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Lipopeptides of the N-terminus of *Escherichia coli* lipoprotein: synthesis, mitogenicity and properties in monolayer experiments

Werner Prass [a], Helmut Ringsdorf [a], Wolfgang Bessler [b], Karl-Heinz Wiesmüller [c] and Günther Jung [c]

[a] *Institut für Organische Chemie, Johannes-Gutenberg-Universität, J.-J. Becher-Weg 18–20, D-6500 Mainz,* [b] *Institut für Immunbiologie, Universität Freiburg, Stefan-Meier-Strasse 8, D-7800 Freiburg,* and [c] *Institut für Organische Chemie der Eberhard-Karls-Universität, Auf der Morgenstelle 18, D-7400 Tübingen (F.R.G.)*

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The N-terminal part of the lipoprotein from the outer membrane of *Escherichia coli*, tripalmitoyl-*S*-glyceryl-L-Cys-Ser and analogs with longer sequences, are polyclonal activators for B-lymphocytes. Triple-chain lipopeptides also constitute efficient low-molecular-weight carrier/adjuvant systems, which can be linked to antigens to yield immunogens for antibody production without further additives. This is the first report of monolayer experiments with chemically well defined, synthetic lipopeptide mitogens with the composition of the N-terminus of an important bacterial membrane protein. Various derivatives of the lipoprotein N-terminus were synthesized. These lipopeptides differed in the length of the peptide moiety, the number of fatty acid residues, and protective groups. In order to obtain the surface areas for the lipopeptides in isotherms and hysteresis isotherms, monolayer experiments with a computer-controlled film balance were performed. To get some information about the interaction of these compounds with typical membrane lipids mixed monolayers were formed from triple-chain lipopeptides with dipalmitoylphosphatidylcholine and cholesterol. A comparison of the mitogenic response of the compounds was made in an in vitro system with B-lymphocytes from Balb/c mice.

Introduction

Lipoprotein from outer membrane of *Escherichia coli* activates B-lymphocytes polyclonally [1]. Enzymatic cleavage with pronase allowed the localization of the mitogenic activity in the N-terminal part of the membrane protein [2] consisting of the unusual amino acid *S*-glyceryl-L-cysteine, which is covalently linked with three fatty acids. The synthetic lipopeptides used in this work contain solely palmitic acid, whereas a mixture of different fatty acids was found in the natural compound [3]. The synthetic lipopentapeptide *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-(*S*)-seryl-(*S*)-seryl-(*S*)-asparaginyl-(*S*)-alanine has been shown to exhibit the same mitogenic properties as the native lipoprotein [4–6]. Even after shortening of the peptide chain from the lipopenta- to the lipodipeptide Pam_3Cys-Ser the immunostimulating properties are still

Abbreviations: Pam_3Cys, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteine; Fmoc, fluorenylmethoxycarbonyl; DPPC, dipalmitoylphosphatidylcholine; FD-MS, field desorption mass spectra.

Correspondence: G. Jung, Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 18, D-7400 Tübingen, F.R.G.

maintained. However, tripalmitoyl-*S*-glyceryl-cysteine (Pam_3Cys) itself exhibited only a weak stimulating effect [7]. The new synthetic mitogens are highly suitable compounds for the study of the early events of the immune response, for the isolation of specific binding proteins for lipoprotein [8], and for structure/activity studies.

Recently, a pronounced carrier and adjuvant function of the lipopeptide Pam_3Cys-Ser was found after its covalent linkage to a partial sequence of the receptor of the epidermal growth factor (EGF-R 514–529) [9,10]. The most promising application of the lipopeptides as a low-molecular weight, clearly defined carrier/adjuvant system for the presentation of antigens on the cell surface [11] prompted us to study their physical properties in monolayer systems with the film balance. For the lipotetrapeptide we report mixture diagrams with cholesterol and dipalmitoylphosphatidylcholine (DPPC). Furthermore the mitogenic activities of the lipopeptides, before and after the deprotection of the bulky and lipophilic *tert*-butyl protecting groups, are reported.

Materials and Methods

Chemicals. Reagents and solvents were from Merck (Darmstadt), if not indicated otherwise. Piperidine, dimethylformamide, 1-hydroxybenzotriazole, and trifluoroacetic acid were from Fluka (Ulm). Acetone, diethyl ether, light petroleum (30–50 °C), and ethyl acetate were freshly distilled before use. Fmoc-Ser(Bu^t)-OH and HCl · H-Asn-OBu^t were from Bachem (Bubendorf). The water used for all film balance studies was purified with a Millipore water purification system (Milli-Q, 4 bowl) fed with distilled water. DPPC and cholesterol used for the studies in mixed monolayers were from Fluka (purissimum grade). While the DPPC could be used without further purification, cholesterol was recrystallized immediately before use. The purity of these compounds was checked by film balance measurements, which is a more precise proof of the purity of the lipids than most other methods, as for example TLC [12].

Monolayer experiments. All isotherms were measured with a computer-controlled film balance as described in Ref. 13, however, with improved programs. Most of the curves were measured in the conventional 'normal mode'. In this mode the compression rate of the moving barrier of the film balance is constant and can be chosen at the beginning of the measurement. We chose a compression rate of 10 min/sweep for all measurements in the 'normal mode'. Some of the curves were measured using the 'thermodynamic mode'. In that mode the barrier does not advance the next step until the surface pressure has reached an equilibrium value, that means that the surface pressure is constant within the resolution of the A/D converter of the pressure pickup system (0.03 mN/m). The results obtained in this way were essentially the same as in the 'normal mode' at very slow compression rates (more than 1 h/sweep). Another approach to measure closer to the thermodynamic equilibrium is to measure hysteresis isotherms. There the monolayer first is compressed to a certain surface pressure (e.g. 20 mN/m) and, after waiting for some time (e.g. 5 min) at this pressure, expanded again to an area much larger than the offset point. After waiting again at this point for a certain time (e.g. 1 min), the film is compressed again beyond the breakdown point. If the compounds are very pure and the monolayer is not too viscous, almost no hysteresis should be found when measuring hysteresis isotherms.

For each curve the substrate water was changed, and the microcomputer did not start the measurement before the temperature reached equilibrium, that means that the temperature drift had to be less than 0.1 K/min. For spreading of the lipopeptide solution we used a gas-tight syringe with a Teflon plunger and an adjustable stopface (Kloehn Co., Whittier, CA, U.S.A.). The amount spread (80–160 µl) was precisely the same for one set of curves. The mixtures were prepared from equimolar stock solutions by mixing appropriate amounts.

Thin-layer chromatography. Silica gel plates 60 F_{254} (Merck, Darmstadt) and the following solvent systems were used in solvent saturated glass chambers for determining R_F values at room temperature.

A: chloroform/methanol/water (65 : 25 : 4, v/v);
B: chloroform/methanol/water/acetic acid (65 : 25 : 4 : 3, v/v);
C: chloroform/methanol/acetic acid (90 : 10 : 1, v/v);

D: ethyl acetate saturated with water;
E: chloroform/methanol (9 : 1, v/v).
For detection the plates were sprayed sequentiallly with water, ninhydrin reagent, and chlorine/4,4′-bis(dimethylamino)-diphenylmethane (TDM reagent). The lipopeptides were also detected with iodine.

