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Model membrane studies for characterization of different antibiotic activities of lipopeptides from *Pseudomonas*

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ABSTRACT

Lipopeptides (LPs) are a structurally diverse class of amphipathic natural products that were in the past mainly known for their surfactant properties. However, the recent discovery of their antimicrobial and cytotoxic bioactivities have fueled and renewed the interest in this compound class. Propelled by the antimicrobial potential of this compound class, in this study a range of six underinvestigated LPs from Pseudomonads were examined with respect to their antibiotic activities towards bacteria. The assays revealed that only the glycosylated lipodipeptide SB-253514, produced by *Pseudomonas* strain SH-C52, showed significant antibacterial activity. Since the bioactivity of LPs is commonly attributed to membrane interactions, we analyzed the molecular interactions between the LPs and bacteria-like lipid model membranes in more detail via complementary biophysical approaches. Application of the quartz crystal microbalance (QCM) showed that all LPs possess a high binding affinity towards the model membranes. Despite their similar membrane affinity, monolayer studies displayed different tendencies of LPs to incorporate into the membrane. The degree of membrane incorporation could be correlated with specific structural features of the investigated LPs, such as distance between the peptidic macrocycle and the fatty acid, but did not fully reflect their respective antibacterial activity. Cyclic voltammetry (CV) experiments further demonstrated that SB-253514 showed no membrane permeabilization effects at inhibitory concentrations. Collectively, these results suggests that the antibacterial activity of SB-253514 cannot be explained by an unspecific detergent-like mechanism generally proposed for amphiphilic molecules but instead appears to occur via a defined structural target.

1. Introduction

Bacterial lipopeptides (LPs) consist of a charged linear or cyclic peptide head and a hydrophobic fatty acid tail of variable length. These amphipathic molecules are predominantly produced by soil bacteria, e.g. actinomycetes, bacilli or pseudomonads and are supposedly secreted to fulfill a suite of natural functions, e.g. host defense, enhancement of cell motility and biofilm formation [1]. Since a number of LPs of microbial origin was reported to possess antibacterial activity, LPs represent a promising class of natural products with antibiotic properties [2,3]. The prospect of this compound class in the field of antibiotics is also underlined by two LPs that reached already the drug market, i.e. the polymyxins and the recently approved daptomycin (Cubicin®) [4].

LPs from *Pseudomonas* species are usually composed of an unbranched-3-hydroxy fatty acid tail linked to a short oligopeptide (2–25 amino acids) which is usually cyclized to form a lactone ring. The presence of D-configurated amino acids and the occurrence of non-proteinogenic amino acids, e.g. homoserine, 4-chloro-threonine or diaminobutyric acid is particularly noteworthy. Based on the number, type, and configuration of the amino acids in the peptide moiety, LPs of *Pseudomonas* spp. were classified into nine major groups [5–8]. Most of the LPs obtained from Pseudomonads have been tested for their surfactant activity or, in a phytopathological context, assayed for their antifungal properties or their involvement in virulence. However, these LPs have been scarcely investigated for their potential to inhibit the growth of bacteria [2]. Antibacterial properties were reported only for some members from four out of the nine major LP groups: LPs of the viscosin [9,10], syringomycin [11,12], tolaasin [13,14] and syringopeptin [15,16] group were shown to be

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active preferentially against towards Gram-positives (e.g. *Bacillus megaterium*, *Staphylococcus aureus* or *Mycobacterium tuberculosis*). Particularly the antimycobacterial activity gained increasingly importance over time since more and more *M. tuberculosis* strains show resistance towards standard treatment [17,18], which impressively underlines the need for new antibiotics. Based on these promising activities, we explored the antibacterial properties of those LPs from Pseudomonads that were related to the nine amino acid containing antimycobacterial LPs but which have not yet been investigated.

