The interaction of cationic antimicrobial peptides with vesicles containing synthetic glycolipids as models of the outer membrane of gram-negative bacteria

MARINA GOBBO,* LAURA BIONDI, FERNANDO FILIRA and RANIERO ROCCHI

Department of Chemical Sciences, University of Padova, Institute of Biomolecular Chemistry of C. N. R. – Section of Padova, via Marzolo 1 – 35131 Padova, Italy

Abstract: Two simple lipid A analogues methyl 2,3-di-*O*-tetradecanoyl-α-D-glucopyranoside (GL1) and methyl 2,3-di-*O*-tetradecanoyl-α-D-glucopyranoside 4-*O*-phosphate (GL2) were synthesized and used for preparing mixed phosphocholine vesicles as models of the outer membrane of gram-negative bacteria. The interaction of these model membranes with magainin 2, a representative of the α-helical membrane active peptides, and apidaecin Ib and drosocin, two insect Pro-rich peptides which do not act at the level of the cellular membrane, were studied by CD and dye-releasing experiments. The CD spectra of apidaecin Ib and drosocin in the presence of GL1- or GL2-containing vesicles were consistent with largely unordered structures, whereas, according to the CD spectra, magainin 2 adopted an amphipathic α-helical conformation, particularly in the presence of negatively charged bilayers. The ability of the peptides to fold into amphipathic conformations was strictly correlated to their ability to bind and to permeabilize phospholipid as well as glycolipid membranes. Apidaecin Ib and drosocin, which are unable to adopt an amphipathic structure, showed negligible dye-leakage activity even in the presence of GL2-containing vesicles. It is reasonable to suppose that, as for the killing mechanism, the two classes of antimicrobial peptides follow different patterns to cross the bacterial outer membrane. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: antimicrobial peptides; Pro-rich peptides; lipid A; liposomes; glycolipids; peptide-membrane interactions

INTRODUCTION

Cationic antimicrobial peptides 12–50 amino acids long, which are widely distributed through the animal and plant kingdoms [1], have been recognized as key elements of innate immunity [2]. Most antimicrobial cationic peptides fold into amphipathic structures [3] and interact with and insert into the negatively charged cytoplasmatic membrane of bacteria [4]. A number of studies [5,6] have utilized various lipid bilayers as models of the cytoplasmatic membrane to elucidate the molecular mechanism of membrane specificity and binding. A direct correlation between antibiotic effect and permeabilization ability has been found for a number of antimicrobial peptides [7]. Besides the cytoplasmatic membrane, bacteria possess a cellular envelope, which in the case of gram-negative bacteria consists of a complex outer membrane characterized by the presence of lipopolysaccharides (LPS) that represent an ideal target for binding cationic antimicrobial peptides [8]. Some antimicrobial peptides either induce deep lesions on the morphology of the outer membrane [9,10], or increase its permeability by causing disorder in the LPS organization [11]. The 2-keto-3-deoxyoctulosonic acid moieties and the phosphorylated glucosamines of lipid A have been shown to play a major role in the binding

of antimicrobial peptides [12–14], and a mechanism of 'self-promoted uptake' [15] has been proposed for the transport of antimicrobial peptides across the complex outer membrane of gram-negative bacteria (OM).

Several studies dealing with the interaction between LPS, or lipid A, and some cationic antimicrobial peptides, such as magainin, cecropin and polymyxin have been reported. In these studies, either dispersed LPS/lipid A [14,16,17], LPS monolayers [18–20] or LPS/lipid A vesicles [20–22] were used. However the heterogeneicity among different LPS preparations and the limited availability of lipid A, either from natural sources or prepared by chemical synthesis, makes it difficult to compare the various results.

We describe here the synthesis of two lipid A analogues, GL1 and GL2 (Scheme 1), as well as a preliminary investigation on their interaction with an α-helical membrane active antimicrobial peptide, magain 2 [23] (Figure 1), and two proline-rich cationic peptides, apidaecin Ib [24] and drosocin [25], which are devoid of a permeabilizing activity [26]. The sugar moiety on the O-glycosylated threonine residue present in the drosocin sequence is involved in the modulation of the peptide antimicrobial activity [27].

MATERIALS AND METHODS

Methyl 4,6-*O*-isopropylidene-α-D-glucopyranoside (28), drosocin and apidaecin Ib (27) were prepared according to the

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* Correspondence to: Marina Gobbo, Department of Chemical Sciences, University of Padova, via Marzolo 1 – 35131 Padova, Italy;
e-mail: marina.gobbo@unipd.it
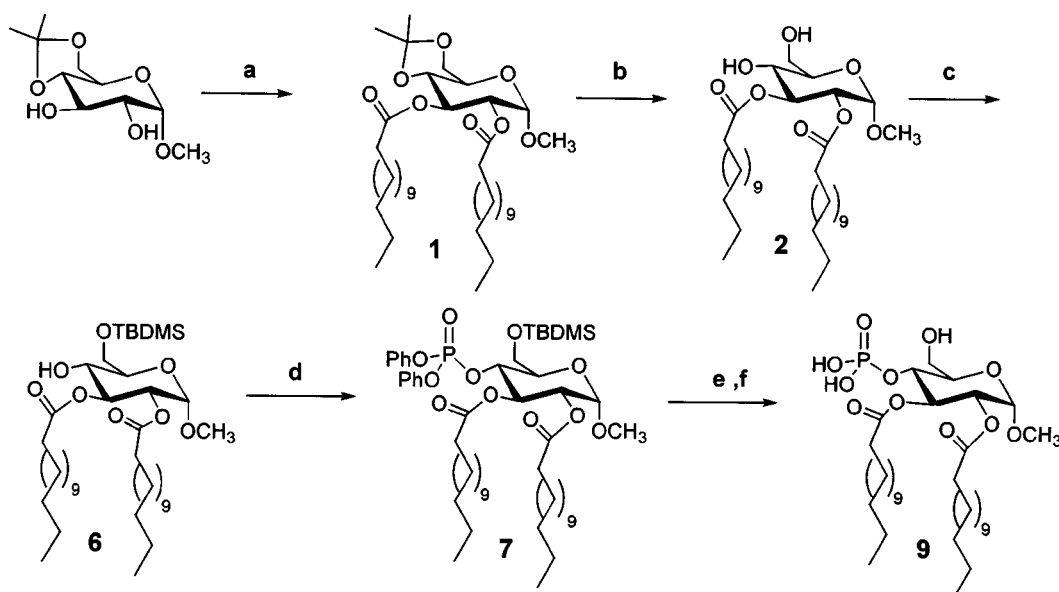
Scheme 1 Synthesis of glycolipids GL1 (**2**) and GL2 (**9**). Reagents: (**a**) myristic acid, DCC/DMAP, DMF; (**b**) 95% acetic acid, reflux; (**c**) t-butyldimethylsilyl chloride (TBDMS-Cl), DMAP, TEA, CH_2Cl_2; (**d**) $(PhO)_2POCl$, DMAP, CH_2Cl_2; (**e**) $BF_3.Et_2O$, CH_2Cl_2; (**f**) H_2, Pt_2O, CH_3OH.

