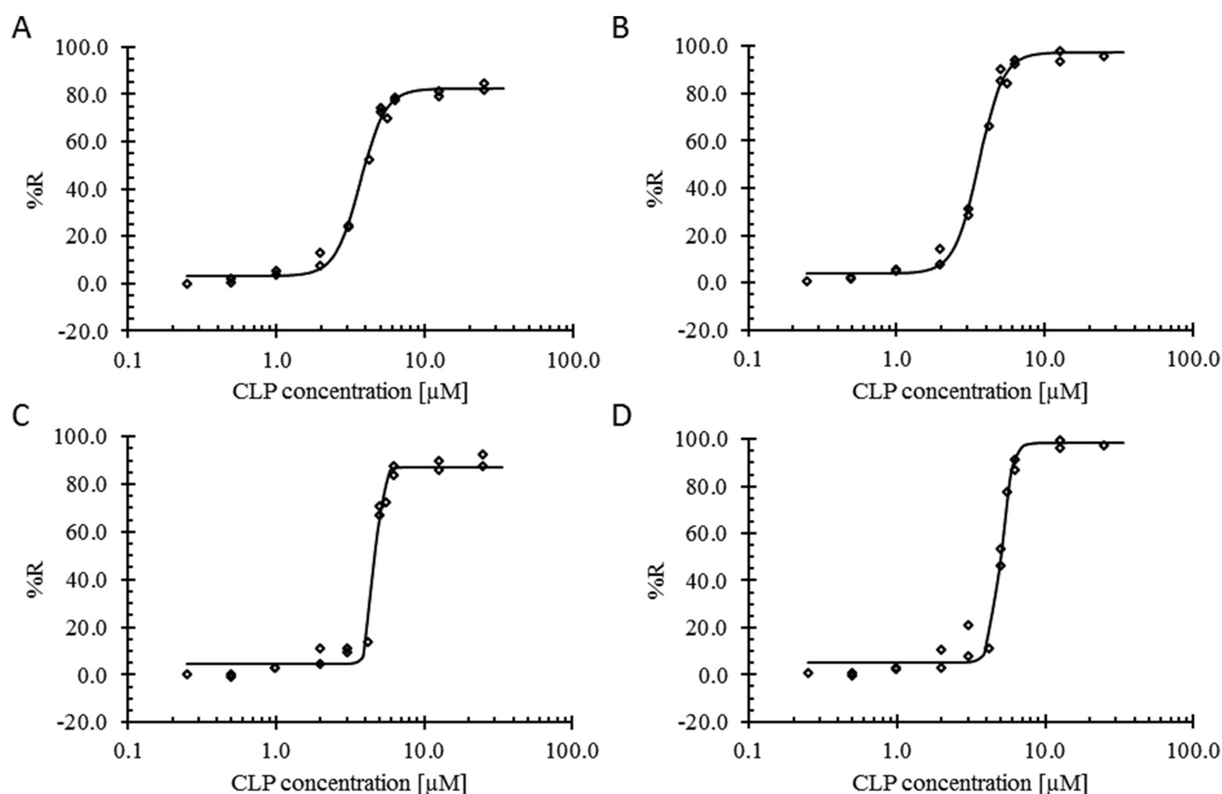

Fig. 2. Results from the fluorescence permeability assay on model membrane vesicles at 25 μM lipid concentration. The release percentage (%R) of the fluorescent dye is shown as a function of the concentration of CLP added to the SUV solution. The solid line is the best fit to the data using the Hill eq. A) pseudodesmin A; B) viscosinamide A; C) WLIP; D) viscosin.

FT-IR to the presence of β-sheets [40]. Due to the high consumption of peptide for FT-IR studies, the study in POPC model membranes was limited to viscosinamide A (QL) alone. However, similar membrane interaction studies using FT-IR were performed for WLIP (ED) by Coraiola *et al.* [24], using PC model membranes, and this study can thus be regarded as complementary.

3.3.1. CD spectroscopy

Fig. 3A shows the CD spectrum of each CLP in TFE solution. The profile mainly resembles what is to be expected for a left-handed helix structure. For instance, the spectrum of viscosinamide A shows two maxima at 225 nm and 209 nm and a large negative band at 195 nm. The assignment of a left-handed helix is in agreement with earlier NMR structure calculations [18]. A limited number of differences are observed between the profiles of the various CLPs, which can be correlated with their primary sequence. The profiles of pseudodesmin A and WLIP (D-subgroup), are slightly shifted to higher wavelengths (≈ 2 nm) and possess a more intense positive Cotton absorption around 208 nm compared to those of viscosinamide A and viscosin (L-subgroup). The E-subgroup (viscosin and WLIP) and Q-subgroup (viscosinamide A and pseudodesmin A) CLPs can be discerned by a distinct intensity of the negative Cotton absorption around 194 nm, with the E-subgroup CLPs displaying more intense absorption.

Table 2
Fitted Hill equation parameters to the permeability fluorescence assay (with 95% confidence intervals).

