

# Physicochemical and biological analysis of synthetic bacterial lipopeptides: Validity of the concept of 'endotoxic conformation'<sup>1</sup>

Andra B. Schromm<sup>\*2</sup>, Jörg Howe<sup>†</sup>, Artur J. Ulmer<sup>‡</sup>, Karl-Heinz Wiesmüller<sup>§</sup>, Tobias Seyberth<sup>\*\*</sup>, Günther Jung<sup>\*\*</sup>, Manfred Rössle<sup>#</sup>, Michel H.J. Koch<sup>#</sup>, Thomas Gutschmann<sup>†</sup>, and Klaus Brandenburg<sup>†</sup>

From the Research Center Borstel, Leibniz-Center for Medicine and Biosciences, Department of Immunochemistry and Biochemical Microbiology, \*Emmy Noether Group of Immunobiophysics,

<sup>†</sup>Division of Biophysics, <sup>‡</sup>Division of Immunology, 23845 Borstel, Germany

<sup>§</sup>EMC microcollections GmbH, 72070 Tübingen, Germany

<sup>\*\*</sup>Institute of Organic Chemistry, University of Tübingen, 72076 Tübingen, Germany

<sup>#</sup>European Molecular Biology Laboratory c/o DESY, Notkestr. 85, 22603 Hamburg

Running title: endotoxic conformation of lipopeptides

<sup>1</sup>This work was funded by DFG grants (SCHR 621/2-2 and UI 68/3-1), the European Commission (ANEPID), and the Federal Ministry of Education and Research (project Biochance no. 0312662).

<sup>2</sup>Address correspondence to: Andra Schromm, Research Center Borstel, Department of Immunochemistry and Biochemical Microbiology, Emmy Noether Group of Immunobiophysics, Parkallee 10, 23845 Borstel, Germany. Phone +49(0)4537-188296; Fax +49(0)4537-188632; E-mail: aschromm@fz-borstel.de

The importance of the biological function and activity of lipoproteins from the outer or cytoplasmic membranes of Gram-positive and Gram-negative bacteria is being increasingly recognized. It is well-established that they are like the endotoxins (lipopolysaccharide, LPS), which are the main amphiphilic component of the outer membrane of Gram-negative bacteria, potent stimulants of the human innate immune system and elicit a variety of proinflammatory immune responses. Investigations of synthetic lipopeptides corresponding to N-terminal partial structures of bacterial lipoproteins defined the chemical prerequisites for their biological activity and in particular the number and length of acyl chains and sequence of the peptide part. Here we present experimental data on the biophysical mechanisms underlying lipopeptide bioactivity. Investigation of selected synthetic diacylated and triacylated lipopeptides revealed, that the geometry of these molecules (i.e. the molecular conformations and supramolecular aggregate structures) and the preference for membrane intercalation provide an explanation for the biological activities of the different lipopeptides. This refers in particular to the agonistic or antagonistic

activity (i.e., their ability to induce cytokines in mononuclear cells or to block this activity, respectively). Biological activity of lipopeptides was hardly affected by the LPS-neutralizing antibiotic polymyxin B (PMB), and the biophysical interaction characteristics were found to be in sharp contrast to that of LPS with PMB. The analytical data show that our concept of 'endotoxic conformation', originally developed for LPS, can be applied also to the investigated lipopeptides, and suggest that the molecular mechanisms of cell activation by amphiphilic molecules are governed by a general principle.

Beside lipopolysaccharides (LPS, endotoxin)<sub>1</sub> which are the major amphiphilic components of the outer membrane of Gram-negative bacteria, other amphiphilic molecules such as lipoproteins and lipopeptides (LP) are found in the cell wall of a large number of microorganisms, including Gram-negative as well as Gram-positive bacteria, mycobacteria, mycoplasmas, and spirochetes (1-5). Like LPS these molecules can be potent activators of lymphocytes and macrophages and give danger signals to the infected host (6;7). A variety of biological activities have been described for native LP as well as for synthetic LP based on the highly conserved lipopeptide moiety of

Braun's lipoprotein derived from *Escherichia coli* (8). The innate immune system plays an essential role in the host defense against bacterial infection. Recognition of bacterial virulence factors is mandatory for the initiation of an inflammatory and anti-bacterial immune response. Bacterial compounds such as LPS and lipoproteins are recognised by type-I transmembrane proteins of the Toll-like receptor (TLR) family (9). Of the more than ten known TLRs, several have been found to participate in the recognition of microbial infections. TLR4 initiates cell activation by LPS in concert with the extracellular protein MD-2 (10-12). TLR4/MD-2 has been found to associate in complexes that include a variety of accessory proteins such as CD14, CD11/CD18, CD55, hsp70, hsp90, GDF5, and CXCR4 (13-20), MOESIN (21), the potassium channel MaxiK (22), and a membrane-associated form of LBP (mLBP) (23;24). For TLR2 a variety of different ligands has been described including lipoteichoic acid, lipoarabinomannan, bacterial lipoproteins/lipopeptides, and also some LPS variants from Gram-negative bacteria, yeast, spirochetes, and fungi (25). TLR2 has also been shown to associate with coreceptors (26). Together with Dectin-1 it recognizes infections by yeasts (27;28) and mycobacteria (29). Via heteromeric receptors complexes with TLR1 and 6, TLR2 also recognizes a variety of bacterial lipopeptides (30-32). Only recently, it was suggested that CD36 may have a role in the recognition of the diacylated bacterial lipopeptide MALP-2 (33).

As far as the structural prerequisites for bioactivity of lipopeptides are concerned, it has been shown that tripalmitoyl-S-glycerol-L-Cys-Ser-Lys-Lys-Lys-Lys (Pam<sub>3</sub>CSK<sub>4</sub>) was highly active, whereas the natural dipeptide part of the outer membrane lipoprotein, tripalmitoyl-S-glycerol-L-Cys-Ser (Pam<sub>3</sub>CS), only has a low activity (34,35). It is known for many amphiphilic molecules like phospholipids and glycolipids that physical parameters are strong determinants of biological activity: Above a certain threshold concentration, i.e. the critical micellar concentration (CMC), these molecules form aggregates in aqueous dispersions, which may adopt different phase states: an ordered gel phase where the acyl chains are in the *all-trans* (zig-zag) conformation, and a liquid crystalline

phase, where the chains are disordered as a result of the introduction of *gauche* conformers. Between these phases a reversible first order transition takes place at a lipid-specific temperature. Furthermore, the type of aggregate structure, uni- and multilamellar, cubic direct or inverted, hexagonal H<sub>I</sub> or inverted H<sub>II</sub> may play a decisive role in biological systems (36). It has been shown for LPS and its lipid moiety, lipid A, the endotoxic principle of LPS (37), that the aggregation type is essential for the expression of bioactivity, and that the state of order of the acyl chains may be a modulator of bioactivity (38-39). For lipopeptides, only for the immunoadjuvants Pam<sub>3</sub>-L-Cys and Pam<sub>3</sub>-L-Cys-Ser experimental data are available, indicating that these molecules form vesicular or tubular aggregates of different sizes in aqueous dispersion depending on the configuration of the glycerol moiety and the head group (40). Furthermore, two Pam<sub>3</sub>Cys-peptides LP1 and LP2, characterized by fluorescence correlation spectroscopy, form large and highly heterogeneous aggregates where the smaller aggregates were shown to have higher bioactivity (unpublished observation).