After evaluation of their antibiotic properties, we investigated the molecular interactions between the selected LPs and lipid model membranes because it is widely accepted that the primary target of LPs is the cytoplasmic membrane. The main destructive action of LPs on cell membranes has been referred to their unspecific detergent-like activity, i.e. their ability to disintegrate the lipid bilayer [10,19,20]. However, more specific mechanisms, e.g. inhibition of the synthesis of cell wall components [21], have also been demonstrated for some LPs.

Considering that the activities of *Pseudomonas* LPs appear to be directed mainly towards Gram-positive bacterial competitors, we designed a model membrane system to mimic properties of the natural bilayer membrane of Gram-positives. To gain deeper insight into their biological activities, the interaction of the selected LPs was studied in detail via the following complementary biophysical methods:

1. The quartz crystal microbalance (QCM) was employed to investigate the intensities and kinetics of LP binding towards supported lipid bilayers. This technique provides time-resolved measurements of mass changes adsorbed at the model membranes and thereby allows the determination of kinetic binding constants of the LPs.
2. The cyclic voltammetry (CV) was used to detect changes in membrane permeability, e.g. pore formation induced by an interaction between the LPs and membranes.
3. Finally, the subphase-assay was used to obtain information about the surface activity, i.e. the capacity of the LP to insert into a phospholipid monolayer by detection of increased lateral membrane surface pressure.

Using these techniques, we addressed the questions, whether LPs affect the structure of model membranes and if this could explain their antibacterial activities. To get a better insight into these activities and affecting parameters, the impact of membrane constituents (ergosterol content) and different sizes of the peptidic moiety of the LPs was investigated in depth.

2. Materials and methods

2.1. Chemicals

The LP syringomycin E was purchased from Sigma Aldrich Chemicals, Deisenhofen, Germany and used as supplied. All chemicals for model membrane preparation were commercially available and used without further purification. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1,2-Dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Ergosterol and hexadecanethiol were purchased from Sigma.

2.2. Bacterial producer strains and culture conditions

The LP producer strains *Pseudomonas fluorescens* Pf-5 [22] (orfamide A), *Pseudomonas* sp. MIS38 [23] (arthrofactin), and *Pseudomonas entomophila* [24] (entolysin B) were kindly provided by Prof. Loper (USDA/Oregon State University, Corvallis, USA), Prof. Morikawa (Hokkaido University, Sapporo, Japan) and Dr. Vallet-Gely (Centre de Génétique, CNRS, Gif-sur-Yvette, France), respectively. *Pseudomonas* sp. SH-C52 (SB-253514) and *P. fluorescens* SS101 [25] (massetolide A) are isolates by one of the authors (J.M.R).

P. fluorescens SS101 was cultivated as previously described [26]. All other strains were grown first in 10 mL Davis Minimal Broth without dextrose containing 20 mM glycerol (DMBgly) in 50 mL Falcon tubes for 24–48 h at room temperature with shaking (100 rpm) using a Gerhardt LS 30 horizontal shaker. Five 5000 mL Erlenmeyer flasks containing 1.5 L DMBgly were inoculated with 2 mL starter culture. Cultures were incubated for 72 h at 26 °C with shaking at 140 rpm using an INFORS HT Multitron incubator shaker.

2.3. Isolation and purification of the lipopeptides

Massetolide A [26] and orfamide A [6] were purified as previously described. The remaining three LPs were obtained according the following procedure: the fermentation broths were acidified to pH 2 and portions (0.5 L) of the broths were extracted two times with 0.5 L ethyl acetate to yield crude extracts. Further fractionation of each extract by RP-SPE (J.T. Baker Bakerbond C18, 2 g) using stepwise gradient elution with MeOH/H_2O (40:60) to 100% MeOH yielded five to six subfractions. ^1H NMR profiling indicated subfractions of further interest due to three clusters of resonances typical for peptides: exchangeable downfield amide signals, α-proton resonances and side chain protons. Purification of these subfractions by RP-HPLC using a linear gradient of 70:30–100:00 MeOH/H_2O (0.05% TFA) over a period of 65 min (Phenomenex Luna, 250 × 10 mm, 5 μm in combination with a Phenomenex SecurityGuard Luna, 10 × 10 mm precolumn; 2 mL/min flow rate; UV monitoring at 215 nm), yielded pure SB-253514 or arthrofactin, respectively. Entolysin B was obtained in this way only in a semi-pure form and had to be rechromatographed two times by RP-HPLC in order to be considered a pure compound as judged by LC-MS.