magainin 2	GIGKFLHSAKKFGKAFVGEIMNS
apidaecin Ib	GNNRPVYIPQPRPPHPRL
drosocin	GKPRPYSPRP(GalNAc-α)TSHPRPIRV

Figure 1 Amino acid sequences of the antimicrobial peptides used in this work.

literature. L-α-phosphatidylcholine (PC) (egg yolk type XI-E) and dimyristoyl-L-α-phosphatidyl-DL-glycerol (DMPG) were purchased from Sigma. The solvents were dried and freshly distilled and evaporations were carried out under reduced pressure at 30–40 °C, using a rotary evaporator. Na_2SO_4 was used for drying purposes. All chemicals were commercial products of the best grade available. Reactions requiring anhydrous conditions were carried out under dry nitrogen. Ascending TLC was routinely performed on TLC plates silica gel 60, UV$_{254}$, Machery-Nagel, using the following solvent systems: E1, ethyl acetate; E2, chloroform; E4, n-hexane : dichloromethane (5 : 1 v/v); E5, n-hexane : chloroform (7 : 1 v/v); E7, dichloromethane; E8, n-hexane : ethyl acetate (8 : 2 v/v). Sugar derivatives were visualized by UV light or by spraying the plates with 10% sulfuric acid in ethanol, followed by heating for 10 min at 100 °C. Low pressure liquid chromatography (LPLC) was performed on silica gel 60 (0.063–0.040 mm, Machery-Nagel, column 26 × 260 mm) by using a Büchi 688 chromatographic pump equipped with a Büchi UV/VIS filter photometer (254 nm) detector. Melting points were taken on a Büchi 150 apparatus and were not corrected. Optical rotations were determined at 25 °C with a Perkin-Elmer model 241 polarimeter. Unless otherwise stated, NMR spectra in CDCl$_3$ were recorded at 298 °K on a Bruker AM-400 spectrometer. ^{1}H and ^{31}P chemical shifts (δ) are expressed in parts per million relative to tetramethylsilane, as internal standard, or 85% phosphoric acid, as external standard, respectively. ESI–MS was performed on a Mariner™ API-TOF Workstation (Perseptive Biosystems) operating in a negative

mode; samples were dissolved in methanol. CD measurements were carried out on a Jasco-715 spectropolarimeter, using a quartz cell of 0.02 cm path length (Hellma). CD spectra were the average of a series of six scans made at 0.1 nm intervals over the 250–190 nm region, recorded at 298 K. Peptide concentrations (0.11–0.15 mM), were determined by amino acid analyses performed on a Carlo Erba 3A 30 amino acid analyzer, interfaced with a Shimadzu C-R4A Chromatopac. The lipid concentration (5 mM) was determined by phosphorous analysis (29). Ellipticity is reported as mean residue ellipticity $[\theta]_R$ (deg.cm^2 dmol^{-1}). Fluorescence measurements were performed on a Perkin-Elmer LS50-B spectrofluorimeter with a thermostated cell holder in disposable polystyrene cuvettes (1 cm × 1 cm, Sigma). Dynamic light scattering measurements, for vesicle sizing, were performed on a Spectra-Physics instrument mod. 2016-04s.

Methyl 4,6-O-isopropylidene-2,3-di-O-tetra-decanoyl-α-D-glucopyranoside (1)

Myristic acid (3.23 g, 14.4 mmol), DCC (2.95 g, 14.31 mmol) and DMAP (0.15 g, 1.2 mmol) were added to an ice-cold solution of methyl 4,6-O-isopropylidene-α-D-glucopyranoside [28] (1.52 g, 6.5 mmol) in CH_2Cl_2 (20 ml). After stirring overnight at room temperature, the precipitated dicyclohexylurea was filtered off and the filtrate was concentrated *in vacuo*. LPLC of the residue (eluant: CH_2Cl_2 : n-hexane 10 : 3 v/v) allowed the isolation of a small amount (0.21 g) of methyl 2,3,4,6-tetra-O-tetradecanoyl-α-D-glucopyranoside and the title compound (1.83 g, 66%). $[\alpha]_D + 52.0°$ (c 0.99, CHCl$_3$); R_f = 0.61 (E2); mp 63 °C; ^{1}H-NMR (400 MHz): 5.41 (t, 1H, $J_{2,3} = J_{3,4} = 9.5$ Hz, H$_3$), 4.89 (d, 1H, $J_{1,2} = 3.76$ Hz, H$_1$), 4.89 (dd, 1H, $J_{1,2} = 3.74$ Hz, $J_{2,3} = 9.81$ Hz, H$_2$), 3.89 (dd, 1H, H$_6$), 3.74 (m, 1H, H$_{6'}$), 3.68 (m, 2H, H$_5$ and H$_4$), 3.37 (s, 3H, OCH$_3$), 2.28 (m, 4H, 2 × $-CH_2-CO$), 1.60 (m, 4H, 2 × $-C\underline{H}_2-CH_3$), 1.45 (s, 1H, CH$_3$ isopropylidene), 1.37 (s, 1H, C'H$_3$ isopropylidene), 1.25

(bs, 20H, $2 \times -(CH_2)_5-$ myristic), 0.87 (m, 6H, J = 6.86 Hz, $2 \times CH_3$ myristic).