To clarify the correlation between physicochemical characteristics and biological activities, we have investigated three selected lipopeptides with different cytokine-inducing activity, with strong agonistic as well as antagonistic activity in human mononuclear cells.

We have found that as in the case of endotoxins, the types of aggregate structures are important determinants of cytokine-inducing activity. Furthermore, we show that our concept of 'endotoxic conformation', which was shown to be applicable so far not only to LPS, but also to particular synthetic phospholipids (41;42), can also be extended to the lipopeptides. In addition, we present data showing that a biologically inactive lipopeptide which antagonizes cell activation by biologically active lipopeptides is also able to antagonize cell activation by LPS, supporting the broad validity of the conformation concept for agonistic as well as antagonistic activity.

## EXPERIMENTAL PROCEDURES

**Synthesis.** Pam<sub>2</sub>CSK<sub>4</sub> and Pam<sub>3</sub>CSK<sub>4</sub> were synthesized and analyzed by EMC microcollections GmbH according to published procedures (43-45). The synthesis and analysis of the lipolanthionine peptide are described elsewhere in detail (46). The structural formula of the synthetic compounds dipalmitoyl-S-glyceryl-L-Cys-Ser-(Lys)<sub>4</sub> (Pam<sub>2</sub>CSK<sub>4</sub>), tripalmitoyl-S-glyceryl-L-Cys-Ser-(Lys)<sub>4</sub> (Pam<sub>3</sub>CSK<sub>4</sub>), and of the lipolanthionine peptide (2R,6R) Pam<sub>2</sub>LanHda-Ser-(Lys)<sub>4</sub>-NH<sub>2</sub> (lipolan) are shown in Fig. 1. The stereochemistry and composition of the lipid tails of lipolan have been optimized for the inhibition of TLR2-dependent IL-8 induction by Pam<sub>3</sub>CSK<sub>4</sub> in myelomonocytic THP-1 cells (46).

**Reagents.** Deep rough mutant LPS (Re LPS) was extracted from *Salmonella enterica* sv. Minnesota strain R595 according to the phenol/chloroform/petrol ether procedure (47). The LPS preparation was lyophilized and used in the natural salt form. The chemical purity of the LPS preparation was confirmed by mass spectrometry. LPS was suspended in PBS (Biochrom, Berlin, Germany) by thorough vortexing. The suspensions were temperature-cycled at least twice between 4 °C and 56 °C, each cycle being followed by intense vortexing for a few min, and then stored at 4 °C for at least 12 h prior to measurement. Suspensions were aliquoted and stored at -20 °C. Phosphatidylcholine (PC), sphingomyelin (SM) and phosphatidylserine (PS) from bovine brain, and phosphatidylethanolamine (PE) from *E. coli* were purchased from Avanti Polar Lipids (Alabaster, AL, USA). All phospholipids were used without further purification. The fluorescent dyes N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-PE (NBD-PE) and N-(rhodamine B sulfonyl)-PE (Rh-PE) were purchased from Molecular Probes (Eugene, OR, USA). Recombinant human LBP (456 amino acid holoprotein rLBP<sub>50</sub>) in 10 mM HEPES, pH 7.5 was a kind gift of XOMA LLC (Berkeley, CA, USA).

**Lipid sample preparation.** All lipid samples were prepared as aqueous suspensions in 20 mM HEPES, pH 7. For this, the lipids were suspended directly in buffer, thoroughly vortexed, temperature-cycled three times

between 5 and 70 °C to enable the formation of stable aggregates and then stored for at least 12 h before measurement. To guarantee physiological conditions, the water content of the samples was usually around 95 %. For preparations of liposomes from a mixture corresponding to the phospholipid composition of the macrophage membrane (PC, PS, PE, and SM in a molar ratio of 1:0.4:0.7:0.5), the lipids were solubilized in chloroform, the solvent was evaporated under a stream of nitrogen, and the lipids were resuspended in the appropriate volume of PBS pH 7.0 and temperature-cycled as described above. Electron microscopy (kindly performed by H. Köhl, Div. of Pathology, Forschungszentrum Borstel) revealed large multilamellar liposomes.

**Activation of human mononuclear cells and macrophages.** Mononuclear cells (MNC) were isolated from human peripheral blood of healthy donors by the Hypaque-Ficoll gradient method and cultivated at 37°C with 6% CO<sub>2</sub> in teflon bags in RPMI 1640 medium (endotoxin ≤ 0.01 EU/ml, Biochrom, Berlin, Germany) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 4% heat-inactivated human serum type AB from healthy donors. Cells were cultured in the presence of 2 ng/ml M-CSF for 7 days to differentiate monocytes to macrophages. To determine cytokine induction after cell stimulation, MNC were seeded at 200 µl aliquots of a suspension of 5 · 10<sup>6</sup> cells/ml and macrophages were seeded at 1 · 10<sup>6</sup> cells/ml in 96-well tissue culture dishes (Nunc, Wiesbaden, Germany) in RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, with or without 4% human serum and stimuli were added as indicated in the respective experiment. Cell-free supernatants were collected 4 h after stimulation and stored at -20 °C until determination of cytokine content. Data shown are mean and standard deviation (±SD) of triplicate samples of one experiment and representative of at least three independent experiments.

**Antagonistic action of the inactive lipopeptide.** Inactive lipopeptides, i.e. compounds which did not induce any cytokines in human mononuclear cells, were investigated with respect to their ability to block the LPS-induced TNFα-production in human

macrophages. For this, LPS Re from *S. minnesota* R595 was prepared at three concentrations 0.5, 1, and 5 ng/ml, and the agonistically inactive lipopeptide was added at a concentration of 1 µg/ml, and the TNFα production of the macrophages was determined.

*Transient transfection and stimulation of HEK293 cells.* HEK293 cells were plated at a density of  $1.5 \cdot 10^5$  cells/ml in 96 well plates in DMEM supplemented with 10% FCS, 0.5 units/ml penicillin, and 0.5 µg/ml streptomycin. The following day, cells were transiently transfected using Polyfect (Quiagen, Hilden, Germany) according to the manufacturer's protocol and plasmids containing huTLR4, huMD2 and/or huCD14 as described elsewhere (30). After 6 h of transfection cells were washed and stimulated with LPS at the indicated concentrations in the absence or presence of 10µM lipolan for further 24 h. Cell-free supernatants were collected and stored at -20 °C until determination of cytokine content. Data shown are mean and standard deviation ( $\pm$  SD) of triplicate samples of one experiment and representative of at least three independent experiments.

*Cytokine determination.* Human TNFα was determined in pooled cell-free supernatants of stimulated cells by sandwich ELISA using monoclonal mouse antibody against human TNFα and POD-conjugated rabbit anti-human TNFα antibody, respectively (Intex, Muttant, Switzerland) as stated in detail elsewhere (48). Human IL-8 was determined by sandwich ELISA using IL-8 cytoset from Biosource (Solingen, Germany) exactly according to the manufacturer's protocol. Data shown are mean  $\pm$  SD of triplicate samples of one representative experiment.