2.4. Detection of antibacterial activity

The antibacterial activity was determined by agar diffusion assays. Culture plates (5% sheep blood Columbia agar, BD) were overlaid with 3 mL tryptic soy soft agar, inoculated with TSB (tryptic soy broth, Oxoid) growth suspension of the bacteria to be tested. Compounds were diluted with DMSO to a concentration of 1 mg/mL (syringomycin E 0.5 mg/mL) and 3 μL of this dilution were placed on the surface of the agar; the diameter of the inhibition zone was measured after 24 h incubation at 37 °C. The indicator strains represent clinical isolates from distinct patients as well as standard laboratory strains and are listed in Table 1. The strains were maintained on Mueller–Hinton (MH) agar or on blood agar.

MIC determinations were carried out in microtiter plates. *Arthrobacter crystallopoietes* DSM 20117 was grown in tryptic soy broth (Oxoid), all other strains were grown in half-concentrated Mueller–Hinton broth (Oxoid). MICs with serial twofold dilution steps were performed (1:2). Bacteria were added to give a final inoculum of 10^5 CFU/ml in a volume of 0.2 mL. After incubation for 24 h at 37 °C, the MIC was read as the lowest compound concentration causing inhibition of visible growth. Results are mean values of three independent determinations. Since some lipopeptides were previously reported to possess a calcium dependency for optimum activity [27], the effect of calcium substituted medium on the MICs was evaluated. Two organisms, *S. aureus* SG511 and *Bacillus subtilis* 168 were used in the test which consisted of a broth dilution MIC determination in supplemented (0.2 mg/mL $CaCl_2$) and unsupplemented half-concentrated Mueller–Hinton broth.

2.5. Model membrane preparation

Cleaning and preparation procedures of the quartz sensors for QCM and CV were already described previously [28]. The supported bilayers were formed by transferring a monolayer consisting of either POPC/DOPG (9/1), POPC/DOPG/ergosterol (6/1/3) or POPC/DOPG/ergosterol

Table 1
Antimicrobial disk diffusion inhibitory activities of the investigated *Pseudomonas*-lipopeptides.

Organism	Zone of inhibition (mm)					
	SB-253514	Massetolide A	Syringomycin E	Orfamide A	Arthrofactin	Entolysin B
Gram-positive bacteria						
Staphylococcus aureus SG 511	3	0	0	0	0	0
Staphylococcus aureus 5185 (MS)[a]	3	0	0	0	0	0
Staphylococcus aureus LT-1334 (MR)[a]	0	0	0	0	0	0
Staphylococcus epidermidis LT-1324 (MR)[a]	0	0	0	0	0	0
Staphylococcus epidermidis ATCC 12228	0	0	0	0	0	0
Staphylococcus simulans 22	0	0	0	0	0	0
Micrococcus luteus ATCC 1856	0	0	0	0	0	0
Enterococcus faecium I-11305b	0	0	0	0	0	0
Bacillus subtilis 168	4	0	0	0	0	0
Bacillus megaterium	0	0	0	0	0	0
Listeria welchimeri DSM 20650	3	0	0	0	0	0
Corynebacterium pseudodiphtheriticum	3	0	0	0	3	0
Corynebacterium diphtheriae	3	0	0	0	3	0
Corynebacterium xerosis Va 167198	3	0	0	0	3	0
Mycobacterium smegmatis ATCC 70084	3	0	0	0	3	0
Arthrobacter crystallopoietes DSM 20117	13	8	5	0	4	3
Gram-negative bacteria						
Escherichia coli DH5 alpha	0	0	0	0	0	0
Escherichia coli O-19592	0	0	0	0	0	0[b]
Klebsiella pneumonia I-10910	0	0	0	0	0	0
Citrobacter freundii I-11090	0	0	0	0	0	0[b]
Pseudomonas aeruginosa I-10968	0	0	0	0	0	0
Serratia marcescens	0	0	0	0	0	0

[a] MS = methicillin-susceptible; MR = methicillin-resistant.
[b] Diffuse inhibition zone of 2 mm visible.