Methyl 2,3-Di-*O*-tetradecanoyl-α-D-gluco-pyranoside, GL1 (2)

Compound **1** (1.8 g, 4.2 mmol) was dissolved in aqueous 90% acetic acid (30 ml) and the solution was warmed at 95 °C for 15 min. The solvent was removed by co-evaporation with toluene and the residue was purified by LPLC (eluant: CHCl$_3$). Yield 1.39 g (86%); $[\alpha]_D$ +65.7° (c 1.2, CHCl$_3$); R_f = 0.20 (E2), R_f = 0.13 (E7); mp 87 °C; ^{1}H-NMR (400 MHz): 5.21 (t, 1H, $J_{2,3} = J_{3,4}$ = 10.08 Hz, H$_3$), 4.91 (d, 1H, $J_{1,2}$ = 3.65 Hz, H$_1$), 4.84 (dd, 1H, $J_{1,2}$ = 3.64 Hz, $J_{2,3}$ = 10.14 Hz, H$_2$), 3.80 (m, 2H, H$_6$ and H$_{6'}$), 3.63 (m, 2H, H$_5$ and H$_4$), 3.33 (s, 3H, OCH$_3$), 2.94 (bs, 1H, OH), 2.25 (m, 4H, $2 \times -CH_2-CO$), 1.66 (bs, 1H, OH), 1.53 (m, 4H, $2 \times -C\underline{H}_2-CH_3$), 1.18 (bs, 20H, $2 \times -(CH_2)_5-$ myristic), 0.81 (m, 6H, J = 6.78 Hz, $2 \times CH_3$ myristic).

Methyl 6-*O*-(1,1-dimethyl-2,2,2-trichloroethoxy-carbonyl)-2,3-di-*O*-tetradecanoyl-α-D-gluco-pyranoside (3) and methyl 4,6-di-*O*-(1,1-di-methyl-2,2,2-tricloroetossicarbonyl)-2,3-di-*O*-tetradecanoyl-α-D-glucopyranoside (4)

A solution of 1,1 dimethyl, 2,2,2 trichloroethyloxycarbonyl (TcBoc)-Cl (0.7 g, 2.9 mmol) in CH$_2$Cl$_2$ (5 ml) was added under nitrogen to an ice-cold solution of GL1 (1.0 g, 2.6 mmol) in dry CH$_2$Cl$_2$ (25 ml) containing pyridine (1 ml). After stirring for 3 h at room temperature, the reaction mixture was diluted with CH$_2$Cl$_2$, extracted with water, dried and evaporated *in vacuo*. LPLC of the oily residue (eluant: *n*-hexane : ethyl acetate 15 : 1 v/v) allowed the isolation of both the mono-TcBoc derivative **3** (0.18 g, 12%) and the di-TcBoc derivative **4** (0.59 g, 28%).

Compound 3. $[\alpha]_D$ +54.5° (c 0.78, CH$_2$Cl$_2$); R_f = 0.80 (E2), R_f = 0.50 (E8); ^{1}H-NMR (400 Mz): δ 5.26 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.43 Hz, H$_3$), 4.90 (m, 2H, H$_1$ and H$_2$), 4.45 (m, 2H, H$_6$ and H$_{6'}$), 3.85 (m, 1H, H$_5$), 3.63 (m, 1H, H$_4$), 3.30 (s, 3H, OCH$_3$), 2.32 (m, 4H, $2 \times -CH_2-CO$), 1.94 (s, 6H, $2 \times CH_3$ TcBoc), 1.62 (m, 4H, $2 \times -C\underline{H}_2-CH_3$), 1.25 (bs, 20H, $2 \times -(CH_2)_5-$ myristic), 0.88 (t, 6H, J = 6.78 Hz, $2 \times CH_3$ myristic).

Compound 4. $[\alpha]_D$ +45.3° (c 1.00, CH$_2$Cl$_2$); R_f = 0.85 (E2), R_f = 0.88 (E8); ^{1}H-NMR (400 Mz): δ 5.59 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.95 Hz, H$_3$), 4.94 (d, 1H, $J_{1,2}$ = 3.63 Hz, H$_1$), 4.89 (dd, 1H, $J_{1,2}$ = 3.64 Hz, $J_{2,3}$ = 10.11 Hz, H$_2$), 4.79 (t, 1H, $J_{3,4} = J_{4,5}$ = 9.45 Hz, H$_4$), 4.43 (dd 1H, $J_{5,6}$ = 5.0 Hz, $J_{6,6'}$ = 12.09 Hz, H$_6$), 4.20 (dd 1H, $J_{5,6'}$ = 2.3 Hz, $J_{6,6'}$ = 12.05 Hz, H$_{6'}$), 4.12 (m, 1H, H$_5$), 3.40 (s, 3H, OCH$_3$), 2.30 (m, 2H, $-CH_2-CO$), 2.18 (m, 2H, $-C'H_2-C'O$), 1.94 (s, 6H, $2 \times CH_3$ TcBoc), 1.90 (s, 6H, $2 \times CH_3$ TcBoc), 1.60 (m, 4H, $2 \times -C\underline{H}_2-CH_3$), 1.28 (bs, 20H, $2 \times -(CH_2)_5-$ myristic), 0.90 (t, 6H,, J = 6.81 Hz, $2 \times CH_3$ myristic).