*Fluorescence resonance energy transfer spectroscopy.* The fluorescence resonance energy transfer (FRET) technique was used as a probe dilution assay (49) to obtain information on the intercalation of synthetic lipopeptides into liposomes resembling the lipid composition of the cytoplasmic membrane of macrophages (PL<sub>MΦ</sub>). For the FRET experiments, liposomes were double-labeled with NBD-PE and Rh-PE in chloroform [PL]:[NBD-PE]:[Rh-PE] at 100:1:1 molar ratios. The solvent was evaporated under a stream of nitrogen, the lipids resuspended in

PBS, mixed thoroughly, and sonicated with a Branson sonicator for 1 min (1 ml solution). Subsequently, the preparation was temperature-cycled at least twice between 4 °C and 56 °C, each cycle followed by intense vortexing for a few min, and then stored at 4 °C for at least 12 h prior to measurement. A preparation of 900 µl of the double-labeled liposomes ( $10^{-5}$  M) at 37 °C was excited at 470 nm (excitation wavelength of NBD-PE), and the intensities of the emission light of the donor NBD-PE (531 nm) and acceptor Rh-PE (593 nm) were measured simultaneously on the fluorescence spectrometer SPEX F1T11 (SPEX Instr., Edison, NY). Compounds Pam<sub>2</sub>CSK<sub>4</sub>, Pam<sub>3</sub>CSK<sub>4</sub>, and lipolan (concentration 1 µM) were added to liposomes after 50 s. Since FRET spectroscopy is used here as a probe dilution assay, intercalation of unlabeled molecules causes an increase of the distance between donor and acceptor and, thus, leads to a reduced energy transfer. This again causes an increase of the donor and decrease of the acceptor intensities. For a qualitative analysis of experiments, the ratio of the intensities of the donor dye and the acceptor dye are plotted against time (denoted in the following as the FRET signal). The data shown are representative for three independent experiments.

*FTIR spectroscopy.* The infrared spectroscopic measurements were performed on an IFS-55 spectrometer (Bruker, Karlsruhe, Germany). For phase transition measurements, the lipid samples were placed between CaF<sub>2</sub> windows with a 12.5 µm teflon spacer. Temperature-scans were performed automatically between -10 and 70 °C with a heating rate of 0.6 °C/min. Every 3 °C, 50 interferograms were accumulated, apodized, Fourier-transformed, and converted to absorbance spectra. For the identification of particular functional groups, infrared spectra of the lipopeptides at 95 % water concentration were analysed. The vibrational bands typical for the hydrophobic region (symmetrical and antisymmetrical)  $\nu_s$  and  $\nu_{as}$  stretching vibration of -CH<sub>2</sub>- groups around 2920 and 2850 cm<sup>-1</sup>, respectively, in the IR-spectra of lipopeptides are sensitive markers of acyl chain order.

*X-ray diffraction.* X-ray diffraction measurements were performed at the European Molecular Biology Laboratory (EMBL) outstation at the Hamburg synchrotron radiation

facility HASYLAB using the SAXS camera X33 (50). Diffraction patterns in the range of the scattering vector  $0.1 < s < 1.0 \text{ nm}^{-1}$  ( $s = 2 \sin \theta / \lambda$ ,  $2\theta$  scattering angle and  $\lambda$  the wavelength = 0.15 nm) were recorded at 40°C with exposure times of 1 min using an image plate detector with online readout (MAR345, MarResearch, Norderstedt, Germany). The s-axis was calibrated with Ag-Behenate which has a periodicity of 58.4 nm. The diffraction patterns were evaluated as described previously (51) assigning the spacing ratios of the main scattering maxima to defined three-dimensional structures. The lamellar and cubic structures are most relevant here. They are characterized by the following features:

(1) Lamellar: The reflections are grouped in equidistant ratios, i.e., 1, 1/2, 1/3, 1/4, etc. of the lamellar repeat distance  $d_l$

(2) Cubic: The different space groups of these non-lamellar three-dimensional structures differ in the ratio of their spacings. The relation between reciprocal spacing

$$s_{hkl} = 1/d_{hkl} \text{ and lattice constant } a \text{ is}$$

$$s_{hkl} = [(h^2 + k^2 + l^2) / a^2]^{1/2}$$

( $hkl$  = Miller indices of the corresponding set of plane).

In the case of very weak diffraction maxima resolution enhancement techniques were applied, in particular Fourier-self deconvolution as described by Kauppinen et al (52).

## RESULTS

*Induction of tumor-necrosis-factor- $\alpha$  (TNF $\alpha$ ) in mononuclear cells by synthetic lipopeptides.* The ability of Pam<sub>2</sub>CSK<sub>4</sub> and Pam<sub>3</sub>CSK<sub>4</sub> to induce the production of TNF $\alpha$  in human mononuclear cells was tested in comparison with LPS deep rough mutant Re (from strain R595 of Salmonella minnesota) (Fig. 2). Clearly, LPS Re is active down to 100 pg/ml, whereas the lipopeptides are active at least down to 10 ng/ml. The lipolanthionine peptide lipolan was completely inactive with respect to cytokine induction up to a concentration of 10  $\mu$ g/ml (data not shown).

*Synthetic lipolanthionine peptide antagonizes cell activation by LPS.* It was recently demonstrated that the lipolanthionine

peptide (2R, 6R)-Pam<sub>2</sub>LanHda-Ser(Lys)<sub>4</sub>-NH<sub>2</sub> (lipolan, Fig. 1) acts as an inhibitor of TLR2-mediated IL-8 secretion induced by the biologically active synthetic lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> (46). Since we did not observe any biological activity of lipolan in human mononuclear cells, we investigated the antagonistic activity of this TLR2-inhibitor with respect to stimulation by LPS (Fig. 3). Using human macrophages differentiated from peripheral blood mononuclear cells, we show here, that lipolan is also able to antagonize cell activation by LPS in human macrophages, as shown by the reduction in TNF $\alpha$  production in the presence of lipolan.

*Antagonistic activity of lipolan does not depend on TLR2.* Cell activation by LPS depends on the presence of TLR4/MD-2 whereas lipopeptides induce cell activation via TLR2/TLR1 or TLR2/TLR6 heterodimer complexes (31). In order to get information concerning the involvement of TLRs in the antagonistic activity of lipolan against LPS we used the defined HEK293 cell transient transfection system. HEK293 cells were transiently transfected with TLR4/MD-2 and the coreceptor CD14 and subsequently stimulated with LPS in the absence or presence of compound lipolan. Cell activation was determined by measuring IL-8 production from the cells. An inhibition of IL-8 secretion from HEK293-TLR4/MD-2/CD14 cells was observed in the presence of lipolan, suggesting that TLR2 is not necessary for the inhibitory effect of lipolan on cell activation by LPS (Fig. 4). In control measurements, lipolan did not affect cell stimulation with TNF $\alpha$  suggesting that the lipopeptide did not exhibit toxic side effects.