Analytic methods. The synthetic lipopeptides were characterized by amino acid analysis after total hydrolysis (6 M HCl, 110 °C, 18 h), whereby the *S*-glycerylcysteine residue is partially decomposed [4]. The same samples of hydrolysates were used for the preparation of pentafluoropropionyl-amino acid-*n*-propyl ester derivatives for the quantitative gas chromatographic determination of the enantiomeric ratios on glass capillaries (20 m) coated with the chiral stationary phase Chirasil-Val [14]. The D-amino acid content of all samples tested was less than 1% after correction of the racemization due to hydrolysis. Elemental analyses were made from the recrystallized and dried products using the elemental analyzer Model 1104 (Carlo Erba). Field desorption mass spectra (FD-MS) were taken on a Varian MAT 711 after dissolving samples in chloroform and applying to carbon whiskers on tungsten wire. ^{13}C-NMR spectra (Table I) were measured on the spectrometer WM 400 (Bruker-Physik, Karlsruhe) in [^{12}C,^{2}H] chloroform/methanol or in [^{2}H]chloroform solution (c = 50 mg/ml) at 25 °C. Signal assignments were made with J-modulated spin-echo spectra and by comparison with a representative data set from related compounds [4,15].

Synthesis of lipopeptides

N^{α}-9-Fluorenylmethyloxycarbonyl-O-tert-butyl-seryl-O-tert-butyl-serine tert-butyl ester. Fmoc-Ser(Bu^{t})-OH (1.3 mmol, 500 mg) in dichloromethane (5 ml), 1-hydroxybenzotriazole (1.3 mmol, 176 mg) in dimethylformamide (1 ml), and *N,N′*-dicyclohexylcarbodiimide (1.3 ml of a 1 M solution in dichloromethane) were stirred 30 min at 0 °C. H-Ser(Bu^{t})-OBu^{t} [16] (1.3 mmol, 283 mg) in dichloromethane (3 ml) was added. After 10 h at room temperature the precipitated dicyclohexylurea was filtered off, the filtrate evaporated in vacuo, the residue dissolved in dichloromethane (20 ml), washed with 5% $KHSO_4$, 5% $NaHCO_3$, and water (3 × 20 ml each), and dried with sodium sulfate. After removal of the solvent the oily residue was taken up in methanol. After precipitation of the dicyclohexylurea at −20 °C, filtration and evaporation of the solvent, the colourless residual oil solidified on drying in vacuo in the presence of diphosphorus pentoxide. Yield 400 mg (53%); m.p. 44 °C; R_F(D) = 0.76; R_F(E) = 0.82. ^{13}C-NMR: see Table I.

$C_{33}H_{46}N_2O_7$ (582.7):
Calcd.: C 68.02, H 7.96, N 4.81
Found: C 68.60, H 8.38, N 4.83.

N^{α}-9-Fluorenylmethyloxycarbonyl-O-tert-butyl-seryl-asparagine tert-butyl ester. Fmoc-Ser(Bu^{t})-OH (1 mmol, 383 mg) in dichloromethane (4 ml), 1-hydroxybenzotriazole (1 mmol, 135 mg) in dimethylformamide (1 ml), and *N,N′*-dicyclohexylcarbodiimide (1 ml of a 1 M solution in dichloromethane) were stirred at 0 °C. After 30 min HCl · H-Asn-OBu^{t} (1 mmol, 225 mg) and *N*-methylmorpholine (1.1 mmol, 11 mg) were added. After 8 h at room temperature the precipitated urea was filtered off and washed with dichloromethane (10 ml). After dilution with dichloromethane (10 ml) the filtrate was washed with 5% $KHSO_4$, 5% $NaHCO_3$, and water (3 × 30 ml each), and dried with Na_2SO_4. After evaporation of the solvent the residue was purified by precipitation from dichloromethane/ethyl acetate (1 : 1, v/v) and from acetone/water to yield colourless Fmoc-Ser(Bu^{t})-Asn-OBu^{t}. Yield 380 mg (69%); m.p. 149 °C; R_F(C) = 0.67; R_F(E) = 0.52. ^{13}C-NMR: see Table I.

$C_{30}H_{39}N_3O_7$ (553.7):
Calcd.: C 65.08, H 7.10, N 7.59
Found: C 65.31, H 7.14, N 7.59.

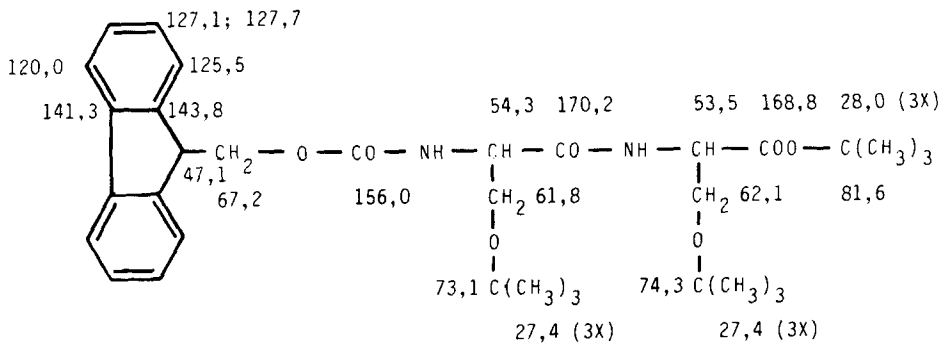
N^{α}-9-Fluorenylmethyloxycarbonyl-O-tert-butyl-seryl-O-tert-butyl-seryl-asparagine tert-butyl ester. Fmoc-Ser(Bu^{t})-Asn-OBu^{t} (580 μmol, 321 mg) in piperidine/dimethylformamide (1 : 10, v/v; 10 ml) was stirred at room temperature, after 1 h the solution was evaporated in vacuo, and the residue was dissolved in dimethylformamide (3 ml). Separately Fmoc-Ser(Bu^{t})-OH (580 μmol, 222 mg) in 5 ml dichloromethane was activated with 1-hydroxybenzotriazole (580 μmol, 78 mg) and *N,N′*-dicyclohexylcarbodiimide (580 μl of a 1 M solution in dichloromethane) at 0 °C for 30 min and added to the dipeptide ester. After 4 h at room temperature the solvent was removed in

TABLE I

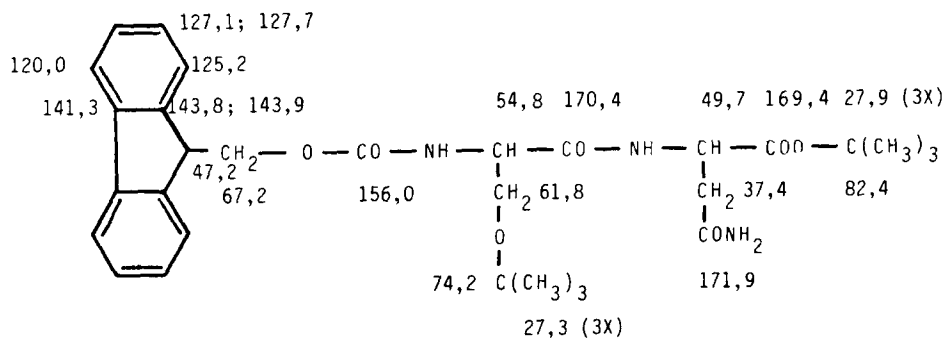
^{13}C-NMR DATA (100.16 MHz) OF THE PROTECTED PEPTIDE INTERMEDIATES

(a) N^{α}-9-Fluorenylmethyloxycarbonyl-*O-tert*-butyl-seryl-*O-tert*-butyl-serine *tert*-butyl-ester.
(b) N^{α}-9-Fluorenylmethyloxycarbonyl-*O-tert*-butyl-seryl-asparagine *tert*-butyl-ester.
(c) N^{α}-9-Fluorenylmethyloxycarbonyl-*O-tert*-butyl-seryl-*O-tert*-butyl-serine-asparagine *tert*-butyl-ester.