	Pseudodesmin A	Viscosinamide A	WLIP	Viscosin
A (%)	3.4 ± 2.6	4.2 ± 2.7	4.3 ± 3.1	5.1 ± 3.5
B	5.1 ± 0.9	5.3 ± 0.9	16.7 ± 6.4	11.9 ± 4.6
$C_{½}$ (μM)	3.7 ± 0.2	3.6 ± 0.2	4.7 ± 0.1	5.0 ± 0.1
D (%)	82.3 ± 3.0	97.4 ± 3.2	86.9 ± 4.0	98.2 ± 5.1

The impact on the CLP conformations upon its insertion into PG:PE:cardiolipin model membranes was also investigated by CD spectroscopy. The CLPs all present a similar CD profile as in TFE solution, with a minimum Cotton absorption around 195 nm and two positive maxima around 210 nm and 224 nm (Fig. 3B). The signal intensity ratio of the minimum and the first maximum absorption does not vary between the TFE solution and the MLVs. However, the second maximum at 224 nm appears slightly more pronounced, possibly indicating a more helical conformation in the membrane environment [41].

The impact on the viscosinamide A conformation upon its insertion into POPC model membranes with and without cholesterol was similarly investigated. In the absence of cholesterol, the Cotton absorption bands appear to be only slightly shifted in POPC vesicles relative to those observed in solution, with a minimum found at 196.5 nm and two maxima at 212.5 nm and 225 nm (Fig. 3C). The insertion of the peptide into a POPC membrane environment thus does not appear to noticeably alter the conformation. When cholesterol is present, the helical content of the CLP appears to slightly decrease, as indicated by the diminished intensity of the positive Cotton absorption at 212 nm.

3.3.2. FT-IR spectroscopy

The 1750–1500 cm^{-1} region of the FT-IR spectrum of viscosinamide A in a D_2O-hydrated film is shown in Fig. 4. Four bands at 1749, 1656, 1641 and 1544 cm^{-1} were observed. The band at 1749 cm^{-1} is assigned to the C = O stretch vibration of the ester bond between L-Ile9 and D-*allo*-Thr3. Those at 1656 and 1641 cm^{-1} are assigned to the C = O stretch vibrations of the peptide bonds (amide-I), while the one at 1544 cm^{-1} originates from the N-H bond vibrations (amide-II) [42]. The 1656 cm^{-1} and 1641 cm^{-1} bands are characteristic for α-helical and β-turns, respectively [40,43,44]

In order to further assess possible changes in CLP conformation upon membrane-insertion, MLVs constituted of POPC, with or without cholesterol, were prepared in the absence and in the presence of CLP for FT-IR analysis (Fig. 5).