*Intercalation of lipopeptides into target cell membranes.* FRET spectroscopy was applied to find out whether the lipopeptides are able to incorporate into artificial liposomes PL<sub>M $\phi$</sub>  made from a composition characteristic for the phospholipid composition of macrophages. Indeed, the lipopeptides are able to intercalate into the liposomes spontaneously (Fig. 5), i.e., without a transport protein such as lipopolysaccharide-binding protein (LBP), which is necessary for the intercalation of LPS (49;53). Interestingly, the TLR2-inhibitory compound lipolan incorporates much better than the two TLR2-dependent active lipopeptides. The

addition of LBP at  $t = 100$  s did not lead to any further increase of the FRET signal, indicating that LBP is not a transport protein for the lipopeptides (data not shown).

*Interaction of lipopeptides with polymyxin B.* The decapeptide polymyxin B (PMB) is a well-known inactivator of endotoxin activity (54). We have found, that concomitantly with this inhibition, PMB leads to a drastic fluidization of the acyl chains of LPS (55). We have tested the fluidity of Pam<sub>3</sub>CSK<sub>4</sub> in the absence and presence of PMB, using the peak position of the symmetric stretching vibrational band at  $2850\text{ cm}^{-1}$  as measure of lipid order (Fig. 6A). The data for the pure lipopeptide Pam<sub>3</sub>CSK<sub>4</sub> are indicative of a broad gel to liquid crystalline phase transition with a mid-temperature around  $T_c = 35^\circ\text{C}$ . Addition of PMB leads to a drastic increase in the  $T_c$ -value (to around  $50^\circ\text{C}$ ) with a sharpening of the transition. Accompanied by this is the strong decrease of the fluidity at  $37^\circ\text{C}$ , as deduced from the decrease of the wavenumbers (see arrow in Fig 6A). This is the opposite of what has been reported for LPS, for which PMB induces a considerable fluidization (55).

We have furthermore tested the ability of PMB to influence the cytokine-inducing capacity of Pam<sub>3</sub>CSK<sub>4</sub>. Fig. 6B shows that at the two higher Pam<sub>3</sub>CSK<sub>4</sub> / PMB ratios there is a slight inhibition of the cytokine response. At the lowest ratio, however, there is no significant change. It can thus be concluded that there is little or no inhibition of the biological activity of the lipopeptides by PMB.

*Aggregate structures of lipopeptides.* All three lipopeptides were investigated by using synchrotron radiation small-angle scattering (SAXS) to elucidate their three-dimensional aggregate structures (Fig. 7). The data for the two TLR2-active lipopeptides Pam<sub>2</sub>CSK<sub>4</sub> and Pam<sub>3</sub>CSK<sub>4</sub> (Fig. 7A and B) are indicative for a complex non-lamellar structure. In both cases, however, the measured peak maxima are in a defined numerical ratio: for example, the reflections at 9.11 , 7.63 , 6.33 , 5.10, 4.55, 3.82, and 3.29 nm for Pam<sub>2</sub>CSK<sub>4</sub> can be grouped according to  $1/\sqrt{3}$ ,  $1/\sqrt{4}$ ,  $1/\sqrt{6}$ ,  $1/\sqrt{9}$ ,  $1/\sqrt{11}$ ,  $1/\sqrt{16}$ , and  $1/\sqrt{22}$  of a periodicity at  $a_Q = 15.5 \pm 0.3$  nm. Thus, an unequivocal assignment to the cubic phase of space group  $Q^{224}$  (56) is possible. The patterns for Pam<sub>3</sub>CSK<sub>4</sub> can be assigned

similarly: by assuming  $a_Q = 12.5 \pm 0.3$  nm, the small reflections superimposed on a broad intensity maximum can be grouped according to  $1/\sqrt{2}$ ,  $1/\sqrt{3}$ ,  $1/\sqrt{4}$ ,  $1/\sqrt{6}$ ,  $\sqrt{8}$ , and  $1/\sqrt{10}$  of the periodicity  $a_Q$ , again allowing an assignment to  $Q^{224}$ .

In contrast, the inhibitory lipolanthionine peptide (2R,6R)-Pam<sub>2</sub>LanHda-Ser-(Lys)<sub>4</sub>-NH<sub>2</sub> (lipolan) has a rather simple diffraction pattern (Fig. 7C), which can readily be interpreted as resulting from a multilamellar structure with a periodicity  $a_l = 6.30$  nm and two further reflections at equidistant ratio  $a_l/2$  and  $a_l/3$ .

## DISCUSSION

Lipopeptides are produced by a broad variety of pathogenic microorganisms and have long been known as inducers of innate and adaptive immune responses by host lymphocytes. They have the capability to stimulate antigen-presenting cells, and these adjuvant effects have recently become important for the development and improvement of vaccines (41). Investigations in the bioactivity and the TLR2/1/6 requirement of a broad panel of synthetic lipopeptides have provided information on the chemical basis of cell activation by lipopeptides (30;31;57). However, in contrast to the well investigated lipopolysaccharides from Gram-negative bacteria, still very little is known about the molecular basis of lipopeptide recognition. The present paper is the first combined systematic physicochemical and biological analysis of lipopeptide structures from bacterial lipoproteins. There are considerable differences in the chemical structures between LPS and lipopeptides. The investigated LP compounds have a positively charged peptide backbone instead of the negatively charged sugar moiety of lipid A and only two or three acyl chains instead of six acyl chains in those lipid A expressing high biological activity. Nevertheless, we found some striking similarities to LPS: The lipopeptides showed high cytokine-inducing ability (Fig. 2) and had a strong preference to adopt cubic structures (Fig. 7A,B), in particular with space group  $Q^{224}$  (also called Pn3m (56)), which has also been reported for lipid A (51). In

contrast, the lipolanthionine peptide (2R,6R)-Pam<sub>2</sub>LanHda-Ser(Lys)<sub>4</sub>-NH<sub>2</sub> (lipolan) was found to adopt a lamellar aggregate structure (Fig. 7C) and as expected did not show cell activation analogously to what has been described for lamellar lipid A structures (38). Furthermore, in analogy to the potent endotoxin antagonist compound 406, a synthetic tetraacyl lipid A which assumes a lamellar structure (58), the lamellar compound lipolan was found to antagonize cell activation by LPS. Although lipolan has been described as an inhibitor for the TLR2-dependent cell activation by lipopeptides (46), the observation that this compound also antagonizes LPS was surprising. When we investigated the TLR-dependence of the inhibition of cell activation by LPS it turned out that the antagonistic activity was independent of TLR2 (Fig. 3), suggesting that besides TLR2-interaction other molecular mechanisms might hold true.

The structural and biological data are very surprising in view of the chemical structures of the compounds (Fig. 1). According to the shape concept of Israelachvili (36), who described the aggregate structures of amphiphiles on the basis of the ratio of the cross-sections of the hydrophilic and hydrophobic moiety, respectively, compounds with a higher value of the former should assume micellar or lamellar structures, in the inverse case non-lamellar cubic or H<sub>II</sub> structures. The hydrophobic moiety of Lipolan, which has three alkyl chains (2x C<sub>12</sub>, 1x C<sub>16</sub>), can be expected to be larger than that of Pam<sub>2</sub>CSK<sub>4</sub> which has two C<sub>16</sub> acyl chains, while the cross-sections of the hydrophilic moieties should be similar. One would thus have expected a stronger tendency towards an inverted cubic structure for Lipolan than for Pam<sub>2</sub>CSK<sub>4</sub> but the experimental results prove the opposite. From these data it can be concluded that when using the geometrical model of Israelachvili one may have to take into account, different water binding capacities in the head groups and the influence of the counterions, for example, but these cannot be readily be deduced from the primary chemical structures.