Methyl 6-*O*-(1,1-dimethyl-2,2,2-trichloroethoxy-carbonyl)-2,3-di-*O*-tetradecanoyl-α-D-gluco-pyranoside 4-*O*-diphenylphosphate (5)

Diphenyl chlorophosphate (0.10 ml, 0.5 mmol) and DMAP (0.60 g, 0.5 mmol) were added to a solution of **3** (0.15 g, 0.25 mmol) in dry CH$_2$Cl$_2$ (4 ml) and the mixture was stirred

for 3 h at room temperature. Methanol (1 ml) was added and after further 15 min stirring, the reaction mixture was concentrated *in vacuo*, diluted with ethyl acetate, washed with 0.1 M KHSO$_4$, 5% NaHCO$_3$, brine and dried. Evaporation of the solvent gave an oily residue that was purified by LPLC (eluant, *n*-hexane : ethyl acetate 10 : 1 v/v). Yield 0.12 g (55%); R_f = 0.70 (E8); ^{1}H-NMR (400 MHz): δ 7.31 (m, 4H, H-*o* aromatics), 7.15 (m, 6H, H-*m,p* aromatics), 5.67 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.26 Hz, H$_3$), 4.96 (d, 1H, $J_{1,2}$ = 3.65 Hz, H$_1$), 4.86 (dd, 1H, $J_{1,2}$ = 3.67 Hz, $J_{2,3}$ = 10.25 Hz, H$_2$), 4.69 (q, 1H, J_P = 19.1 Hz, J = 9.21 Hz, H$_4$), 4.32 (2s, 2H, H$_6$ and H$_{6'}$), 4.07 (m, 1H, H$_5$), 3.38 (s, 3H, OCH$_3$), 2.38 (m, 2H, $-CH_2-CO$), 2.12 (m, 2H, $-C'H_2-C'O$), 1.94 and 1.83 (2s, 6H, $2 \times CH_3$ TcBoc), 1.59 (m, 4H, $2 \times -C\underline{H}_2-CH_3$), 1.35 (bs, 20H, $2 \times -(CH_2)_5-$ myristic), 0.88 (t, 6H, J = 6.58 Hz, $2 \times CH_3$ myristic). ^{31}P-NMR (161.9 MHz): δ -10.68.

Methyl 6-*O*-*t*-butyldimethylsilyl-2,3-di-*O*-tetra-decanoyl-α-D-glucopyranoside (6)

t-Butyldimethylsilyl chloride (TBDMS-Cl; 1.2 ml, 7.2 mmol) was added to a solution of **2** (1.58 g, 4.1 mmol) and DMAP (26 mg, 0.2 mmol) in dry DMF (10 ml). After stirring overnight at room temperature, the solvent was evaporated *in vacuo* and the residue was purified by LPLC (eluant: CH$_2$Cl$_2$: *n*-hexane, 10 : 3 v/v). Yield 1.5 g, (73%, oil); $[\alpha]_D$ +52.6° (c 0.95, CHCl$_3$); R_f = 0.80 (E2), R_f = 0.44 (E4); ^{1}H-NMR (400 Mz): δ 5.33 (t, 1H, $J_{2,3} = J_{3,4}$ = 9.3 Hz, H$_3$), 4.88 (d, 1H, $J_{1,2}$ = 3.5 Hz, H$_1$), 4.83 (dd, 1H, $J_{1,2}$ = 3.6 Hz, $J_{2,3}$ = 10.1 Hz, H$_2$), 3.88 (m, 2H, H$_6$ and H$_{6'}$), 3.68 (m, 2H, H$_4$ and H$_5$), 3.38 (s, 3H, OCH$_3$), 3.05 (bs, 1H, OH$_4$), 2.32 (m, 4H, $2 \times -CH_2-CO$), 1.66 (m, 4H, $2 \times -C\underline{H}_2-CH_3$), 1.26 (bs, 20H, $2 \times -(CH_2)_5-$ myristic), 0.89 (m, 15H, $2 \times CH_3$ myristic and (CH$_3$)$_3$C– TBDMS), 0.01 (s, 6H, $2 \times CH_3$ TBDMS).

Methyl 6-*O*-*t*-butyldimethylsilyl-2,3-di-*O*-tetra-decanoyl-α-D-glucopyranoside 4-*O*-diphenyl-phosphate (7)

Diphenyl chlorophosphate (0.43 ml, 2 mmol) was added to a solution of **6** (0.51 g, 1 mmol) and DMAP (0.25 g, 2 mmol) in dry CH$_2$Cl$_2$ (10 ml). After stirring for 3 h at room temperature, methanol (1.5 ml) was added and the reaction mixture was further stirred for 15 min. The solvent was evaporated *in vacuo* and the residue (1.05 g) was used, without further purification, for the preparation of **8** (see below). For characterization, a small amount of crude **7** (200 mg) was chromatographed on the silica gel column (eluant: CH$_2$Cl$_2$) yielding a consistent amount of **8** (45 mg) and the title compound **7** (60 mg, 30%); $[\alpha]_D$ +33.3° (c 0.45, CHCl$_3$); R_f = 0.61 (E5); ^{1}H-NMR (400 MHz): δ 7.30 (m, 4H, H-*o* aromatics), 7.18 (m, 6H, H-*m, p* aromatics), 5.69 (t, 1H, $J_{2,3}$ = 9.89 Hz, $J_{3,4}$ = 9.58 Hz, H$_3$), 4.92 (d, 1H, $J_{1,2}$ = 3.63 Hz, H$_1$), 4.82 (dd, 1H, $J_{1,2}$ = 3.65 Hz, $J_{2,3}$ = 10.23 Hz, H$_2$), 4.66 (q, 1H, J_P = 18.8 Hz, J = 9.62 Hz, H$_4$), 3.89–3.78 (m, 2H, H$_5$ and H$_6$), 3.72 (q, 1H, $J_{6,6'}$ = 11.54 Hz, $J_{5,6}$ = 5.74 Hz, H$_{6'}$), 3.41 (s, 3H, OCH$_3$), 2.31 (m, 2H, CH$_2$-CO), 2.14 (m, 2H, $-C'H_2-C'O$), 1.6 (m, 4H, $2 \times -C\underline{H}_2-CH_3$), 1.26 (bs, 20H, $2 \times -(CH_2)_5-$), 0.88 (m, 15H, $2 \times CH_3$ myristic and (CH$_3$)$_3$C– TBDMS), 0.01 (s, $2 \times CH_3$– TBDMS).