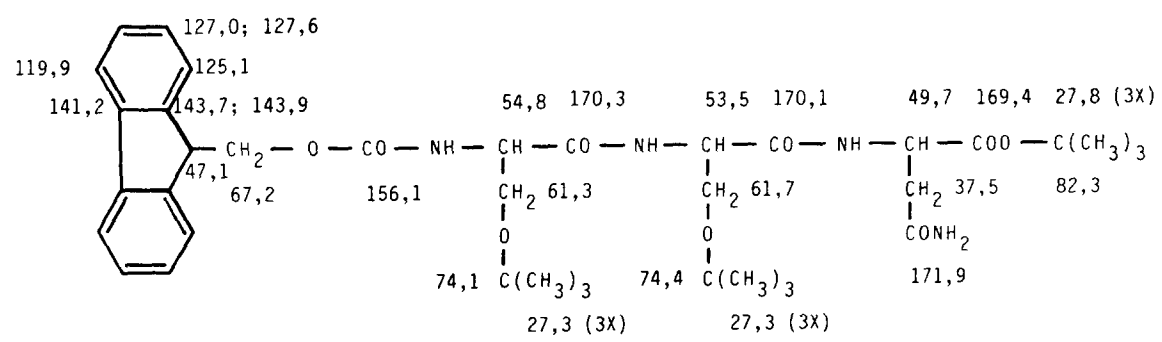
(a)



(b)



(c)



vacuo, the residue was taken up in dichloromethane (80 ml), filtered, washed with 5% $KHSO_4$, 5% $NaHCO_3$, water (3 × 80 ml each), dried with Na_2SO_4, and the solvent evaporated. The residue was stirred in diethyl ether/light petroleum (30–50 °C), and after 12 h at 4 °C the colourless tripeptide was collected by filtration, washed with cold light petroleum and dried in vacuo with diphosphorus pentoxide. Yield 340 mg (84%); m.p. 139 °C; R_F(D) = 0.40; R_F(E) = 0.51. ^{13}C-NMR: see Table I. Amino acid analysis: Asp 1.00; Ser 1.8; ammonia 0.72.

$C_{37}H_{52}N_4O_9$ (696.9):

Calcd.: C 63.77, H 7.52, N 8.04

Found: C 64.02, H 8.02, N 7.99.

S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-O-tert-butyl-(S)-serine tert-butyl ester. Pam_3Cys-OH (99 μmol, 90 mg) was activated in dichloromethane (2 ml) with *N,N'*-dicyclohexylcarbodiimide (0.1 mmol, 0.1 ml of a 1 M solution in dichloromethane) and 1-hydroxybenzotriazole (0.1 mmol, 13.5 mg) in dimethylformamide (1 ml) at 0 °C. After 30 min H-Ser(Bu^t)-OBu^t (0.1 mmol, 22 mg) was added. After 4 h at room temperature the precipitated dicyclohexylurea was removed by centrifugation. The supernatant was chromatographed on Sephadex LH 20 (column 80 × 1.5 cm; dichloromethane/chloroform (1 : 1, v/v); 2 drops/s; 50 fractions of 1 ml). After TLC analysis (silica gel plates, system E, chlorine/TDM detection) fractions 25–35 were evaporated in vacuo and dried with diphosphorus pentoxide. Yield 88 mg (79%); m.p. 50 °C; R_F(C) = 0.94; R_F(D) = 0.87; ^{13}C-NMR: see Table II.

$C_{65}H_{124}N_2O_9S$ (1109.8):

Calcd.: C 70.34, H 11.26, N 2.52, S 2.88

Found: C 70.34, H 11.26, N 2.60, S 2.97.

S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-(S)-serine. Pam_3Cys-Ser(Bu^t)-OBu^t (45 μmol, 50 mg) was deprotected in trifluoroacetic acid (1 ml). After 1 h at room temperature the acid was removed in vacuo, and the residue dissolved in warm chloroform (2 ml). After addition of methanol (5 ml) Pam_3Cys-Ser-OH precipitated at 4 °C. After filtration and washing with cold methanol the lipopeptide was dried with diphosphorus pentoxide. Yield 42 mg (94%); m.p. 78 °C; R_F(B) = 0.84; R_F(C) = 0.49; R_F(E) = 0.82. Amino acid analysis: Ser 1.0; *S*-(2,3-dihydroxypropyl)cysteine (uncorr. remaining amount after hydrolysis): 0.37; FD-MS: M^+ 998.

$C_{57}H_{108}N_2O_7S$ (997.6):

Calcd.: C 68.60, H 10.89, N 2.81, S 3.20

Found: C 68.36, H 10.74, N 2.70, S 3.47.

S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-O-tert-butyl-(S)-seryl-O-tert-butyl-(S)-serine tert-butyl ester. Pam_3Cys-OH (0.33 mmol, 303 mg) in dichloromethane (5 ml) was activated with *N,N'*-dicyclohexylcarbodiimide (0.33 mmol, 0.33 ml of a 1 M solution in dichloromethane) and 1-hydroxybenzotriazole (0.33 mmol, 51 mg) in 1 ml dimethylformamide at 0 °C for 30 min. Meanwhile Fmoc-Ser(Bu^t)-Ser(Bu^t)-OBu^t (0.33 mol, 183 mg) was treated with 3 ml of piperidine/dimethylformamide (1 : 10, v/v) at room temperature for 1 h. After removal of the solvent and dissolving the residue in dichloromethane (3 ml) the dipeptide ester was combined with the activated Pam_3Cys-OH. After 4 h at room temperature the mixture was centrifuged and the supernatant chromatographed on Sephadex LH 20 (65 × 3 cm) in dichloromethane/methanol (1 : 1, v/v), 2 drops/s, 110 fractions a 2 ml; fractions 75–85 contained the product. Yield 290 mg (70%); m.p. 55 °C; R_F(C) = 0.91; R_F(E) = 0.82; ^{13}C-NMR: see Table II.

$C_{72}H_{137}N_3O_{11}S$ (1253.0):

Calcd.: C 69.02, H 11.02, N 3.35, S 2.56

Found: C 68.83, H 11.17, N 3.55, S 2.48.