An important aspect of cell activation by LPS is its transport by the serum-protein LPS-binding protein (LBP) to the surface antigen CD14 on myelomonocytic cells and the transport and intercalation into target cell membranes

(49;53;59). Investigation of the interaction of lipopeptides with phospholipid liposomes as a model for the lipid matrix of the cytoplasmic membrane showed that these compounds intercalate by themselves into phospholipid membranes (Fig. 5), whereas for endotoxins (agonistic as well as antagonistic structures) the transport protein LBP is necessary. The membrane activity of the lipopeptide lipolan might be the key to their antagonistic activity towards the TLR4-ligand LPS as the intercalated acyl-chains of the lipopeptide might diffuse laterally to membrane proteins and thus interfere with assembly and signal transduction by the TLR-receptor complexes. It has been reported (60) that cell activation by di- and triacylated lipopeptides can be enhanced in the presence of LBP, suggesting that LBP might nevertheless play a role in lipopeptide cell activation. A recent publication (33) showed that the surface antigen CD36 is involved in the recognition of the diacylated lipopeptide MALP-2, however, cell activation by the triacylated Pam<sub>3</sub>CSK<sub>4</sub> appeared to be independent of CD36. The authors suggest that CD36 functions analogously to CD14 by concentrating lipopeptides on the cell surface. Whether this involves transport by serum-proteins such as LBP remains to be elucidated.

The importance of membrane binding of lipopeptides was demonstrated previously by Boncheva et al. (61) who investigated the behaviour of a triacylated lipopeptide in phosphatidylcholine liposomes and monolayers. They found complete flexibility of the peptide part, thus enabling it to interact with the environment such as antibodies. Wolf *et al.* (62;63) applied electron energy loss spectroscopy to study the interaction of cells with lipopeptides Pam<sub>3</sub>Cys-Ser and fluorinated derivatives. They observed a distribution of the lipopeptides in different cell compartments, in particular also in the plasma membranes of macrophages and lymphoid cell lines. These data were supported also by findings with ultrathin cryosections of macrophages derived from bone marrow (64). The interaction of Pam<sub>3</sub>Cys peptides was also investigated using spin labels and fluorescent markers with phosphatidylcholine and immune cells (65). It was found that the lipopeptide incorporates into the vesicles and cells, with exposure of the

peptide moiety to the hydrophilic compartment and the Pam<sub>3</sub>Cys part as membrane anchor. This membrane intercalation of the lipopeptide was assumed to be an early step of interaction and subsequent stimulation of immunocompetent cells, which is in complete accordance to the findings presented here.

The behavior of lipopeptides with PMB is completely different from that of endotoxins. In contrast to the fluidization observed when PMB interacts with endotoxins (55;66), PMB clearly causes a rigidification of the acyl chains of the lipopeptide (Fig. 6A). If at all the bioactivity of the lipopeptide, is only very slightly reduced by PMB (Fig. 6B) in contrast to the massive reduction with endotoxins (54). These findings may be related to the positive charge of the lipopeptides, opposed to the negative charges of endotoxins. An interaction of the lipopeptides with the positively charged PMB could work via bridging of negative counterions (Cl<sup>-</sup>), which in fact results in the change of the phase transition shown in Fig. 6A; in this interaction the aggregate structure of Pam<sub>3</sub>CSK<sub>4</sub> is, however, not changed into a multilamellar structure, which has been found to be a necessary step for the inactivation for endotoxins (55).

Our data provide a physicochemical characterisation of synthetic analogues of lipoprotein N-termini and reveal that molecular and supramolecular conformations are important

determinants for their bioactivity. This refers in particular to the structural prerequisites for agonistic or antagonistic activity. In addition, we found some striking characteristics of the interaction of lipopeptides with lipid membranes and their interaction with the antibiotic PMB, which are in sharp contrast to what has been described for LPS.

Only recently, a mutant of *Staphylococcus aureus* defective in the synthesis of lipopeptides was described (3). Isolation of lipoteichoic acid (LTA) from these mutants resulted in a preparation which was dramatically attenuated in its biological activity compared to LTA preparations from wild type strains. The authors concluded that not LTA but lipoproteins are the dominant immunobiologically active compounds in cell wall preparations of *S.aureus*. These findings once more underline the importance of lipopeptides as immunostimulatory active microbial compounds. The molecular principles underlying lipopeptide recognition are crucial for an understanding of the role of these molecules in health and disease.

*Acknowledgements.* We thank K. Stephan, S. Groth, G. von Busse, and C. Hamann for performing the cytokine induction assay, the infrared, and FRET measurements, respectively.

## REFERENCES

1. Gioffre, A., Caimi, K., Zumarraga, M. J., Meikle, V., Morsella, C., Bigi, F., Alito, A., Santangelo, M. P., Paolicchi, F., Romano, M. I., and Cataldi, A. (2006) *J. Vet. Med. B Infect. Dis. Vet. Public Health* **53**, 34-41
2. Wardenburg, J. B., Williams, W. A., and Missiakas, D. (2006) *Proc. Natl. Acad. Sci. U.S.A* **103**: 13831-6
3. Hashimoto, M., Tawaratsumida, K., Kariya, H., Kiyohara, A., Suda, Y., Krikae, F., Kirikae, T., and Gotz, F. (2006) *J. Immunol.* **177**, 3162-3169
4. Kataoka, H., Yasuda, M., Iyori, M., Kiura, K., Narita, M., Nakata, T., and Shibata, K. (2006) *Cell Microbiol.* **8**, 1199-1209
5. Yang, X. F., Alani, S. M., and Norgard, M. V. (2003) *Proc. Natl. Acad. Sci. U.S.A* **100**, 11001-11006
6. Melchers, F., Braun, V., and Galanos, C. (1975) *J. Exp. Med.* **142**, 473-482
7. Sellati, T. J., Waldrop, S. L., Salazar, J. C., Bergstresser, P. R., Picker, L. J., and Radolf, J. D. (2001) *J. Immunol.* **166**, 4131-4140