Methyl 2,3-di-*O*-tetradecanoyl-α-D-glucopyranoside 4-*O*-diphenylphosphate (8)

From compound 7. The crude **7** (1.05 g) was taken up with an ethyl acetate : water mixture (100 ml, 7 : 3 v/v) and vigorously stirred at room temperature for 15 min. The quantitative removal of the TBDMS group occurred during this procedure probably because of the low acidity (pH 3–4) of the aqueous layer. The organic layer was collected, washed with water (2×30 ml), dried, concentrated *in vacuo* and purified by LPLC (eluant: CH_2Cl_2). Yield 0.37 g (70%); $[\alpha]_D$ +22.6° (c 1.03, $CHCl_3$); $R_f = 0.32$ (E2); ^{1}H-NMR (400 MHz): δ 7.35 (m, 4H, H-*o* aromatics), 7.18 (m, 6H, H-*m,p* aromatics), 5.70 (t, 1H, $J_{2,3} = J_{3,4} = 9.71$ Hz, H$_3$), 4.96 (d, 1H, $J_{1,2} = 3.67$ Hz, H$_1$), 4.88 (dd, 1H, $J_{1,2} = 3.67$ Hz, $J_{2,3} = 10.16$ Hz, H$_2$), 4.74 (q, 1H, $J_P = 19.29$ Hz, J = 9.65 Hz, H$_4$), 3.75 (d, 1H, $J_{4,5} = 9.86$ Hz, H$_5$), 3.66 (2s, 2H, H$_6$ and H$_{6'}$), 3.39 (s, 3H, OCH$_3$), 2.29 (m, 2H, CH$_2$–CO), 2.11 (m, 2H, –C'H2–C'O), 1.57 (m, 2H, –C$\underline{H}_2$–CH$_3$), 1.45 (m, 2H, –C'$\underline{H}_2$–C'H$_3$), 1.26 (bs, 20H, $2 \times$ –(CH$_2$)$_5$–), 0.88 (t, 6H, J = 6.64 Hz, $2 \times$ CH$_3$ myristic). ^{31}P-NMR (161.9 MHz): δ −8.24. Alternatively, the quantitative removal of TBDMS group from **7** can be achieved by treatment with BF$_3$.Et$_2$O [29].

From compound 5. Zinc dust (35 mg) was added to a solution of **5** (100 mg, 0.12 mmol) in 95% aqueous acetic acid (4 ml). The resulting suspension was vigorously stirred for 2 h at 60 °C and filtered through Celite. Toluene was added to the filtrate, the solvent was removed *in vacuo* and the residue was purified by LPLC (eluant: CH_2Cl_2). The yield was 45 mg (60%) of a product analytically undistinguishable from that obtained from **7**.

Methyl 2,3-di-*O*-tetradecanoyl-α-D-glucopyranoside 4-*O*-phosphate, GL2 (9)

Compound **8** (96 mg, 0.15 mmol) was dissolved in methanol (10 ml) and hydrogenated at room temperature for 20 h, in the presence PtO$_2$. The catalyst was removed by filtration and the solvent was removed *in vacuo*. Yield 69 mg (95%); $[\alpha]_D$ +68.3° (c 1.04, $CHCl_3$: methanol, 75 : 15 v/v); $R_f = 0.58$ (E1); ^{1}H-NMR (400 MHz, 10% CD$_3$OD in CDCl$_3$): δ 5.44 (t, 1H, $J_{2,3} = J_{3,4} = 9.64$ Hz, H$_3$), 4.85 (d, 1H, $J_{1,2} = 3.62$ Hz, H$_1$), 4.77 (dd, 1H, $J_{1,2} = 3.69$ Hz, $J_{2,3} = 10.23$ Hz, H$_2$), 4.27 (q, 1H, $J_P = 19.29$ Hz, J = 9.75 Hz, H$_4$), 3.86 (d, 1H, $J_{6,6'} = 11.76$ Hz, H$_6$), 3.67 (m, 2H, H$_6$ and H$_5$), 3.31 (s, 3H, OCH$_3$), 2.25 (m, 4H, $2 \times$ CH$_2$–CO), 1.50 (m, 4H, $2 \times$ –C$\underline{H}_2$–CH$_3$), 1.26 (bs, 20H, $2 \times$ –(CH$_2$)$_5$–), 0.80 (t, 6H, J = 6.70 Hz, $2 \times$ CH$_3$ myristic). ^{31}P-NMR (161.9 MHz): δ 2.87; ESI-MS: [M − H] m/e 693.38 (calcd 693.43).

Preparation of Phospholipid Vesicles

Small unilamellar vesicles (SUV) were prepared by sonication using a titanium microtip ultrasonicator (Ultrasonic Processor Gex400, Vibracell). The lipids (20 mg) were dissolved by mixing in a chloroform : methanol mixture (2 ml, 1 : 1 v/v). The solvent was removed by passing a stream of nitrogen through the solution and the lipid film was thoroughly dried *in vacuo*, suspended in 5 ml of buffer (10 mM tris(hydroxymethyl)aminomethane (Tris), 100 mM NaCl, pH 7.4), and kept at 37 °C for 30 min and overnight at room temperature. The suspension was sonicated for 15 min at 40 °C

(under nitrogen, at 0 °C, in the case of PC containing multilayers) and the titanium debris were removed by centrifugation at 26 500 g.