S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-N-pal-

TABLE II

^{13}C-NMR DATA (100.16 MHz) OF THE LIPOPEPTIDES

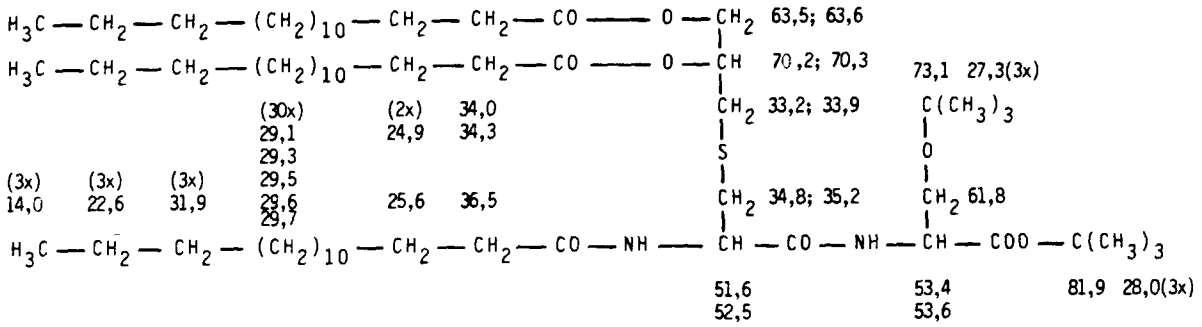
(a) *S*-[2,3-Bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-*O*-*tert*-tubyl-(*S*)-serine *tert*-butyl ester.

(b) *S*-[2,3-Bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-seryl-*O*-*tert*-butyl-serine *tert*-butyl ester.

(c) *S*-[2,3-Bis(palimotyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-*O*-*tert*-butyl-(*S*)-seryl-*O*-*tert*-butyl-seryl-asparagine *tert*-butyl ester.

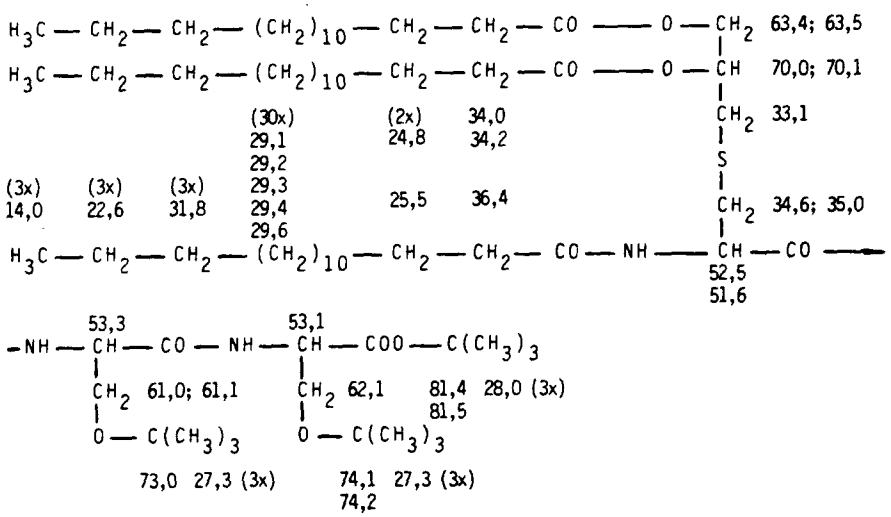
(*c* = 50 mg/ml [^{2}H]chloroform, 25 °C)

(a)



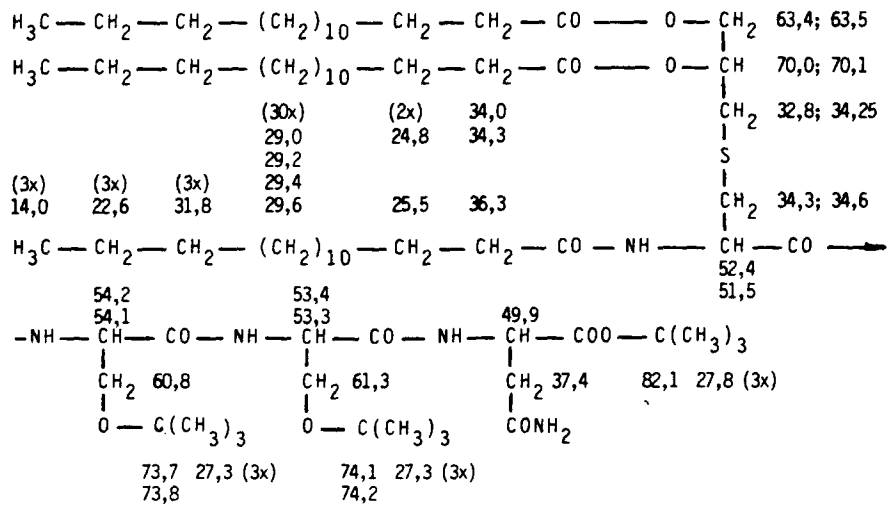
Carbonyl signals (not assigned): 168,7; 169,9; 172,9; 173,1; 173,2.

(b)



Carbonyl signals (not assigned): 168,7; 168,8; 169,2; 169,6; 170,0; 170,1; 173,0; 173,1; 173,2.

(c)



Carbonyl signals (not assigned). 169,3; 169,6; 169,9; 170,6; 170,7; 172,0; 173,2; 173,3; 173,4.

mitoyl-(R)-cysteinyl-(S)-seryl-(S)-serine. Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-OBu^t (0.13 mmol, 160 mg) was deprotected with trifluoroacetic acid (2 ml) at room temperature within 1 h. After removal of the acid the lipotripeptide was precipitated from chloroform/methanol (1 : 3, v/v) in the ice bath and dried with diphosphorus pentoxide. Yield 130 mg (92%); m.p. 183 °C; $R_F(B) = 0.79$; $R_F(C) = 0.20$; $R_F(E) = 0.70$. Amino acid analysis: Ser 2.0; *S*-(2,3-dihydroxypropyl)cysteine (uncorr.) 0.35; FD-MS: M^+ 1086.

$C_{60}H_{113}N_3O_{11}S$ (1084.6):

Calcd.: C 66.44, H 10.50, N 3.87, S 2.96

Found: C 66.27, H 10.68, N 3.83, S 3.17.

S-[2,3-Bis(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-O-tert-butyl-(S)-seryl-O-tert-butyl-(S)-seryl-(S)-asparagine-tert-butyl-ester. Pam_3Cys-OH (0.22 mmol, 200 mg) and 1-hydroxybenzotriazole (0.22 mmol, 30 mg) in 2 ml of dichloromethane/dimethylformamide (1 : 1, v/v) were activated with *N,N'*-dicyclohexylcarbodiimide (0.22 mmol, 0.22 ml of a 1 M solution in dichloromethane) at 0 °C for 30 min. Meanwhile Fmoc-Ser(Bu^t)-Ser(Bu^t)-Ser(Bu^t)-Asn-OBu^t (0.22 mmol, 153 mg) was deprotected in 3 ml of piperidine/dimethylformamide (1 : 10, v/v) at room temperature within 1 h. After evaporating to dryness and suspending in dichloromethane (2 ml), the tripeptide ester was added to activated Pam_3Cys-OH. After 4 h at room temperature methanol (20 ml) was added, and the lipotetrapeptide precipitated at 0 °C; after washing with cold methanol it was recrystallized again from dichloromethane/methanol to yield a colourless product. Yield 182 mg (61%); m.p. 163 °C; $R_F(C) = 0.93$; $R_F(E) = 0.64$; ^{13}C-NMR: see Table II.