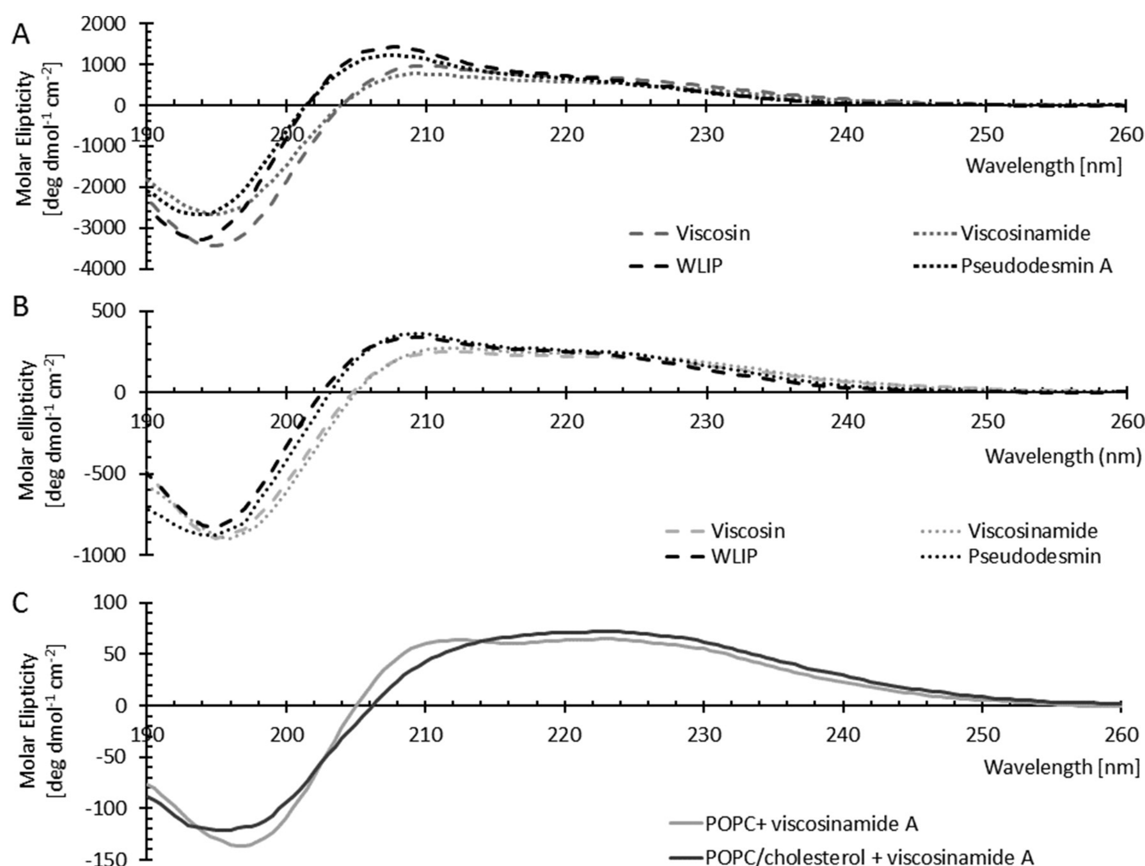

Fig. 3. A) CD spectrum of the CLPs in a TFE solution (0.22 mM). B) CD spectra of CLPs in MLVs consisting of PG:PE:cardiolipin. C) CD spectrum of viscosinamide A in the presence of POPC vesicles (grey) or cholesterol containing POPC vesicles (black).

The spectra without cholesterol will first be analyzed. In the absence of viscosinamide A, the 3000–2800 cm^{-1} region of the spectra (Fig. 5A) shows three bands centered at 2956, 2923 and 2852 cm^{-1}, corresponding respectively to the $–CH_3$ asymmetric stretching, $-CH_2$ asymmetric stretching and $–CH_2$ symmetric stretching vibrations of the POPC alkyl tail [27,45]. The introduction of viscosinamide A induces a clear shift to higher wavenumbers (blue shift) in the location of the asymmetric (~ 3 cm^{-1}) and the symmetric (~ 2 cm^{-1}) C–H stretching vibrations of the POPC alkyl chains. The contribution of the hydroxydecanoic acid tail of viscosinamide A to this shift can safely be neglected, since it only contributes 2.5% of the total signal. A blue shift of the $–CH_2$ stretching vibrations is known to indicate a fluidification of the phospholipid bilayer [46]. In the amide-I region of the spectra of POPC in the absence of viscosinamide A (Fig. 5B), one broad band centered at 1733 cm^{-1} can be observed corresponding to the C = O ester groups of the phospholipid [27,45]. When viscosinamide A is inserted within the MLVs, the location of this band does not undergo any noteworthy change, and thus provides no indication that the C = O ester groups are involved in the interaction with the CLP. Fig. 5C shows the 1300- 900 cm^{-1} regions of the spectra. The band centered at 1230 cm^{-1} corresponds to the asymmetrical stretching of P = O groups of phospholipids, while the band at 1087 cm^{-1} corresponds to their symmetrical stretching. In the presence of viscosinamide A, the band at 1230 cm^{-1} shifts to lower wavenumbers, indicating a more hydrogen bonded state of the P = O groups and thus an involvement of phosphate groups within the interactions. For viscosinamide A, the spectral bands found in the POPC environment are very similar to those found in the D_2O-hydrated film (Fig. 5B). The amide-I region also features a band at 1658 cm^{-1} with a shoulder at 1641 cm^{-1}. The relative intensity of the band at 1658 cm^{-1} compared to the band at 1641 cm^{-1} has increased however, indicating the peptide part of viscosinamide A to be somewhat more helical in the membrane environment.

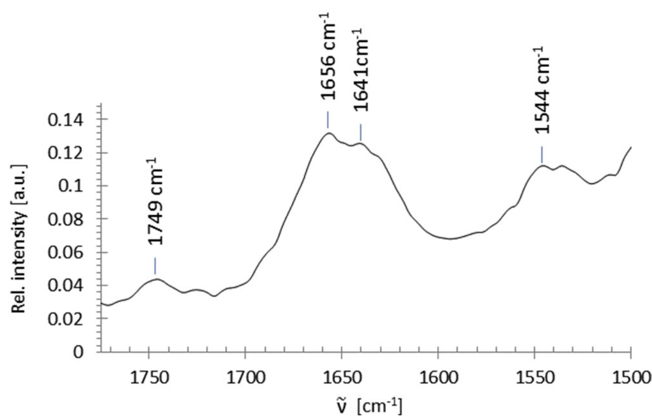

Fig. 4. Amide I region of the IR spectrum of viscosinamide A in D_2O (as hydrated film). The spectrum is dominated by a wide band at 1656 cm^{-1} (α-helix) with a broad shoulder at 1641 cm^{-1} (β-turn). The band at 1749 cm^{-1} indicates the presence of the ester bond in the CLP structure. The band at 1544 cm^{-1} (amide II) originates from the N-H bond vibrations in the peptide.