Large unilamellar vesicles (LUV) were prepared by vortexing the dried lipid film in the appropriate buffer (10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes), 150 mM NaCl, 1 mM EDTA, pH 7.4) or in a 70 mM calcein solution (pH 7.4) The resulting suspension was freeze-thawed for 10 cycles and extruded at 37 °C through polycarbonate filters (Nucleopore) (two times through two stacked 0.2 µm pore size filters followed by 10 times through two stacked 0.1 µm pore size filters). The calcein-entrapped vesicles were separated from free calcein by gel filtration on a Sephadex G-75 column (eluant: 10 mM Hepes, 150 mM NaCl, 1 mM EDTA, pH 7.4). Lipid concentration was determined by phosphorous analysis [30]. If necessary, calcein-free LUV were mixed with dye-loaded liposomes to adjust the lipid concentration to the desired value. According to the dynamic light scattering experiments, the average diameter of the vesicles was 47–57 nm for SUV and 100–107 nm for LUV.

Dye-leakage Assay

The peptide-induced release of calcein from LUV was fluorometrically monitored at an excitation wavelength of 490 nm and at an emission wavelength of 520 nm, at 37 °C. The lipid concentration was constant (50 µM), and the increasing [peptide]/[lipid] molar ratio was obtained by adding aliquots of peptide solution. The percentage of released calcein at time t (5 min) was determined as $(F_t − F_0)/(F_T − F_0) \times 100$, where 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 10% aqueous 4-(1,1,3,3-tetramethylbutyl)-phenyl-polyethylene (10) glycol (Triton X-100; 20 µl) after 5 min of fluorescence registration.

RESULTS AND DISCUSSION

Synthesis of Glycolipids and Vesicles Formation

The glycolipids GL1 (**2**) and GL2 (**9**) were synthesized starting from methyl 4,6-*O*-isopropylidene-α-D-glucopyranoside, as shown in Scheme 1. Reaction with myristic acid in the presence of DCC and DMAP afforded the methyl 4,6-*O*-isopropylidene-2,3-di-tetradecanoyl-α-D-glucopyranoside **1**. The isopropylidene group was removed by acid hydrolysis and the crude GL1 was isolated in good yield (86%), after chromatographic purification. An attempt to protect selectively the primary hydroxyl group as TcBoc derivative [31] afforded the 4,6-di-TcBoc derivative as the major product. Better selectivity was achieved (Scheme 1) by using the TBDMS-Cl [32]. Phosphorylation of the resulting 6-O-TBDMS derivative (73% yield, after chromatographic purification) was carried out by reaction with diphenyl chlorophosphate in the presence of DMAP. Spontaneous cleavage of the acid-labile TBDMS group occurred during the workup of the reaction mixture. The phosphorylated derivative **8** was purified by LPLC

and the phenyl groups were removed by catalytic hydrogenolysis. The overall yield of the GL1–GL2 transformation was 53%. SUV and LUV of egg phosphatidylcholine containing either the synthesized glycolipids or anionic phospholipids (DMPG) were prepared by sonication or extrusion through 100 nm pure polycarbonate membranes, respectively, and characterized on the base of dynamic light scattering measurements. LUV entrapping a fluorescent dye (calcein) were prepared for membrane permeabilization experiments. They were generally stable at room temperature for 48 h.

Circular Dichroism Measurements

According to CD measurements, most amphipatic antimicrobial peptides exhibit an unordered structure in aqueous solution but adopt an ordered α-helical conformation in the presence of liposomes composed of negatively charged phospholipids [33,34] or lipid A [17,21]. The CD spectra of Pro-rich peptides in aqueous solution exhibit a strong negative band at, or a little above, 200 nm, which has been attributed either to a lack of conformational preferences [27,35] or to a poly(L-proline) type II conformation [36,37]. Membrane mimicking solvents scarcely affect the CD