$C_{76}H_{143}N_5O_{13}S$ (1367.1):

Calcd.: C 66.77, H 10.54, N 5.12, S 2.35

Found: C 66.84, H 10.74, N 4.93, S 2.58.

S-[2,3-Bis-(palmitoyloxy)-(2RS)-propyl]-N-palmitoyl-(R)-cysteinyl-(S)-seryl-(S)-seryl-(S)-asparagine. Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-Asn-OBu^t (0.12 mmol, 160 mg) was deprotected with trifluoroacetic acid (2 ml) at room temperature within 1 h. After evaporation in vacuo the residue was twice taken up in dichloromethane (5 ml) and evaporated again. The residual product was precipitated from chloroform (2 ml) by addition of methanol (6 ml) in the ice bath. The colourless lipotetrapeptide was washed with cold methanol and dried with diphosphorus pentoxide in vacuo. Yield 136 mg (95%); m.p. 176 °C; $R_F(B) = 0.49$; $R_F(C) = 0.93$; $R_F(E) = 0.64$. Amino acid analysis: Asp 1.0, Ser 1.63, *S*-(2,3-dihydroxypropyl)-cysteine (uncorr.) 0.34; FD-MS: M^+ 1199.

$C_{64}H_{119}N_5O_{13}S$ (1198.7):

Calcd.: C 64.13, H 10.00, N 5.84, S 2.67

Found: C 64.07, H 10.18, N 5.64, S 2.77.

TABLE III

COMPOUNDS USED FOR MONOLAYER CHARACTERISATION

(1)	Pam-Cys-OMe
(2)	(Pam-Cys-OMe)$_2$
(3a)	Pam-Cys(S-dihydroxypropyl)-OMe
(3)	Pam-Cys(S-dihydroxypropyl)-OH
(4)	Pam_3Cys-OH
(5a)	Pam_3Cys-Ser(Bu^t)-OBu^t
(5)	Pam_3Cys-Ser-OH
(6a)	Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-OBu^t
(6)	Pam_3Cys-Ser-Ser-OH
(7a)	Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-Asn-OBu^t
(7)	Pam_3Cys-Ser-Ser-Asn-OH
(8a)	Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-Asn-Ala-OBu^t
(8)	Pam_3Cys-Ser-Ser-Asn-Ala-OH

Results and Discussion

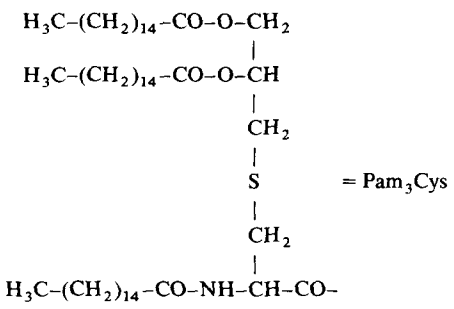
Synthesis and analysis of the lipopeptides

Based on the experimental experiences on the synthesis of the lipopentapeptide Pam_3Cys-Ser-Ser-Asn-Ala [4], the new lipopeptides Pam_3Cys-Ser, Pam_3Cys-Ser-Ser, and Pam_3Cys-Ser-Ser-Asn were prepared as summarized in Fig. 1.

Monolayer characterization of the lipopeptides

The lipophilic cystine and cysteine derivatives (1), (2) and (3a) were dissolved in chloroform, spread on the air/water interface, and the isotherms (Fig. 2) were measured. All three compounds exhibit a fluid analogue phase at 20 °C (Fig. 2). At 10 °C compounds (1) and (3a) show also a solid analogue phase. The cystine derivative (2) with two fatty acid chains at 10 °C still shows no crystalline monolayer phase, probably because of the quite bulky and not very polar headgroup.

Fig. 3 shows two different isotherms taken from



L-Ser(Bu^t)-OBu^t → Pam$_3$Cys-Ser(Bu^t)-OBu^t $\xrightarrow{CF_3COOH}$ Pam$_3$Cys-Ser-OH
(5a) (5)

Fmoc-L-Ser(Bu^t)-Ser(Bu^t)-OBu^t → Pam$_3$Cys-Ser(Bu^t)-Ser(Bu^t)-OBu^t $\xrightarrow{CF_3COOH}$ Pam$_3$Cys-Ser-Ser-OH
(6a) (6)

L-Asn-OBu^t → Fmoc-L-Ser(Bu^t)-Asn-OBu^t → Fmoc-L-Ser(Bu^t)-Ser(Bu^t)-Asn-OBu^t →

→ Pam$_3$Cys-Ser(Bu^t)-Ser(Bu^t)-Asn-OBu^t $\xrightarrow{CF_3COOH}$ Pam$_3$Cys-Ser-Ser-Asn-OH
(7a) (7)

Fig. 1. Scheme of synthesis of the new-triple chain lipopeptides.

compound (3) at 20 °C. Curve a represents an isotherm taken in the 'normal' mode with a sweeptime of 10 min. The isotherm looks like a normal isotherm of an extremely fluid monolayer, but the area at the collapse point (0.09 nm^2/molecule) is just half of the value that can be expected for a most densely packed monolayer of a single-chain compound (0.18 nm^2/molecule). This behaviour suggests that compound (3) is partially water soluble, and thus the monolayer is not stable. Therefore the stability of these monolayers was checked using the 'thermodynamic' mode of isotherm measurement. In this mode the moving barrier of the film balance does not advance the next step until the surface pressure has reached a constant value. If the monolayer is stable and not too viscous, the isotherms in the 'thermodynamic' mode are very similar to those measured in the 'normal' mode [1], whereas in stable monolayers give saw toothed isotherms shifted to smaller areas. Curve b in Fig. 3, which represents an isotherm taken from compound (3) in the 'thermodynamic' mode, is both shifted to smaller areas compared to curve a and saw toothed. Therefore it is obvious

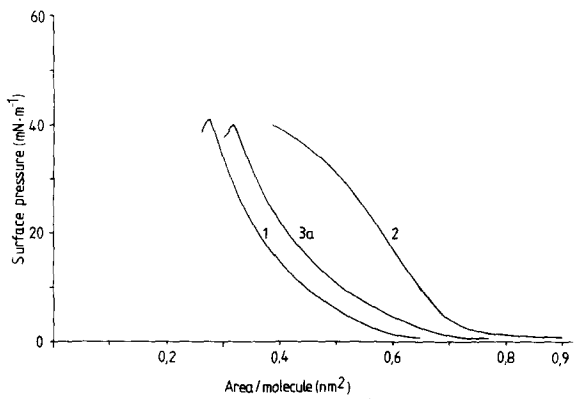


Fig. 2. Monolayer isotherms from Pam-Cys-OMe (1), (Pam-Cy-OMe)$_2$ (2) and Pam-Cys(*S*-dihydroxypropyl)-OMe (3a) at 20 °C.