8. Braun, V. and Wolff, H. (1970) *Eur. J. Biochem.* **14**, 387-391
9. Underhill, D. M. and Ozinsky, A. (2002) *Curr. Opin. Immunol.* **14**, 103-110
10. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282**, 2085-2088
11. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) *J. Exp. Med.* **189**, 1777-1782
12. Schromm, A. B., Lien, E., Henneke, P., Chow, J. C., Yoshimura, A., Heine, H., Latz, E., Monks, B. G., Schwartz, D. A., Miyake, K., and Golenbock, D. T. (2001) *J. Exp. Med.* **194**, 79-88
13. Delude, R. L., Savedra, R., Jr., Zhao, H., Thieringer, R., Yamamoto, S., Fenton, M. J., and Golenbock, D. T. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 9288-9292
14. Blondin, C., Le Dur, A., Cholley, B., Caroff, M., and Haeflner-Cavaillon, N. (1997) *Eur. J. Immunol.* **27**, 3303-3309
15. Correia, J. D., Soldau, K., Christen, U., Tobias, P. S., and Ulevitch, R. J. (2001) *J. Biol. Chem.* **276**, 21129-21135
16. Kielian, T. L. and Blecha, F. (1995) *Immunopharmacology* **29**, 187-205
17. El-Samalouti, V. T., Schletter, J., Chyla, I., Lentschat, A., Mamat, U., Brade, L., Flad, H.-D., Ulmer, A. J., and Hamann, L. (1999) *FEMS Immunol. Med. Microbiol.* **23**, 259-269
18. Triantafilou, K., Triantafilou, M., and Dedrick, R. L. (2001) *Nature Immunol.* **2**, 338-345
19. Triantafilou, M., Brandenburg, K., Gutschmann, T., Seydel, U., and Triantafilou, K. (2002) *Crit. Rev. Immunol.* **22**, 251-268
20. Triantafilou, M., Miyake, K., Golenbock, D. T., and Triantafilou, K. (1999) *J. Cell Sci.* **115**, 2603-2611
21. Iontcheva, I., Amar, S., Zawawi, K. H., Kantarci, A., and Van Dyke, T. E. (2004) *Infect. Immun.* **72**, 2312-2320
22. Blunck, R., Scheel, O., Mueller, M., Brandenburg, K., Seitzer, U., and Seydel, U. (2001) *J. Immunol.* **166**, 1009-1015
23. Gutschmann, T., Mueller, M., Carroll, S. F., MacKenzie, R. C., Wiese, A., and Seydel, U. (2001) *Infect. Immun.* **69**, 6942-6950
24. Mueller, M., Scheel, O., Lindner, B., Gutschmann, T., and Seydel, U. (2002) *J. Endotoxin Res.* **9**, 181-186
25. Heine, H. and Lien, E. (2003) *Int. Arch. Allergy Immunol.* **130**, 180-192
26. Ozinsky, A., Underhill, D. M., Fontenot, J. D., Hajjar, A. M., Smith, K. D., Wilson, C. B., Schroeder, L., and Aderem, A. (2000) *Proc. Natl. Acad. Sci. U.S.A* **97**, 13766-13771
27. Gantner, B. N., Simmons, R. M., Canavera, S. J., Akira, S., and Underhill, D. M. (2003) *J. Exp. Med.* **197**, 1107-1117
28. Viriyakosol, S., Fierer, J., Brown, G. D., and Kirkland, T. N. (2005) *Infect. Immun.* **73**, 1553-1560
29. Yadav, M. and Schorey, J. S. (2006) *Blood* **108**, 3168-75
30. Buwitt-Beckmann, U., Heine, H., Wiesmueller, K. H., Jung, G., Brock, R., and Ulmer, A. J. (2005) *FEBS J.* **272**, 6354-6364
31. Buwitt-Beckmann, U., Heine, H., Wiesmuller, K. H., Jung, G., Brock, R., Akira, S., and Ulmer, A. J. (2006) *J. Biol. Chem.* **281**, 9049-9057
32. Liu, H., Komai-Koma, M., Xu, D., and Liew, F. Y. (2006) *Proc. Natl. Acad. Sci. U.S.A* **103**, 7048-7053
33. Hoebe, K., Georgel, P., Rutschmann, S., Du, X., Mudd, S., Crozat, K., Sovath, S., Shamel, L., Hartung, T., Zaehring, U., and Beutler, B. (2005) *Nature* **433**, 523-527
34. Bessler, W. G., Cox, M., Lex, A., Suhr, B., Wiesmuller, K. H., and Jung, G. (1985) *J. Immunol.* **135**, 1900-1905
35. Prass, W., Ringsdorf, H., Bessler, W., Wiesmuller, K. H., and Jung, G. (1987) *Biochim. Biophys. Acta* **900**, 116-128
36. Israelachvili, J. N. (1991) *Intermolecular and surface forces*, 2<sup>nd</sup> Ed., Academic Press, London, 366-394

37. Rietschel, E. T. and Brade, H. (1992) *Sci. Am.* **267**, 54-61
38. Brandenburg, K., Mayer, H., Koch, M. H. J., Weckesser, J., Rietschel, E. Th., and Seydel, U. (1993) *Eur. J. Biochem.* **218**, 555-563
39. Brandenburg, K. and Wiese, A. (2004) *Curr. Top. Med. Chem.* **4**, 1127-1146
40. Reichel, F., Roelofsen, A. M., Geurts, H. P., Der Gaast, S. J., Feiters, M. C., and Boons, G. J. (2000) *J. Org. Chem.* **65**, 3357-3366
41. Seydel, U., Hawkins, L., Schromm, A. B., Heine, H., Scheel, O., Koch, M. H., and Brandenburg, K. (2003) *Eur. J. Immunol.* **33**, 1586-1592
42. Brandenburg, K., Hawkins, L., Garidel, P., Andrä, J., Müller, M., Heine, H., Koch, M. H. J., and Seydel, U. (2004) *Biochemistry* **43**, 4039-4046
43. Metzger, J., Wiesmuller, K. H., Schaudé, R., Bessler, W. G., and Jung, G. (1991) *Int. J. Pept. Protein Res.* **37**, 46-57
44. Wiesmueller, K. H., Bessler, W. G., and Jung, G. (1992) *Int. J. Pept. Protein Res.* **40**, 255-260
45. Metzger, J. W., Beck-Sickinger, A. G., Loleit, M., Eckert, M., Bessler, W. G., and Jung, G. (1995) *J. Pept. Sci.* **1**, 184-190
46. Seyberth, T., Voss, S., Brock, R., Wiesmueller, K. H., and Jung, G. (2006) *J. Med. Chem.* **49**, 1754-1765
47. Galanos, C., Lüderitz, O., and Westphal, O. (1969) *Eur. J. Biochem.* **9**, 245-249
48. Mueller, M., Brandenburg, K., Dedrick, R., Schromm, A. B., and Seydel, U. (2005) *J. Immunol.* **174**, 1091-1096
49. Schromm, A. B., Brandenburg, K., Rietschel, E. Th., Flad, H.-D., Carroll, S. F., and Seydel, U. (1996) *FEBS Lett.* **399**, 267-271
50. Koch, M. H. J. (1988) *Makromol. Chem. Macromol. Symp.* **15**, 79-90
51. Brandenburg, K., Richter, W., Koch, M. H. J., Meyer, H. W., and Seydel, U. (1998) *Chem. Phys. Lipids* **91**, 53-69
52. Kauppinen, J. K., Moffat, D. J., Mantsch, H. H., and Cameron, D. G. (1981) *Appl. Spectrosc.* **35**, 271-276
53. Gutschmann, T., Schromm, A. B., Koch, M. H. J., Kusumoto, S., Fukase, K., Oikawa, M., Seydel, U., and Brandenburg, K. (2000) *Phys. Chem. Chem. Phys.* **2**, 4521-4528
54. Gough, M., Hancock, R. E. W., and Kelly, N. M. (1996) *Infect. Immun.* **64**, 4922-4927
55. Brandenburg, K., Moriyon, I., Arraiza, M. D., Lehwarck-Yvetot, G., Koch, M. H. J., and Seydel, U. (2002) *Thermochim. Acta* **382**, 189-198
56. Luzzati, V., Vargas, R., Mariani, P., Gulik, A., and Delacroix, H. (1993) *J. Mol. Biol.* **229**, 540-551
57. Spohn, R., Buwitt-Beckmann, U., Brock, R., Jung, G., Ulmer, A. J., and Wiesmueller, K. H. (2004) *Vaccine* **22**, 2494-2499
58. Schromm, A. B., Brandenburg, K., Loppnow, H., Moran, A. P., Koch, M. H. J., Rietschel, E. Th., and Seydel, U. (2000) *Eur. J. Biochem.* **267**, 2008-2013
59. Mathison, J., Wolfson, E., Steinemann, S., Tobias, P., and Ulevitch, R. (1993) *J. Clin. Invest.* **92**, 2053-2059
60. Schroder, N. W., Heine, H., Alexander, C., Manukyan, M., Eckert, J., Hamann, L., Gobel, U. B., and Schumann, R. R. (2004) *J. Immunol.* **173**, 2683-2691
61. Boncheva, M., Duschl, C., Beck, W., Jung, G., and Vogel, H. (1996) *Langmuir* **12**, 5636-5642
62. Wolf, B., Hauschildt, S., Uhl, B., Metzger, J., Jung, G., and Bessler, W. G. (1989) *Immunol. Lett.* **20**, 121-126
63. Wolf, B., Uhl, B., Hauschildt, S., Metzger, J., Jung, G., and Bessler, W. G. (1989) *Immunobiol.* **180**, 93-100
64. Uhl, B., Wolf, B., Schwinde, A., Metzger, J., Jung, G., Bessler, W. G., and Hauschildt, S. (1991) *J. Leukoc. Biol.* **50**, 10-18
65. Metzger, J. W., Sawyer, W. H., Wille, B., Biesert, L., Bessler, W. G., and Jung, G. (1993) *Biochim. Biophys. Acta* **1149**, 29-39
66. Brandenburg, K., David, A., Howe, J., Koch, M. H., Andra, J., and Garidel, P. (2005) *Biophys. J.* **88**, 1845-1858