In the case of MLVs constituted by POPC and cholesterol, no significant shift was observed for the C–H stretching vibrations bands of the POPC aliphatic tails upon addition of viscosinamide A (Fig. 5D), in contrast to what was observed without cholesterol. This could be explained by a hindered insertion of the CLP into the lipid bilayer. Furthermore, in contrast to MLVs without cholesterol, the absorbance originating from the POPC C = O ester group is shifted to lower wavenumbers, indicating a more hydrated state of these groups (Fig. 5E). The C = O ester groups thus appear to be more involved in the interactions between lipids and

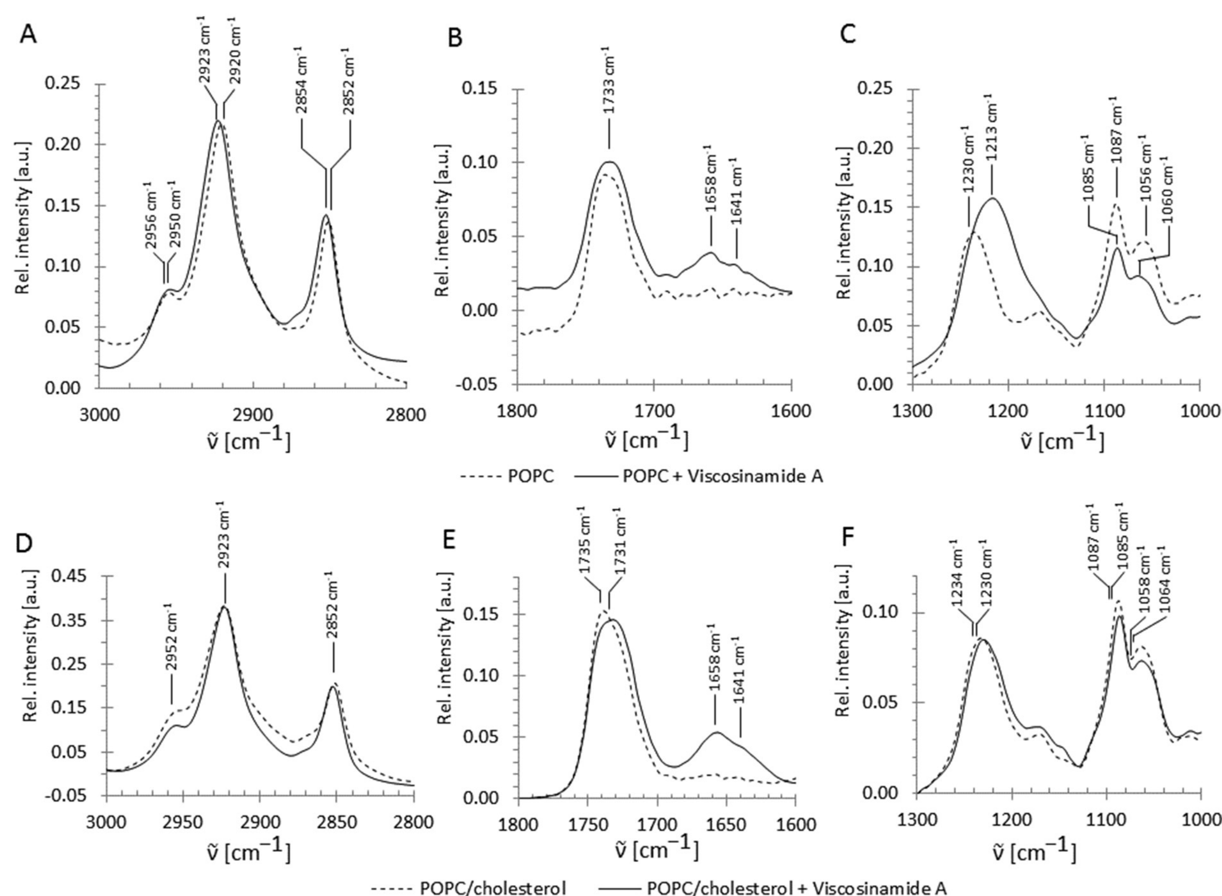

Fig. 5. Different regions of interest (alkyl (A,D), amide I (B,E) and phosphate (C,F) regions) of the IR spectra of viscosinamide A in POPC vesicles (A, B, and C) and cholesterol containing POPC vesicles (D, E, F). Dashed lines and full lines are spectra in the absence and the presence of viscosinamide A respectively.

CLPs when cholesterol is present. Also the POPC P = O absorptions show different behavior in the presence of cholesterol: only a slight shift was observed for the asymmetrical stretching vibrations, indicating reduced involvement of phosphate groups in the interaction (Fig. 5F). Finally, the bands associated with the peptide part of viscosinamide A showed no significant difference compared to what was found for POPC bilayers (Fig. 5E).