(4/1/5, molar ratio) by Langmuir–Blodgett technique onto the covalently fixed first monolayer of hexadecanethiol.

2.6. Biophysical methods

QCM experiments were performed at 25 °C using the LiquiLab21 system (ifak e.V., Barleben, Germany). After reaching a constant frequency, 3 mL of a 0.5 μM peptide solution were injected into the flow system. The run was stopped after reaching a constant frequency. The binding constants were calculated from the frequency traces as described before [29].

CV experiments to detect membrane barrier disturbances have already been described [29]. LP solution (0.5 μM) was injected into the CV chamber after completing 35 scans. Potential current changes were calculated from the CV curves by integrating each complete cycle.

For the *monolayer subphase-assay* the phospholipid mixtures were spread on a Langmuir trough filled with ultra-pure water. After recovery for 15 min, the monolayer was compressed to 1/2–2/3 of the collapse pressure. Five injections of each 0.1 μM LP (final concentration 0.5 μM) into the aqueous subphase at intervals of at least two minutes were monitored in real time by changes in the lateral surface pressure.

The logP values for the six lipopeptides were estimated by using the ACD-LogD-Suite 5.0 software and are given in Fig. 1.

3. Results

3.1. Selection criteria for LPs

To date, all LPs of Pseudomonads that have been investigated for antibacterial activity represent either 9- or 22-amino acid (AA)-containing cyclic LPs. Based on and inspired by the antimycobacterial activity of the 9-AA-containing LPs of the viscosin and syringomycin class, we wanted to probe the chemical space around these molecules. To this end, we included the 2-AA-containing SB-253514 [8], the 10-AA-containing orfamide A [6], the 11-AA-containing arthrofactin and the 14-AA-containing entolysin B [7] in this study (Fig. 1). These LPs possess special features like glycosylation, different cyclization schemes, and different fatty acid chain lengths. Each of these LPs has not been examined for antibacterial properties yet, either due to paucity of available material or because the substance class was discovered only recently. SB-253514 represents the smallest member of the *Pseudomonas*-LP family. Its biosynthesis pathway unambiguously groups it with this compound class; however, its peptide moiety is rearranged to a cyclocarbamate structure during biosynthesis (J.M.R. and H.G., unpublished). Furthermore, it is the only *Pseudomonas*-LP known so far with a glycosylated 3-hydroxy-fatty acid side chain. Arthrofactin was originally isolated from a bacterium of the genus *Arthrobacter* [23], however its taxonomic classification was later revised to *Pseudomonas* sp. MIS38 [30]. Therefore, arthrofactin is now considered a true *Pseudomonas*-LP. Additionally, there was confusion about the structure of arthrofactin, since two versions of the molecule were reported in the literature, differing in the cyclization scheme and stereochemistry [23,31]. We reinvestigated this compound spectroscopically and corrected the structure as depicted in Fig. 1 (details of the structure revision will be published elsewhere). Massetolide A and syringomycin E were included as reference compounds and to deduce possible structure-activity relationships.