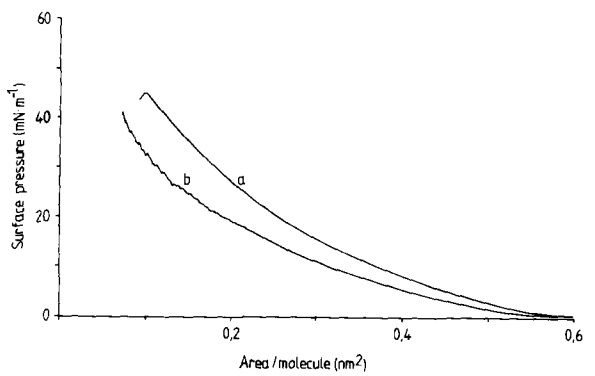


Fig. 3. Monolayer isotherms from Pam-Cys(*S*-dihydroxypropyl)-OH (3) at 20 °C. (a) 'normal' mode, sweeptime 10 min; (b) 'thermodynamic' mode.

that compound (3) gives no stable monolayers, which is probably due to its large and very hydrophilic headgroup.

The isotherms from the *tert*-butyl protected and from the lipopeptides are summarized in Figs. 4 and 5. All these isotherms have been measured at 20 °C. The most obvious difference between the isotherms of the protected and the unprotected lipopeptides is, that all protected compounds exhibit both a liquid expanded and a solid analogue phase at 20 °C, whereas the unprotected lipopeptides show only a crystalline monolayer even at 30 °C. Obviously, for the protected lipopeptides the appearance of the liquid analogue phase is due to the hydrophobicity and the bulkiness of the *tert*-butyl protecting groups. On the other hand the unprotected compounds can be packed much closer in the monolayer, thus they give only a solid analogue phase.

In the liquid expanded phase of a monolayer, only the headgroups of the lipids interact with each other, whereas the hydrocarbon tails still have an almost undisturbed mobility. Thus the area required from the protected lipopeptides in the liquid expanded phase allows an estimation of the bulkiness of the lipid headgroups. As expected, the area required by the headgroups of the lipopeptide increases on prolongation of the peptide chain (Fig. 4). In the crystalline phase all compounds are packed quite densely. The area required at the collapse point is in all cases quite close to the minimal value of closest chain packing for these hydrocarbon chains (0.54 nm^2/molecule) [17].

The monolayers of the deblocked lipopeptides show a much smaller compressibility in the solid analogue phase than that of the protected compounds, as expressed by the increased steepness of the curve (Figs. 4 and 5). These films also are extremely viscous; the Wilhelmi plate of the measuring system did not hang down vertically any more when the film was compressed to values close to the collapse point of the monolayer. So the real values for the surface pressure at the collapse point all are higher than the measured values. The monolayer was too viscous to flow around the Wilhelmi plate fast enough. The effect of this high viscosity of the monolayer on the results of measurements taken under different conditions is demonstrated for compound (4) as one example (Fig. 6).

A hysteresis isotherm was measured from compound (4) at 20 °C, and the monolayer first was compressed to 20 mN/m (Fig. 6A, dashed line).

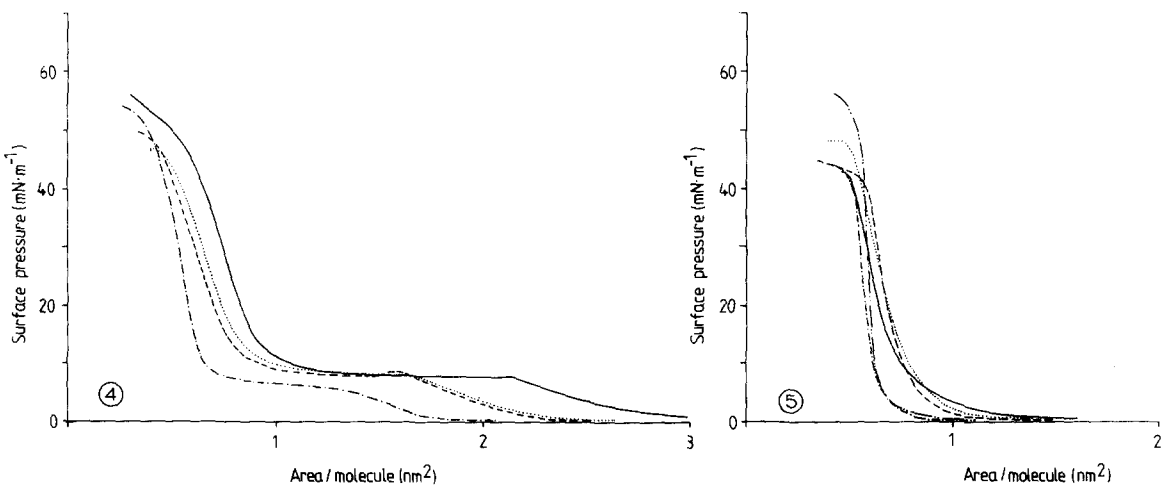
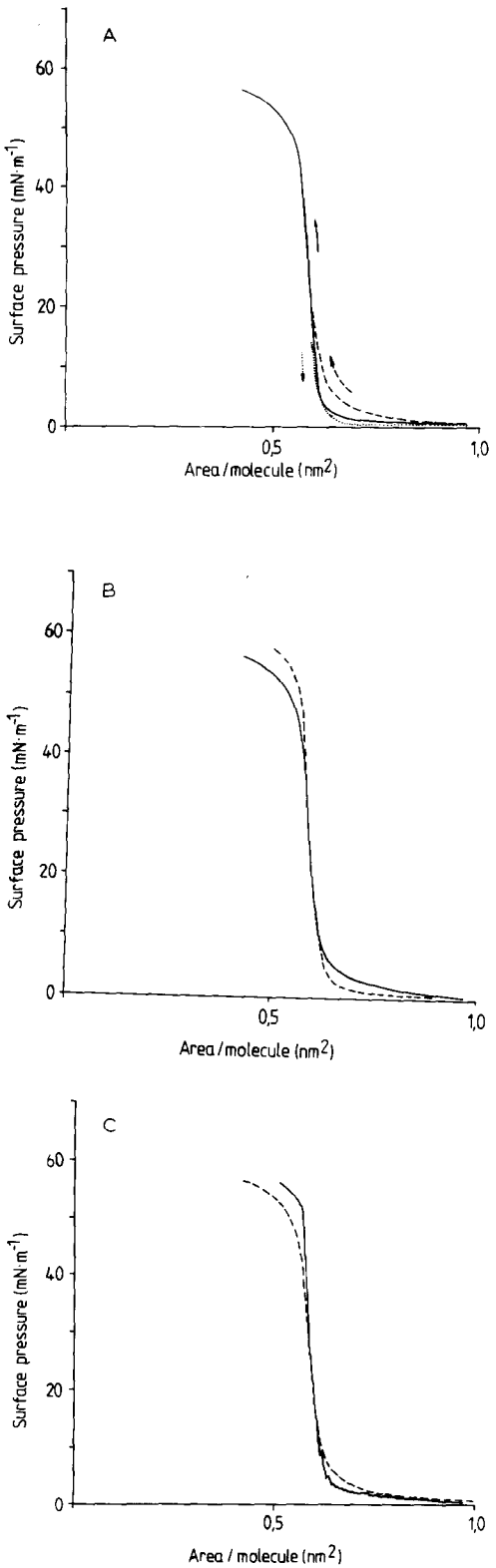