4. Discussion

This study provides the first comparative analysis of the principal viscosin group CLPs in terms of antimicrobial activity and interaction with model membranes using a range of methodologies. Typically, antimicrobial (lipo)peptides, fold into an α-helix upon binding to the lipid membrane [47], providing the driving force for membrane insertion. In contrast, viscosin group CLPs already possess a rigid helical structure in free solution, as established by NMR spectroscopy [13,18], without undergoing a substantial change upon membrane insertion, as demonstrated by CD and FT-IR spectroscopy in both PG/PE/cardiolipin and POPC model membranes. Both methods demonstrated only a limited increase in helical content. A possible explanation of the latter could be a stabilization of the less rigid exocyclic N-terminal part of the oligopeptide, as within a membrane environment there would be less competition from solvent molecules to disrupt the conformation. The retention of CLP conformation contradicts the findings of Coraiolo *et al.* [24], where WLIP was reported to undergo some slight conformational changes after insertion into lipid vesicles. These authors concluded this from an observed reduced number of protons available for deuterium exchange after membrane insertion, and thus increased intramolecular hydrogen bonding. We previously reported that viscosin group CLPs can self-assemble to large supramolecular structures in non-polar organic solvent environment that involve the formation of intermolecular hydrogen bonds [18,21,26,48], which led us to conclude that CLP self-assembly could provide an alternative explanation for these findings. Lo Cantore *et al.* [25] indeed proposed that WLIP, which is too small to span the lipid bilayer, forms aggregates in order to form pores in cellular membranes so as to explain the marked concentration-dependence of the observed pore diameter.

FT-IR analysis of POPC MLVs in the presence of viscosinamide A indicated that both the polar (P = O groups) and hydrophobic parts of POPC are involved in the interaction, and that an increase in bilayer fluidity occurs. Alternatively, the involvement of hydrophobic groups could be also explained by the modification of their packing, which increase the area per lipid head group and hence increase hydration and mobility. This suggests at least a partial insertion of viscosinamide A into the bilayer rather than an adsorption on the surface. In the presence of cholesterol however, the insertion depth of the CLP in the bilayer and the CLPs impact on bilayer fluidity appear negatively affected. This is in agreement with the findings of Coraiola *et al.*, who observed a reduced membrane permeabilizing activity for WLIP when cholesterol or ergosterol was present [24]. Cholesterol is indeed known to significantly decrease bilayer fluidity, resulting in a less extensive penetration of membrane interacting peptides [49]. Cholesterol does not actually prevent CLP activity however, as viscosin group CLPs are known to possess hemolytic [25] and antifungal activity [11,28,39]. The slight modifications observed on the lipopeptide conformation in the presence of cholesterol could be due to an increase of hydrogen bonds within the peptide as already observed in the literature [50]. This could be due to the changes occurring in the membrane systems especially concerning the packing of head groups in the presence of cholesterol or to the direct interactions

between cholesterol and the peptide part of lipopeptide inducing conformational modification.

Fluorescence spectroscopy experiments in PG/PE/cardiolipin showed that the viscosin group CLPs do not induce vesicle fusion. At higher concentrations, from 20 μM and higher, membrane vesicle destruction is observed, indicating a detergent-like effect. This is in agreement with observations made for WLIP using dynamic light scattering where vesicle size was found to sharply decrease at WLIP concentration above 24 μg mL^{-1} [24].

Further fluorescence spectroscopy experiments clearly demonstrated that the various CLPs permeabilize PG/PE/cardiolipin membranes at concentrations around 4 μM. The stereochemistry of the Leu5 residue appears to have no impact on the concentration where permeabilization occurs, with no significant difference observed between the CLPs of the L- and D-subgroups. The presence of a Gln2 or Glu2 residue does appear to have a slight effect on the concentration where permeabilization occurs, with the Q-subgroup CLPs permeabilizing membranes about 1 μM earlier. A possible explanation of this difference could be a difference in partitioning between the aqueous phase and the membrane [51], implying that the charged E-subgroup CLPs are less partitioned in the membrane than those from the uncharged Q-subgroup. This would be consistent with observations that were made before, in that the uncharged CLPs stick more to the cellular envelope of their producers, impairing their isolation from planktonic *Pseudomonas* cultures. However, it cannot be excluded that the Glu/Gln substitution could also lead to a difference in peptide-lipid interaction.

The antimicrobial activity tests showed that the CLPs inhibit growth of various Gram-positive bacteria, in agreement with previous results. No activity was found against the two human pathogenic fungi tested (*A. fumigatus* and *C. albicans*), although activity against plant-pathogenic fungi (*Rhizoctonia solani* and *Pythium ultimum*) has previously been observed [11,28]. Comparison of the four different CLPs reveals that, in the majority of cases, the uncharged Q-subgroup CLPs, are more active than the charged E-subgroup ones. In contrast, a clear effect was found in neither the antimicrobial activity nor the permeability study that reflects the D/L stereo inversion of the Leu5 residue. These observations correlate with the results from the membrane permeability studies, where the same structural variation appeared to affect the model membrane permeability $C_{½}$ values. This is in agreement with the paradigm that membrane interaction is central to the molecular mechanism of the antimicrobial activity for these compounds. However, it should be noted that a direct, analytical comparison between the antimicrobial MIC½ and permeability $C_{½}$ values is complicated by the difference in experimental set-up. In the permeability studies, one has perfect control over the used model membrane systems, while Gram-positive bacteria possess more intricate membrane compositions. Also, the membrane compositions vary from organism to organism, implying that care must be taken when comparing the activities of a particular bacterium with the model membrane study, since membrane compositions vary and the impact of CLP structural differences may subtly differ. To determine whether the observed differences between bacteria correlate with membrane permeability, further model membrane studies with variable composition will be required. Nevertheless, the observed trend that E-subgroup CLPs are overall less active than Q-subgroup CLPs is clear.