3.2. Antibacterial activity of LPs

The effect of all LPs on the growth of a large collection of bacterial genera and species is shown in Table 1. In general, only Gram-positive bacteria were affected by the six investigated LPs. All tested LPs displayed inhibitory activities against *A. crystallopoietes*. In addition, arthrofactin was observed to moderately inhibit the growth of *Corynebacteria* and a mycobacterial strain. The antibacterial activities of SB-253514 paralleled those of arthrofactin, but were stronger against *A. crystallopoietes* and additionally affected staphylococci, bacilli and *Listeria*. The MIC values of the LPs showed a good correlation with the respective inhibition zones, obtained from the agar-diffusion assay. However, this analysis also revealed that only SB-253514, massetolide A and entolysin B displayed significant

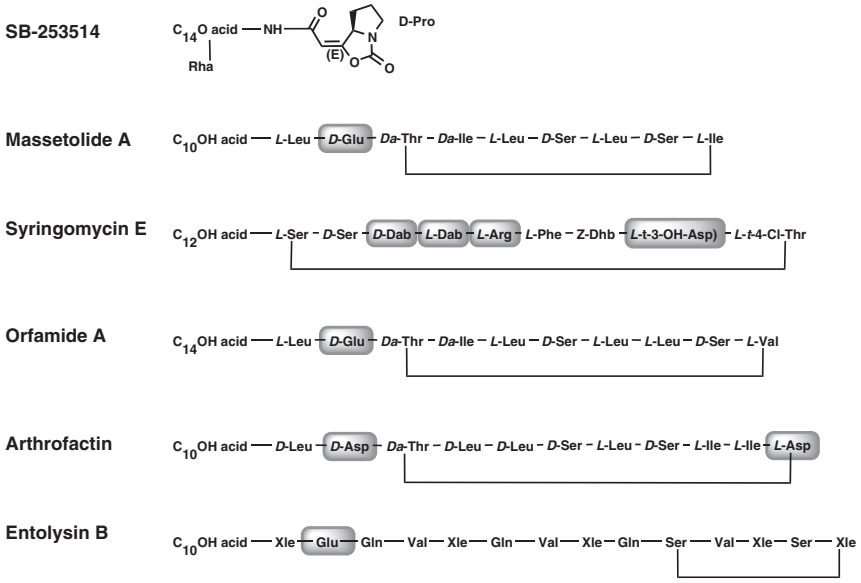

Fig. 1. Chemical structures of the investigated lipopeptides. Abbreviations: Dab = 2,4 diaminobutyric acid, Dhb = 2,3-dehydroaminobutyric acid. All other amino acids are identified by standard three-letter biochemical notation. The absolute configuration of each amino acid is indicated by the following stereodescriptors: *D/L*, *E/Z*, *a* = *allo*, *t* = *threo*. The absolute configuration of entolysin B has not been determined. Gray boxes indicate amino acids with chargeable side chains. Basic amino acids (Dab, Arg) were positively charged (protonated) under the test conditions of the model membrane assays (pH 5.5). Acidic amino acids (Asp, Glu) were not affected by the given pH and remained neutral. The logP values were estimated as follows: SB-253514 (+5.21), massetolide A (−2.26), syringomycin E (−9.24), orfamide A (−0.50), arthrofactin (−3.86), entolysin B (−2.69).

antibacterial activities towards *B. subtilis* 168, *A. crystallopoietes* DSM 20117 and *Corynebacterium xerosis* Va 167198, respectively (Table 2). Since it became recently evident that some LPs heavily depend on the presence of Ca^{2+} ions for full antibacterial activity [27], all *Pseudomonas*-LPs of this study were also re-tested for their antibacterial activity in the presence of $CaCl_2$. However, for all tested lipopeptides no increase in potency was observed in the presence of added calcium (data not shown).