## FOOTNOTES

<sup>1</sup>Abbreviations used in this paper: FRET, fluorescence resonance energy transfer; IL, interleukin; LBP, lipopolysaccharide binding protein; lipolan, lipolanthionine peptide (2R,6R)Pam<sub>2</sub>LanHda-Ser-(Lys)<sub>4</sub>-NH<sub>2</sub>; LP, lipopeptide; LPS, lipopolysaccharide; Pam<sub>2</sub>CSK<sub>4</sub>, dipalmitoyl-S-glycerol-L-Cys-Ser-(Lys)<sub>4</sub>; Pam<sub>3</sub>CSK<sub>4</sub>, tripalmitoyl-S-glycerol-L-Cys-Ser-(Lys)<sub>4</sub>; PL<sub>MΦ</sub>, phospholipid mixture resembling the macrophage membrane; PMB, polymyxin B; SD, standard deviation; TLR, Toll-like receptor; TNF, tumor necrosis factor.

## FIGURE LEGENDS

**FIGURE 1:** Chemical structures of synthetic dipalmitoyl-S-glycerol-L-Cys-Ser-(Lys)<sub>4</sub> (Pam<sub>2</sub>CSK<sub>4</sub>), tripalmitoyl-S-glycerol-L-Cys-Ser-(Lys)<sub>4</sub> (Pam<sub>3</sub>CSK<sub>4</sub>), and lipolanthionine peptide amide (2R,6R)-Pam<sub>2</sub>LanHde-Ser(Lys)<sub>4</sub>-NH<sub>2</sub> (lipolan).

**FIGURE 2:** Activation of human macrophages by synthetic lipopeptides. Human mononuclear cells from healthy donors were stimulated with LPS and the lipopeptides Pam<sub>2</sub>CSK<sub>4</sub> and Pam<sub>3</sub>CSK<sub>4</sub> for 5 h and TNF $\alpha$  was determined in the supernatants. The data represent mean and standard deviation ( $\pm$  SD) from one representative experiment out of three, and the error bars result from the determination of TNF $\alpha$  in triplicate.

**FIGURE 3:** Synthetic lipolanthionine peptide lipolan inhibits activation of human macrophages by LPS. Human macrophages were incubated with the biologically inactive lipolanthionine peptide lipolan at a concentration of 1  $\mu$ g/ml for 30 min. Subsequently, LPS was added at the indicated concentrations, and cells were stimulated for 5 h before supernatants were harvested for the determination of TNF $\alpha$ . Data shown are mean ( $\pm$  SD) from one representative experiment out of three and the error bars result from the determination of TNF $\alpha$  in triplicate.

**FIGURE 4:** Antagonistic activity of synthetic lipolanthionine peptide does not depend on TLR2. HEK293 cells were transiently transfected with plasmids coding for huTLR4, huMD2, and huCD14 and incubated for 6 h to allow heterologous expression of the receptor proteins. Subsequently, cells were left unstimulated or stimulated with LPS (1  $\mu$ g/ml) or TNF $\alpha$  (10 ng/ml) in the absence or presence of lipolan (10  $\mu$ M). After 24 h, supernatants were collected for the determination of the chemokine IL-8. Data shown are mean  $\pm$  SD of triplicate samples of one experiment representative of at least three independent experiments. \*  $p < 0.05$  (p-value determined by students t-test).

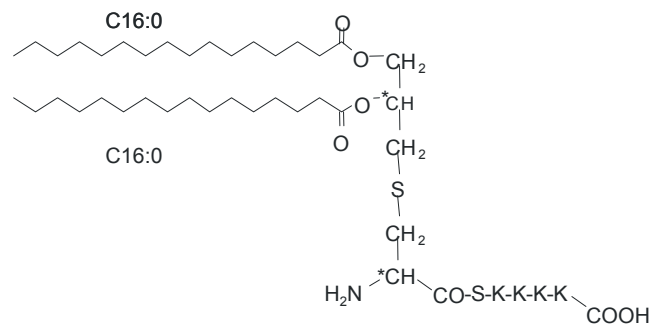
**FIGURE 5:** Synthetic lipopeptides interact with reconstituted phospholipid membranes. Incorporation of lipopeptides into liposomes PL<sub>MΦ</sub> corresponding to the phospholipid composition of the macrophage membrane by fluorescence resonance energy spectroscopy (FRET). The FRET signal I<sub>D</sub>/I<sub>A</sub> is plotted versus time. After 50 s, lipopeptides were added to the liposomes to a final concentration of 1  $\mu$ M. Data are representative for three independent experiments.

**FIGURE 6:** Interaction of synthetic lipopeptides with polymyxin B. (A) Peak position of the symmetric stretching vibrational band of the methylene groups versus temperature for Pam<sub>3</sub>CSK<sub>4</sub> in the absence and presence of polymyxin B (PMB) with Fourier-transform infrared spectroscopy (FTIR). In the gel phase, the peak position lies around 2850 cm<sup>-1</sup>, in the liquid crystalline phase between 2852.5 and 2853.0 cm<sup>-1</sup>. (B) Production of TNF $\alpha$  from human mononuclear cells induced by Pam<sub>3</sub>CSK<sub>4</sub> in the absence and presence of PMB. Data shown are of one representative experiment out of three, and the error bars ( $\pm$  SD) result from the determination of TNF $\alpha$  in triplicate.