In conclusion, we assessed for the first time whether the structural variations between the principal CLPs from the viscosin group result in differences in the antimicrobial activity and membrane interactions. We demonstrated that each of these CLPs exhibits antagonistic activity against various Gram-positive bacteria and can permeabilize PG/PE/cardiolipin model membrane bilayers. The Gln/Glu structural variation appears to impact both of these properties, while the inversion of the stereocenter at Leu5 does not. Furthermore, the CLPs retain their solution conformation upon membrane insertion. In POPC model membranes, it was found that CLPs increase the bilayer fluidity, and this effect is reduced by the presence of cholesterol. The exact molecular details of how these small lipopeptides permeabilize membrane bilayers, the precise reason for the observed impact of the Gln/Glu structural variation, as well as the observed differences in antimicrobial activities between the different bacteria remain subjects of further research.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

N.G., M.N.N., J.M.C. performed the spectroscopic experiments and sample preparations. T.C. performed and supervised the antimicrobial assays. N.G., M.N.N., D.S. and M.D. interpreted and analyzed the results. J.M.R. and K.F. participated in the scientific discussions. L.L., J.C.M., D.S. and M.D. supervised the research. All authors contributed to the writing of the manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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References

[1] J.M. Raaijmakers, I. De Bruijn, O. Nybroe, M. Ongena, Natural functions of lipopeptides from Bacillus and Pseudomonas, FEMS Microbiol. Rev. 34 (2010) 1037–1062.

[2] N. Roongsawang, K. Washio, M. Morikawa, Diversity of nonribosomal Peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants, Int. J. Mol. Sci. 12 (2010) 141–172.

[3] H. Gross, J.E. Loper, Genomics of secondary metabolite production by *Pseudomonas spp*, Nat. Prod. Rep. 26 (2009) 1408–1446.

[4] J. Raaijmakers, I. De Bruijn, M. De Kock, Cyclic lipopeptides production by plant-associated *Pseudomonas spp*, Mol. Plant-Microbe Interact. 19 (2006) 699–710.

[5] H. Gross, V.O. Stockwell, M.D. Henkels, B. Nowak-Thompson, J.E. Loper, W.H. Gerwick, The genomisotopic approach: a systematic method to isolate products of orphan biosynthetic gene clusters, Chem. Biol. 14 (2007) 53–63.

[6] A.S. Alsohim, T.B. Taylor, G.A. Barrett, J. Gallie, X.X. Zhang, A.E. Altamirano-Junqueira, L.J. Johnson, P.B. Rainey, R.W. Jackson, The biosurfactant viscosin produced by *Pseudomonas fluorescens* SBW25 aids spreading motility and plant growth promotion, Environ. Microbiol. 16 (2014) 2267–2281.

[7] C. Song, K. Aundy, J. van de Mortel, J.M. Raaijmakers, Discovery of new regulatory genes of lipopeptide biosynthesis in *Pseudomonas fluorescens*, FEMS Microbiol. Lett. 356 (2014) 166–175.