3.3. Membrane binding affinity of LPs

Since LPs generally display surfactant properties, one might associate their antibiotic activity with the capacity to interact with the bacterial membranes. This capacity should mainly be reflected by the binding affinity of the peptides towards the membrane. Quartz crystal microbalance (QCM) was applied to determine the constants (association rate k_{ass}, dissociation rate k_{diss}, overall binding affinity k_D) of the six LPs binding to model membranes. The lipid composition was adjusted to imitate the predominant membrane components of Gram-positive bacteria and mycobacteria. POPC was used as the main phospholipid representing the most common fatty acid in bacterial membranes (C16:0 and C18:1) [32–35]. Although PGs are more common in bacterial membranes than PCs, we consciously decided to use PCs as main components (for stability reasons) and added 10 mol% DOPG to confer a net negative charge to the membranes. All bacteria possess membrane stabilizing hopanoids. The activity of these sterol-like components were simulated by adding certain fractions of ergosterol (either 30 mol% or 50 mol%).

All LPs displayed considerable high overall binding affinities to the three different types of model membranes (Fig. 2), which are in the low micromolar or high nanomolar range (2.22×10^{-7}–1.28×10^{-6} M). Considering the k_D values (Fig. 2A), ergosterol has obviously no significant influence on the peptide binding intensities. The QCM technique allows for a detailed view on the association and dissociation rates of the peptides. Fig. 2B and C reveal that the high affinities of the LPs mainly result from rapid association rates (3589–8777 $M^{-1}\,s^{-1}$), whereas the dissociation rates are in a median range. It is worth mentioning that the binding affinity of SB-253514 is slightly lower compared to the other LPs mainly due to a diminished association rate.

3.4. Membrane incorporation of LPs

The rapid and comparable association rates of the LPs binding to membranes could suggest that besides the polar interaction of the peptide with the phospholipid head group, a membrane insertion is dominant. An insertion of the lipid chains of the LPs into bacterial membranes could induce antibacterial effects by changing the lateral distribution and function of membrane components, such as carriers of cell wall precursors.

In this study, we performed a monolayer subphase assay to investigate whether the LPs in general are able to incorporate into the membrane and to quantify this capacity for a correlation with their

Table 2
Antimicrobial minimal inhibition concentrations (MICs) of the investigated *Pseudomonas*-lipopeptides.

Organism	MIC (μg/mL)					
	SB-253514	Arthrofactin	Massetolide A	Syringomycin E	Entolysin B	Orfamide A
Staphylococcus aureus SG 511	25	>50	>50	50	>50	>50
Bacillus subtilis 168	12.5	50	>50	25	>50	>50
Listeria welchimeri DSM 20650	25	>50	>50	>50	>50	50
Corynebacterium xerosis Va 167198	50	50	>50	>25	12.5	>50
Mycobacterium smegmatis ATCC 70084	50	>50	>50	>50	50	>50
Arthrobacter crystallopoietes DSM 20117	12.5	50	12.5	50	50	50