Fig. 4. Monolayer isotherms from the *tert*-butyl protected lipopeptides at 20 °C. Pam_3Cys-Ser(Bu^t)-OBu^t (5a) (·–·–·), Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-OBu^t (6a) (- - - - - -), Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-Asn-OBu^t (7a) (· · · · · ·), Pam_3Cys-Ser(Bu^t)-Ser(Bu^t)-Asn-Ala-OBu^t (8a) (———).

Fig. 5. Monolayer isotherms from the unprotected lipopeptides at 20 °C. Pam_3Cys-OH (4) (· ·–· ·–· ·), Pam_3Cys-Ser-OH (5) (·–·–·), Pam_3Cys-Ser-Ser-OH (6) (- - - - - -), Pam_3Cys-Ser-Ser-Asn-OH (7) (· · · · · ·), Pam_3Cys-Ser-Ser-Asn-Ala-OH (8) (———).



At this surface pressure the film was allowed to stand for 5 min, and then the monolayer was expanded again (dotted line). During the 5 min relaxation time the measured surface pressure of the monolayer, which was kept at constant area, decreased to 15 mN/m due to a more dense packing of the monolayer. This dense packing in monolayers with normal viscosity, such as monolayers from palmitic acid, appears already during the normal measurement. Therefore in the hysteresis isotherms the surface pressure normally is not decreased when the barrier stops moving at a certain surface pressure. Here, because of the high viscosity of the film, the monolayer needs some time to relax. After the expansion of the film and waiting again for one minute, the film was compressed a second time (Fig. 6A, straight line). On this second compression the compressibility of the monolayer is much smaller than in the first compression, and also the breakdown point is shifted towards higher surface pressure compared to the measurement in the 'normal' mode.

In Fig. 6B the isotherms measured at 20 °C (straight line) and 30 °C (dashed line) are compared. The isotherms at higher temperature have a higher collapse pressure, and the compressibility is smaller than at lower temperature. This kind of behavior is also very typical for highly viscous monolayers, because the real collapse pressure always decreases with increasing temperature. If the monolayer is very viscous during the compression, the surface pressure is not constant all over the film surface. Near the moving barrier the surface pressure always is higher than near the measuring system when the viscosity of the film is high, because the material needs some time to flow from the moving barrier to the measuring plate. Therefore the film already can reach the collapse pressure near the moving barrier, when the measured surface pressure still is far below breakdown. Due to the slow flow of material in these monolayers, the measured compressibility is smaller than the

Fig. 6. Monolayer isotherms from Pam_3Cys-OH (4). (A) Hysteresis isotherm at 20 °C; compression to 20 mN/m (- - - - - -), expansion after 5 min (· · · · · ·); compression again after 1 min (———). (B) Isotherms at 20 °C (———) and 30 °C (- - - - - -). (C) Comparison of 'normal' (- - - - - -) and 'thermodynamic' mode (———) at 20 °C.

real one. At higher temperatures, however, the viscosity has decreased, the flow of material being faster and the measured values for the collapse pressure and the compressibility being closer to the real values.

Fig. 6C finally compares isotherms from compound (4) measured in the 'normal' and the 'thermodynamic' mode. Because in the 'thermodynamic' mode the isotherm measurement is carried out in a way that the measured values are more close to the real 'thermodynamic' values, the monolayer has a smaller compressibility and a higher collapse pressure. As pointed out above, this result can be expected for highly viscous films.

Mixed monolayers

All the triple chain lipopeptides did not form very stable vesicles by themselves. In many cases vesicle formation of lipids, which do not form vesicles by themselves, is possible after mixing them with other lipids. Therefore the mixing behavior of the lipopeptides in monolayers was investigated with compound (7) in mixtures with cholesterol and dipalmitoylphosphatidylcholine (DPPC). From this mixing behavior it may be possible to estimate the ability to form liposomes as well as to get some information about the interactions of the lipopeptides with membrane forming lipids.

In Fig. 7 the isotherms of mixtures of compound (7) with cholesterol are shown together with the areas required at certain surface pressures plotted versus the cholesterol content of the mixture (area-composition curves). In these area-composition curves two distinct breaks in slope can be recognized at about 10 and 50% cholesterol content. These breaks mark phase boundaries between different phases in the mixed monolayer. The condensing effect of cholesterol, that has been reported for cholesterol-phosphatidylcholine mixtures already in 1925 [18], also seems to occur for mixtures containing less than 10% cholesterol.

The monolayer mixture of compound (7) with DPPC shows a different behavior (Fig. 8). As the isotherms of different mixtures overlap, only a few representative curves are given in Fig. 8. The inset in Fig. 8 shows area-composition curves for a surface pressure below and above the phase transition of DPPC (15 mN/m and 25 mN/m, respectively). Breaks in the area-composition curves indicating phase boundaries are visible only at low surface pressures where DPPC still is in the fluid phase. At higher surface pressures, the area-composition curves give only straight lines indicating either ideal mixing of the lipids or total phase separation [19]. In the fluid phase, however, there is some interaction detectable between compound (7) and DPPC. This interaction is indicated by the breaks in the area-composition curves below 20 and around 70 mol% of DPPC. The break at low concentrations of DPPC is not expressed very

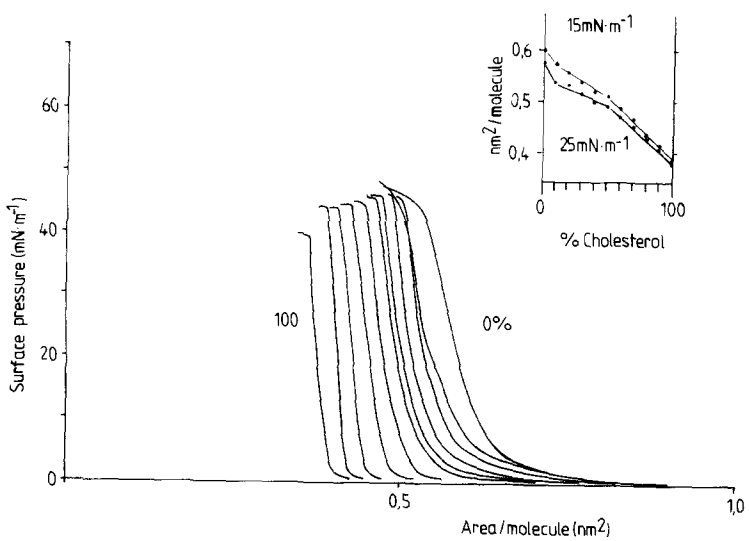


Fig. 7. Monolayer isotherms at 20 °C from mixtures of Pam_3Cys-Ser-Ser-Asn-OH (7) and 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% cholesterol (molar ratio). The inset shows the surface area-composition diagrams of the mixtures at 15 and 25 mN/m.