[8] J. D'Aes, N.P. Kieu, V. Leclere, C. Tokarski, F.E. Olorunleke, K. De Maeyer, P. Jacques, M. Hofte, M. Ongena, To settle or to move? The interplay between two classes of cyclic lipopeptides in the biocontrol strain *Pseudomonas* CMR12a, Environ. Microbiol. 16 (2014) 2282–2300.
[9] M. Hofte, N. Altier, Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems, Res. Microbiol. 161 (2010) 464–471.
[10] J. D'Aes, K. De Maeyer, E. Pauwelyn, M. Hofte, Biosurfactants in plant-*Pseudomonas* interactions and their importance to biocontrol, Environ. Microbiol. Rep. 2 (2010) 359–372.
[11] T.H. Nielsen, C. Christophersen, U. Anthoni, J. Sorensen, Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54, J. Appl. Microbiol. 86 (1999) 80–90.
[12] J. Gerard, T. Barsby, P. Haden, M.T. Kelly, R.J. Anderson, A.-H. Massetolides, antimycobacterial cyclic depsipeptides produced by two Pseumonads isolated from marine habitats, J. Nat. Prod. 60 (1997) 223–229.
[13] D. Sinnaeve, C. Michaux, J. Van Hemel, J. Vandenkerckhove, E. Peys, F.A.M. Borremans, B. Sas, J. Wouters, J.C. Martins, Structure and X-ray conformation of pseudodesmins A and B, two new cyclic lipodepsipeptides from *Pseudomonas* bacteria, Tetrahedron 65 (2009) 4173–4181.
[14] J.Y. Jang, S.Y. Yang, Y.C. Kim, C.W. Lee, M.S. Park, J.C. Kim, I.S. Kim, Identification of Orfamide A as an Insecticidal Metabolite Produced by *Pseudomonas protegens* F6, J. Agric. Food Chem. 61 (2013) 6786–6791.
[15] V. Groupé, L.H. Pugh, D. Weiss, M. Kochi, Observations on Antiviral Activity of Viscosin, Exp. Biol. Med. 78 (1951) 354–358.
[16] J.E. Van De Mortel, H. Tran, F. Govers, J.M. Raaijmakers, Cellular responses of the late blight pathogen *Phytophthora infestans* to cyclic lipopeptide surfactants and their dependence on G proteins, Appl. Environ. Microbiol. 75 (2009) 4950–4957.
[17] M. Laycock, P.D. Hildebrand, P. Thibault, J. Walter, J. Wright, Viscosin, a potent peptidolipid biosurfactant and phytopathogenic mediator produced by a pectolytic strain of *Pseudomonas fluorescens*, J. Agric. Food Chem. 39 (1991) 483–489.
[18] N. Geudens, M. De Vleeschouwer, K. Feher, H. Rokni-Zadeh, M.G. Ghequire, A. Madder, R. De Mot, J.C. Martins, D. Sinnaeve, Impact of a stereocentre inversion in cyclic lipodepsipeptides from the viscosin group: a comparative study of the viscosinamide and pseudodesmin conformation and self-assembly, ChemBioChem 15 (2014) 2736–2746.
[19] C. Soler-Rivas, N. Arpin, J.M. Olivier, H.J. Wichers, WLIP, a Lipodepsipeptide of *Pseudomonas reactans*, as Inhibitor of the Symptoms of the Brwn Blotch Disease of *Agaricus bisporus*, J. Appl. Microbiol. 86 (1999) 635–641.
[20] J.W. Quail, N. Ismail, M. Soledade, C. Pedras, S.M. Boyetchko, Pseudophomins A and B, a class of cyclic lipodepsipeptides isolated from a *Pseudomonas* species, Acta Crystallogr. C 58 (2002) 268–271.
[21] D. Sinnaeve, P.M. Hendrickx, J. Van Hemel, E. Peys, B. Kieffer, J. Martins, The Solution Structure and Self-Association Properties of the Cyclic Lipodepsipeptide Pseudodesmin A Support Its Pore-Forming Potential, Chem. Eur. J. 15 (2009) 12653–12662.
[22] M. Mazzola, I. de Bruijn, M.F. Cohen, J.M. Raaijmakers, Protozoan-induced regulation of cyclic lipopeptide biosynthesis is an effective predation defense mechanism for *Pseudomonas fluorescens*, Appl. Environ. Microbiol. 75 (2009) 6804–6811.
[23] K. Reder-Christ, Y. Schmidt, M. Dorr, H.G. Sahl, M. Josten, J.M. Raaijmakers, H. Gross, G. Bendas, Model membrane studies for characterization of different antibiotic activities of lipopeptides from Pseudomonas, Biochim. Biophys. Acta, Biomembr. 1818 (2012) 566–573.
[24] M. Coraiola, P. Lo Cantore, S. Lazzaroni, A. Evidente, N.S. Iacobellis, M. Dalla Serra, WLIP and tolaasin I, lipodepsipeptides from *Pseudomonas reactans* and *Pseudomonas tolaasii*, permeabilise model membranes, Biochim. Biophys. Acta, Biomembr. 1758 (2006) 1713–1722.
[25] P. Lo Cantore, S. Lazzaroni, M. Coraiola, M. Dalla Serra, C. Cafarchia, A. Evidente, N.S. Lacobellis, Biological characterization of White Line-Inducing Principle (WLIP) produced by *Pseudomonas reactans* NCPPB1311, Mol. Plant-Microbe Interact. 19 (2006) 1113–1120.
[26] M. De Vleeschouwer, D. Sinnaeve, J. Van den Begin, T. Coenye, J.C. Martins, A. Madder, Rapid Total Synthesis of Cyclic Lipodepsipeptides as a Premise to Investigate their Self-Assembly and Biological Activity, Chem. Eur. J. 20 (2014) 7766–7775.
[27] M. Deleu, J.M. Crowet, M.N. Nasir, L. Lins, Complementary biophysical tools to investigate lipid specificity in the interaction between bioactive molecules and the plasma membrane: A review, Biochim. Biophys. Acta, Biomembr. 1838 (2014) 3171–3190.