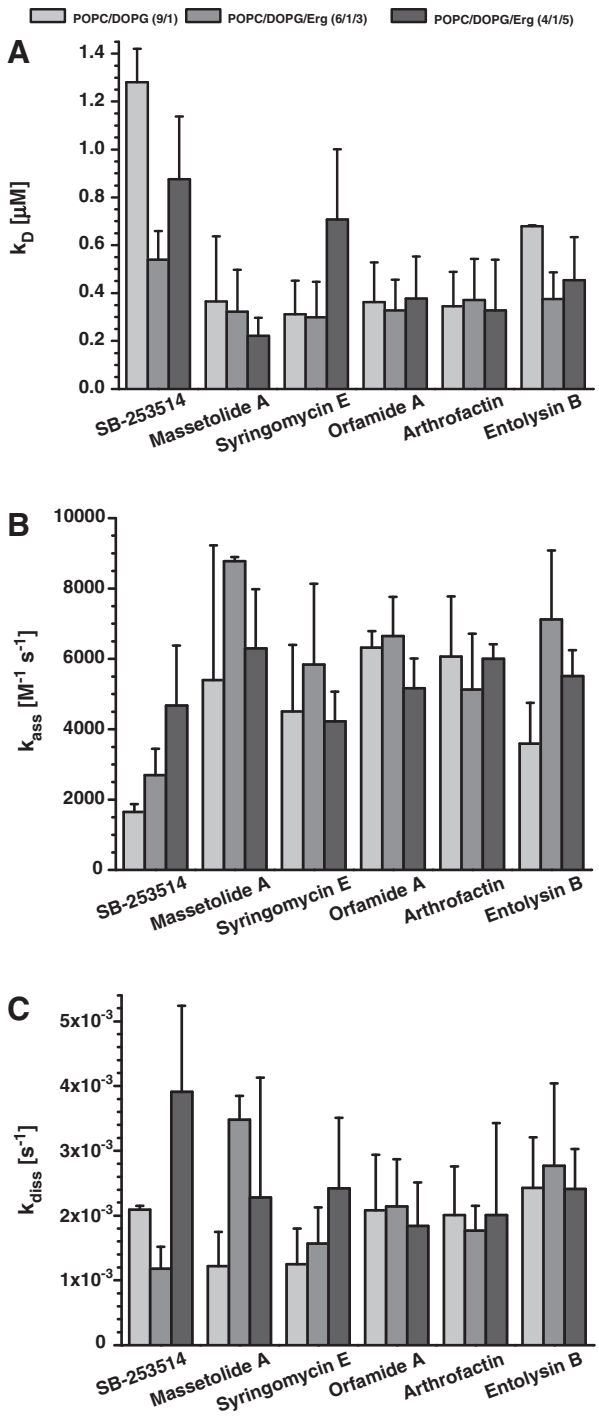

Fig. 2. Kinetic constants of lipopeptide binding affinities to model membranes. (A) Independent on the model membrane composition (POPC/DOPG/ergosterol in different ratios), all lipopeptides display strong binding affinities (k_D) in the lower micromolar range comparable to that of target-mediated membrane interaction of other antibiotic active peptides. These high affinities mainly result from rapid association rates k_{ass} (B), whereas the dissociation rates k_{diss} (C) are in a median range.

structural properties. The monolayers (POPC/DOPG/ergosterol; 9/1/0; 6/1/3; 4/1/5, molar ratio, resp.) were spread on the surface of a water filled Langmuir trough. After lateral compressing the monolayer, 0.1 µM peptide solutions were injected into the subphase, repeatedly for five times each after reaching an equilibrated surface pressure. Changes in the surface pressure were monitored in real time. An example for LP injection into the aqueous subphase and the time course of the resulting changes in lateral surface pressure of the monolayer is given in Fig. 3A. It is evident that each injection further increases the surface pressure which can be related to the extent of peptide incorporation into the membrane, reaching a saturation around the highest concentration of 0.5 µM. Overall Δp represents the total increase in surface pressure after five injection (0.5 µM) and can thus be regarded as the degree of membrane insertion capacity of each of the LPs (Fig. 3B).

In contrast to the QCM data, differences in the extent of incorporation became obvious. Orfamide A and entolysin B had the highest tendency for membrane incorporation, followed by massetolide A, arthrofactin, and SB-253514. However, syringomycin E had only a minor tendency to insert into the membrane. With exception of arthrofactin and entolysin B showing slightly decreased Δp values with increasing amounts of ergosterol, the general tendency of peptide monolayer insertion appears to occur relatively independent on membrane composition. On a first view, these data do not directly reflect the binding affinity data and need further discussion with respect to the structural features of the peptides.

3.5. Membrane permeabilization effects of LPs

The subphase data cannot explain the different antibacterial activities of the LPs. However, membrane insertion of the LPs could be regarded as a prerequisite for modifying the membrane permeability in light of the already reported membrane disintegrating activities of some LPs [36]. To focus on this feature, we applied a cyclic voltammetry (CV) assay in combination with the model membranes used. By detecting changes in Faradaic currents after peptide addition, this technique allows for the determination of membrane permeability changes. However, it is not possible to differentiate between unspecific membrane permeabilization and formation of defined pores. To compare the CV with the QCM data and refer to the saturation concentration in the subphase assay, 0.5 µM peptide solution was used. For all LPs it became evident that the addition of 0.5 µM peptide solution did not lead to any permeabilization of the model membranes, independent of the membrane composition (data not shown).