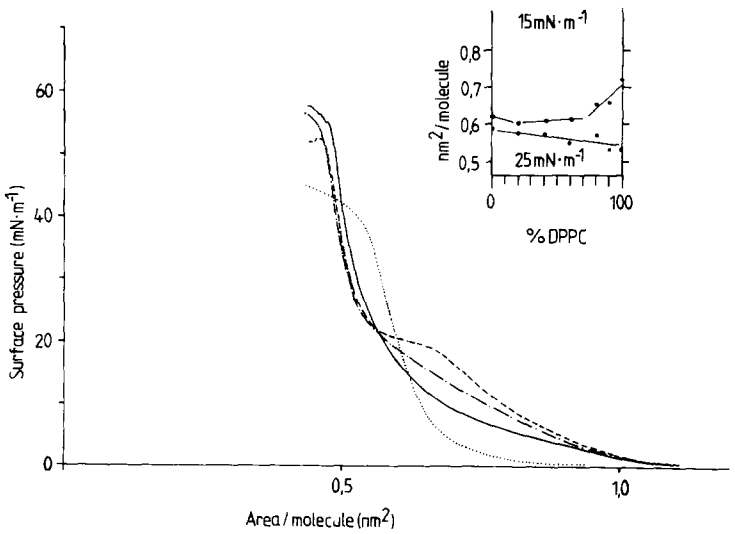


Fig. 8. Monolayer isotherms at 30 °C from mixtures of Pam_3Cys-Ser-Ser-Asn-OH (7) and 0 (·····), 40 (———), 90 (·–·–·) and 100% (- - - - -) DPPC (molar ratio). The inset shows the surface area-composition diagrams of the mixtures at 15 and 25 mN/m.

distinctly, but the deviation from the straight line exceeds the experimental error. At high concentrations of DPPC the effect of the lipid–lipid interaction becomes visible also in the isotherms directly. The DPPC phase transition is broadened already at 10% of compound (7) and seems to be shifted to lower values of surface pressure with increasing content of compound (7).

Polyclonal activation of B-lymphocytes

In order to obtain information on a possible correlation between the physico-chemical properties and the biological activity of the compounds, we tested some of the lipopeptides in vitro for the activation of murine spleen cells (Fig. 9). The results indicate, that triple-chain compounds with more than one amino acid can efficiently activate splenocytes, as determined by the incorporation of [^{3}H]thymidine into DNA. The comparison of the protected with the deprotected lipopeptides clearly demonstrates that polar head groups are essential

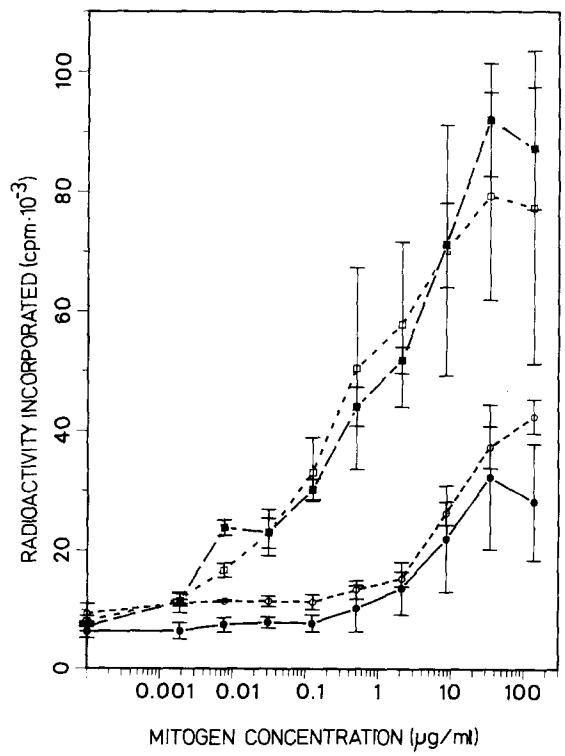


Fig. 9. Dose-response curves for [^{3}H]thymidine incorporation in splenocytes of Balb/c mice induced by Pam_3Cys-Ser-Ser-Asn-Ala (■), Pam_3Cys-Ser (□), Pam_3Cys-Ser(Bu^t)-OBu^t (○), and Pam_3Cys (●) (means of triplicate determinations ± S.D.). Cells were cultured for 48 h at 37 °C at a cell density of $3.3 \cdot 10^6$/ml in Falcon 3040 microtiter plates, in a moist CO_2 incubator (200 µl aliquots). Culture medium: RPMI 1640 with 10% fetal calf serum (FCS, Seromed, Munich), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 µg/ml), and 2-mercaptoethanol ($5 \cdot 10^{-5}$ M). The lipopeptides were added to the cultures after suspension in Minimal Essential Medium containing 10% FCS by sonification (6 × 10 s, 100 W, Labsonic 1510 sonifier, Braun, Melsungen). 24 h before harvesting, 1 µCi [^{3}H]thymidine (Amersham, Braunschweig, spec. act. 185 GBq/mol) was added to each well. The cultures were harvested (Mash-II-harvester), collected on glass fibre filters, and measured by liquid scintillation counting.

for obtaining strong biological activities: The lipophilic, fully protected Pam_3-Cys-Ser(Bu^t)-OBu^t shows much less mitogenic activation than the amphiphilic, deprotected Pam_3Cys-Ser. As shown previously, only B lymphocytes are activated and the activation optimum is already obtained with the lipodipeptide Pam_3Cys-Ser, carrying only one polar amino acid attached to Pam_3Cys; a prolongation to the lipopentapeptide Pam_3Cys-Ser-Ser-Asn-Ala or even to the parent lipoprotein from *E. coli* does not evoke better activation [7]. Accordingly, all Pam_3Cys conjugates synthesized so far for direct presentation of polar antigens [9] exhibited comparable B-cell activation. The hydrophobic triple-chain Pam_3Cys anchor alone shows a weak effect comparable to the one obtained with the fully protected lipopeptide Pam_3Cys-Ser(Bu^t)-OBu^t.

Our physico-chemical studies on lipopeptides certainly contribute to a better knowledge of the role of these compounds in B-lymphocyte activation. Both changes in membrane fluidity or permeability, and interactions with specific binding proteins may contribute to the observed cellular response. Hereby, the polar headgroups of the Pam_3Cys-lipopeptides probably play an important role for the specificity of the activation process, since the unspecific insertion of the Pam_3Cys anchor into the lipid membrane of lymphocytes alone is not sufficient for signalling activation.

In this context it is of significance that after attachment of a fluorescence label (FITC) the low molecular weight lipopeptides were shown to induce membrane changes such as patching and capping on lymphocytes. The labeled lymphocytes could be studied in the fluorescence activated cell sorter (FACS) (Metzger, J. et al., to be published).

Acknowledgement

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