[28] J.T. De Souza, M. De Boer, P. De Waard, T.A. Van Beek, J.M. Raaijmakers, Biochemical, Genetic, and Zoosporicidal Properties of Cyclic Lipopeptide Surfactants Produced by *Pseudomonas fluorescens*, Appl. Environ. Microbiol. 69 (2003) 7161–7172.
[29] Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing, Twentieth second Informational Supplement M100-S22. Wayne, PA, USA, 2012.
[30] I. Vandecandelaere, N. Matthijs, H.J. Nelis, P. Depuydt, T. Coenye, The presence of antibiotic-resistant nosocomial pathogens in endotracheal tube biofilms and corresponding surveillance cultures, Pathog. Dis. 69 (2013) 142–148.
[31] F. Van Bambeke, A. Kerkhofs, A. Schanck, C. Remacle, E. Sonveaux, P.M. Tulkens, M.-P. Mingeot-Leclercq, Biophysical studies and intracellular destabilization of pH-sensitive liposomes, Lipids 35 (2000) 213–223.
[32] M.-P. Mingeot-Leclercq, L. Lins, M. Bensliman, F. Van Bambeke, P. Van Der Smissen, J. Peuvot, A. Schanck, R. Brasseur, Membrane destabilization induced by β-amyloid peptide 29-42: Importance of the amino-terminus, Chem. Phys. Lipids 120 (2002) 57–74.
[33] H. Ellens, J. Bentz, F.C. Szoka, Proton- and calcium-induced fusion and destabilization of liposomes, Biochemistry 24 (1985) 3099–3106.
[34] D. Hoekstra, T. De Boer, K. Klappe, J. Wilschut, Fluorescence method for measuring the kinetics of fusion between biological membranes, Biochemistry 23 (1984) 5675–5681.
[35] L. Lins, L. Chapelle, P.J. Talmud, A. Thomas, R. Brasseur, Lipid-interacting properties of the N-terminal domain of human apolipoprotein C-III, Protein Eng. 15 (2002) 512–520.
[36] M.N. Nasir, A. Thawani, A. Kouzayha, F. Besson, Interactions of the natural antimicrobial mycosubtilin with phospholipid membrane models, Colloids Surf. B Biointerfaces 78 (2010) 17–23.
[37] A. Savitzky, M.J.E. Golay, Smoothing and Differentiation of Data by Simplified Least Squares Procedures, Anal. Chem. 36 (1964) 1627–1639.
[38] R.F. Epand, P.B. Savage, R.M. Epand, Bacterial lipid composition and the antimicrobial efficacy of cationic steroid compounds (Ceragenins), Biochim. Biophys. Acta 1768 (2007) 2500–2509.
[39] C. Thrane, T.H. Nielsen, M. Neiendam Nielsen, J. Sorensen, S. Olsson, Viscosinamide-producing *Pseudomonas fluorescens* DR54 exerts a biocontrol effect on *Pythium ultimum* in sugar beet rizosphere, FEMS Microbiol. Ecol. 33 (2000) 139–146.
[40] E. Vass, M. Hollosi, F. Besson, R. Buchet, Vibrational Spectroscopic Detection of Beta- and Gamma-Turns in Synthetic and Natural Peptides and Proteins, Chem. Rev. 103 (2003) 1917–1954.
[41] R. Cerpa, F.E. Cohen, I.D. Kuntz, Conformational switching in designed peptides: the helix/sheet transition, Fold. Des. 1 (1996) 91–101.
[42] S. Timasheff, G.D. Fasman, Structure and stability of biological macromolecules, Dekker, New York, 1969.
[43] L.K. Tamm, S.A. Tatulian, Infrared spectroscopy of proteins and peptides in lipid bilayers, Q. Rev. Biophys. 30 (1997) 365–429.
[44] A. Kouzayha, M.N. Nasir, R. Buchet, O. Wattraint, C. Sarazin, F. Besson, Conformational and Interfacial Analysis of $K_3A_{18}K_3$ and Alamethicin in Model Membranes, J. Phys. Chem. B 113 (2009) 7012–7019.
[45] J.L.R. Arrondo, F.M. Goñi, Infrared studies of protein-induced perturbations of lipids in lipoproteins and membranes, Chem. Phys. Lipids 96 (1998) 53–68.
[46] H.H. Mantsch, R.N. McElhaney, Phospholipid phase transitions in model and biological membranes as studied by infrared spectroscopy, Chem. Phys. Lipids 57 (1991) 213–226.
[47] S. Thennarasu, D.K. Lee, A. Tan, U. Prasad Kari, A. Ramamoorthy, Antimicrobial activity and membrane selective interactions of a synthetic lipopeptide MSI-843, Biochim. Biophys. Acta 1711 (2005) 49–58.
[48] D. Sinnaeve, M.-A. Delsuc, J.C. Martins, B. Kieffer, Insight into peptide self-assembly from anisotropic rotational diffusion derived from ^{13}C NMR relaxation, Chem. Sci. 3 (2012) 1284–1292.
[49] E.J. Prenner, R.N. Lewis, M. Jelokhani-Nairaki, R.S. Hodges, R.N. McElhaney, Cholesterol attenuates the interaction of the antimicrobial peptide gramicidin S with phospholipid bilayer membranes, Biochim. Biophys. Acta 1510 (2001) 83–92.
[50] M.N. Nasir, F. Besson, Specific Interactions of Mycosubtilin with Cholesterol-Containing Artificial Membranes, Langmuir 27 (2011) 10785–10792.
[51] S. Fiedler, H. Heerklotz, Vesicle Leakage Reflects the Target Selectivity of Antimicrobial Lipopeptides from Bacillus subtilis, Biophys. J. 109 (2015) 2079–2089.