pattern of Pro-Arg rich peptides, but a somewhat greater effect was observed in the presence of acidic phospholipid vesicles at low saline concentration [36–38]. As shown in Figure 2A,B, the CD spectra of apidaecin Ib and drosocin do not change significantly moving from the buffer to mixed acidic phospholipid vesicles (PC-DMPG or PC-GL2) suggesting that, even in the presence of negatively charged vesicles, peptides possess a considerable conformational freedom. GL2 vesicles and SDS micelles, but not DMPG vesicles (Figures 2C and D) significantly affect the position and intensity of the negative maximum, suggesting a tighter interaction of peptides with the glycosidic head groups of the glycolipid vesicles. Magainin 2 is very sensitive to the composition of the lipid environment (Figure 3A) and the acidic vesicles are the most effective promoters of a helical conformation. The helicity induced by GL2 is similar to that induced by DMPG and comparable to that induced by LPS and lipid A [21]. The conformational change is driven by an electrostatic interaction between the cationic peptide and the negatively charged surface of the lipid aggregate, and in the presence of zwitterionic vesicles containing the neutral glycolipid GL1 the peptide helical content is sensibly lower than in PC alone (Figure 2B).
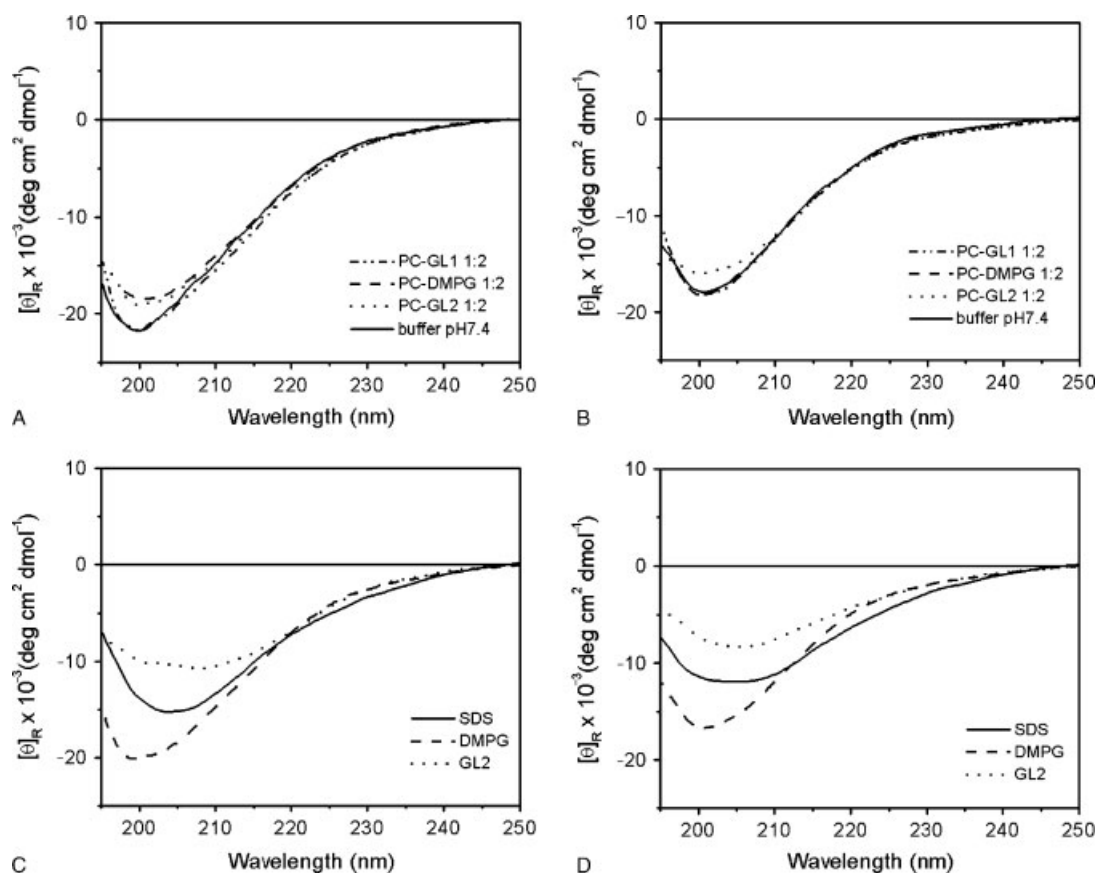
Figure 2 CD spectra at 25 °C of apidaecin Ib (A and C) and drosocin (B and D) (peptide concentration 0.1 mM) in buffer (Tris/NaCl 10/100 mM, pH 7.4), 1% SDS in buffer, and in the presence of SUV in the indicated composition (molar ratio). Lipid concentration is 5 mM.
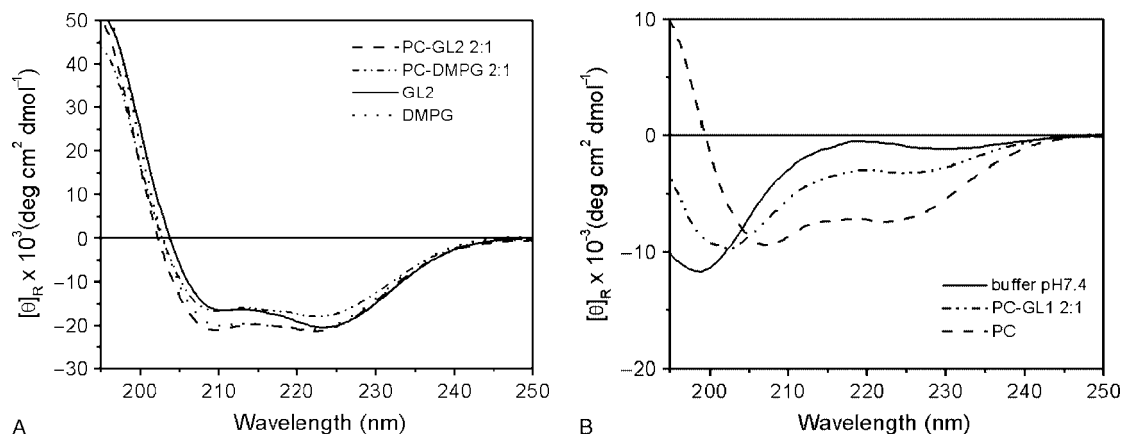
Figure 3 CD Spectra at 25 °C of magainin 2. Experimental conditions as in Figure 2.
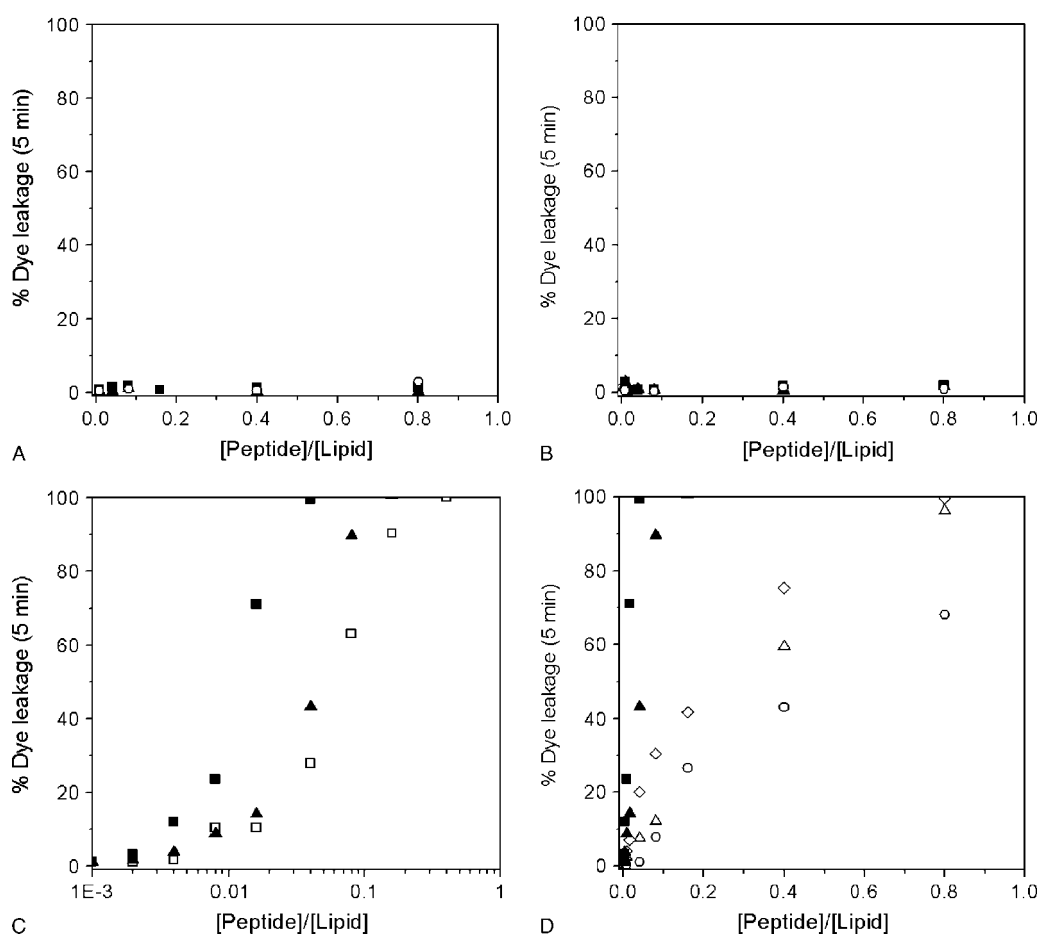
Figure 4 Release of the fluorescent probe calcein from LUV: ■ PC-GL2 1 : 2; ▲ PC-DMPG 1 : 2; ○ PC-GL1 2 : 1; □ PC-GL2 2 : 1; ◇ PC-GL2 1 : 2 + 10 mM MgCl$_2$; △ PC-DMPG 1 : 2 + 10 mM MgCl$_2$ (lipid concentration 50 μM), measured after 5 min after the addition of increasing amounts of peptide. (A) apidaecin Ib, (B) drosocin, (C and D) magainin 2.