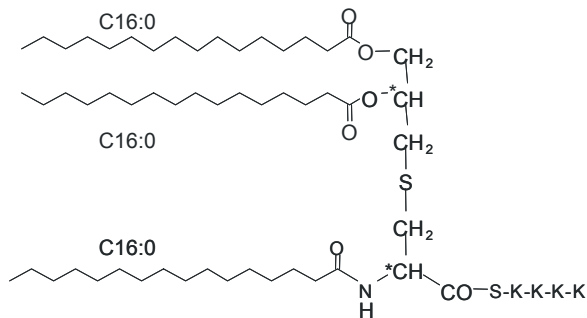
**FIGURE 7:** Molecular conformation of agonistic and antagonistic synthetic lipopeptides. Synchrotron radiation small-angle X-ray diffraction patterns of the three lipopeptides at 40 °C and 95 % water content. The band positions of the small peaks shown in Fig. 7A and B could be confirmed by applying Fourier self-deconvolution (52).

Figure 1

**Pam<sub>2</sub>CSK<sub>4</sub>**



**Pam<sub>3</sub>CSK<sub>4</sub>**



**Lipolan**

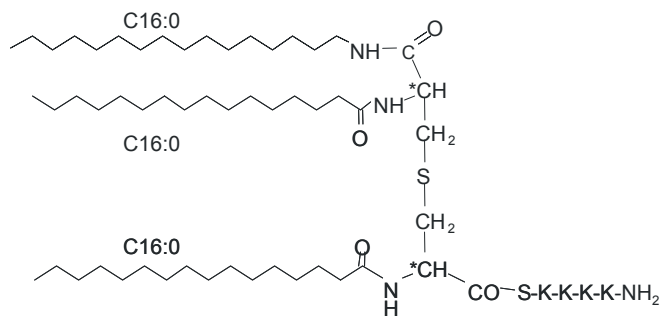


Figure 2

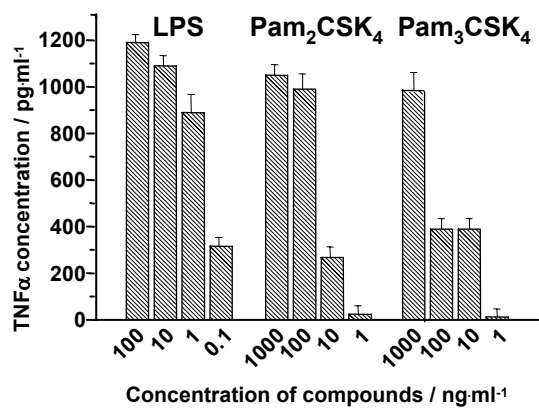


Figure 3

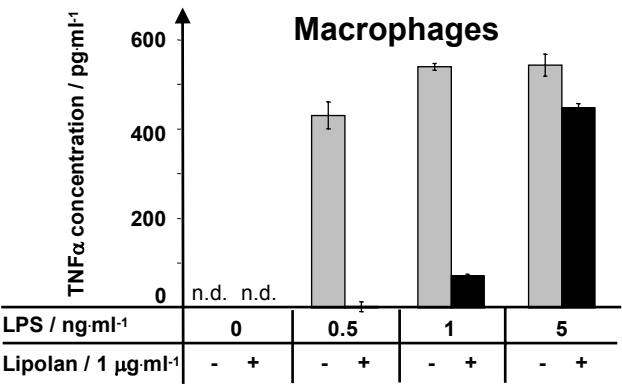


Figure 4

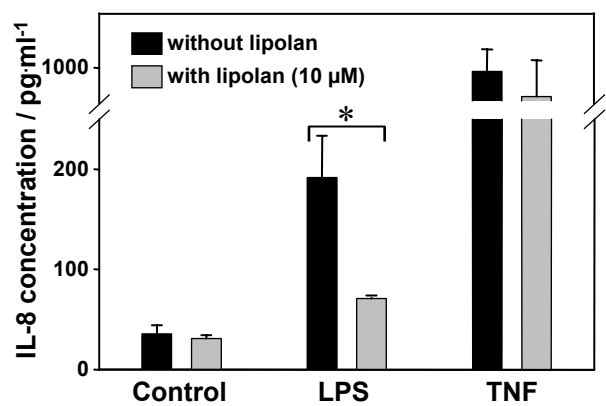


Figure 5

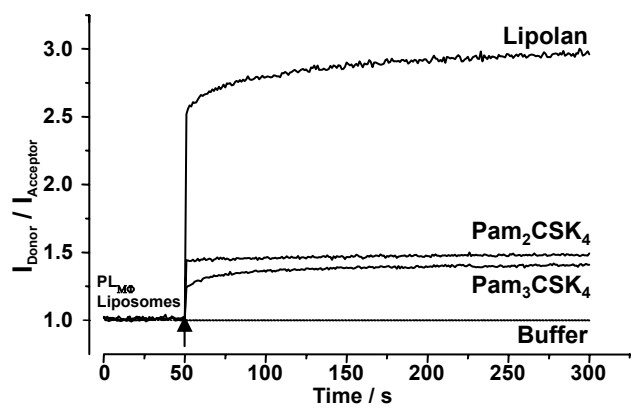
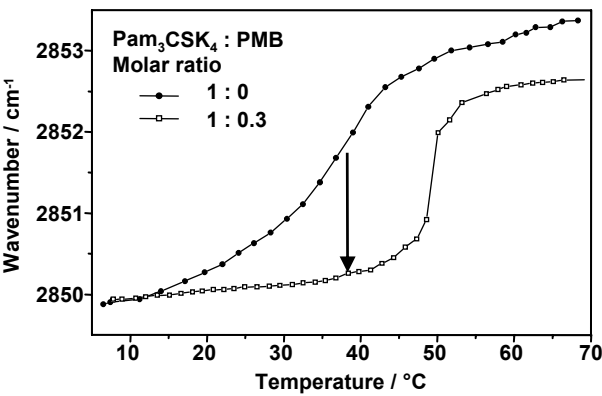


Figure 6

A



B

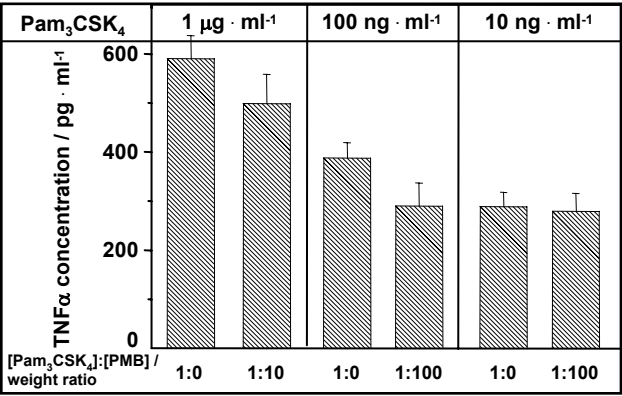


Figure 7