Membrane Permeabilization Activity against Acidic Phospholipid- and Phosphoglycolipid-containing Liposomes

Whether antibacterial peptides act at the level of the cytoplasmatic membrane or on intracellular targets, they have to cross the bacterial OM. The size of antimicrobial peptides is not compatible with an uptake mechanism based on diffusion through porins, proteins which span the OM and induce the formation of a water-filled channel accessible to small hydrophilic molecules [39]. A self-promoted uptake mechanism [15] has been proposed for polycationic antimicrobial peptides, in which they displace the divalent cations (primarily Mg^{2+}

and Ca^{2+}) that bridge adjacent LPS and stabilize the OM. The peptide-induced disorder of the OM structure is accompanied by the permeabilization to a variety of compounds, including the peptide itself. Lipid A, the glycolipid membrane component of LPS, plays a major role in the binding of polycationic antimicrobial peptides, and it has been demonstrated that magainin 2 can lead to disorder into the LPS organization [13] and destabilise bilayers containing either LPS or lipid A [21].

The permeabilization-inducing ability of apidaecin Ib, drosocin and magainin 2 on PC-GL2 vesicles was evaluated by measuring the efflux of a fluorescent dye, calcein, entrapped within LUV of a different composition. The results were compared with those obtained with neutral and anionic phospholipid bilayers, lacking glycosidic head groups, often used to investigate the mechanism of action of antimicrobial cationic peptides [5,6]. The profiles of the calcein leakage, 5 min after the peptide addition, as a function of peptide/lipid ratio, are shown in Figure 4 for some selected vesicles. Apidaecin Ib (Figure 4A) and drosocin (Figure 4B) did not show any leakage activity on either GL2- or DMPG-containing vesicles, in agreement with the results previously obtained on simple phospholipid bilayers [27]. On the contrary, magainin 2 effectively permeabilized PC-GL2 vesicles and, according to previously reported data [33], the leakage activity was related to the amount of anionic glycolipid in the bilayer (Figure 4C). Both electrostatic and hydrophobic interactions are important for membrane binding and permeabilization. Drosocin and apidaecin Ib, which are unable to adopt such an amphipathic arrangement, do not perturbe the bilayer. Addition of magnesium ions (10 mM MgCl$_2$) reduced in a similar way the magainin 2 leakage activity on both PC/GL2 and PC/DMPG vesicles (Figure 4D), indicating that only the electrostatic peptide-membrane interaction was inhibited by divalent cations.

CONCLUSION

The interaction of cationic antimicrobial peptides with vesicles containing charged glycolipids closely resembles that of simple anionic phospholipid membranes. Peptides such as magainin 2 when in contact with negatively charged membranes, can adopt an amphipathic secondary structure and insert their non-polar amino acid side-chains into the hydrophobic core of the membrane. This can lead to disorder in the supramolecular architecture of the bilayer. On the contrary, peptides such as apidaecin Ib and drosocin, which are devoid of this potential amphipathicity, cannot effectively destabilize the membrane. Among the various hypotheses involving the crossing of the OM, the self-promoted uptake mechanism is compatible with the first class of peptides, but pathways similar to that proposed for some cell-penetrating peptides [40] are more suitable for Pro-Arg rich antimicrobial peptides.

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