To investigate whether higher and MIC relevant concentrations induce permeability changes, we selected the most effective LP SB-253514 and repeated the CV experiments at concentrations representing two-, four- and tenfold its MIC (25; 50; 125 µg mL^{-1}). Furthermore, orfamide A, massetolide A, and arthrofactin were also analyzed for their permeabilization ability in the MIC relevant range (50 µg mL^{-1}) and compared with syringomycin E as a reference, which is known for pore formation in concentrations explicitly above 0.5 µM. Since data from Dalla Serra and coworkers [37] pointed out the positive influence of sterols for membrane permeabilization, we used a model membrane composed of POPC/DOPG/ergosterol (4/1/5). As expected, syringomycin E permeabilized the membrane at concentrations comparable to its MIC (40.8 µM), which became evident by an increased Faradaic current by 71%. In contrast, SB-253514 did not show any increase in the Faradaic current, neither at 45 µM (twofold MIC), 90 µM (fourfold MIC) nor at 225 µM (tenfold MIC) indicating that membrane permeabilization is obviously not the key to explain the bactericidal activity of this glycosylated lipodipeptide. The other three LPs tested did not show effects on the membrane barrier function at MIC concentrations, which was partly surprising as discussed below.

4. Discussion

4.1. Antibacterial activity of the LPs

Consistent with previous observations [10,14–16], none of the Gram-negative bacterial species tested were inhibited in growth by the six *Pseudomonas*-LPs investigated here. This lack of activity of many LPs against Gram-negative bacteria has been ascribed to protective effects of

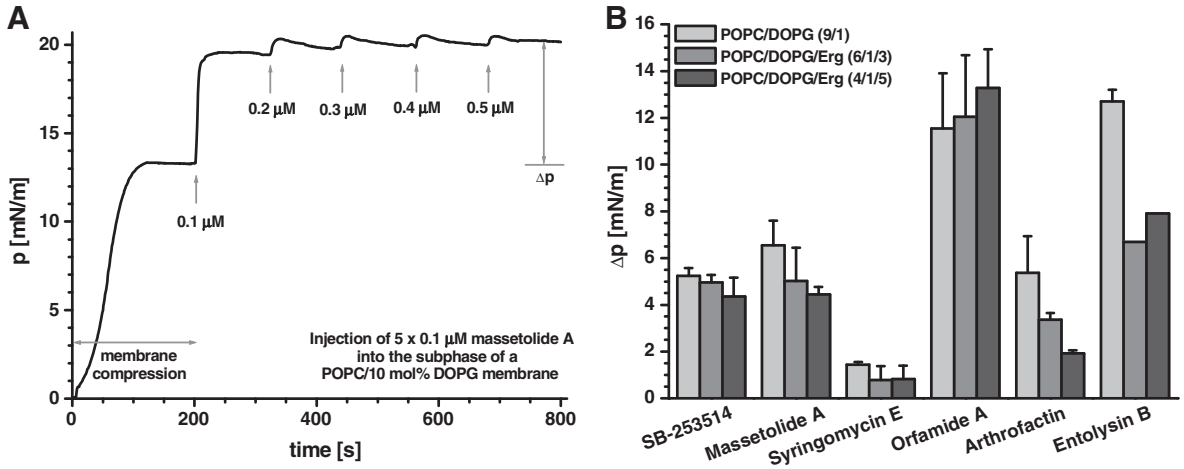

Fig. 3. Membrane incorporation of the lipopeptides detected by Langmuir monolayer assay. A) Each lipopeptide injection further increases the lateral surface pressure which can be related to the extent of peptide incorporation into the membrane, reaching saturation around the highest concentration. B) Overall Δp represents the total increase in surface pressure after five injection (0.5 µM). The amount of membrane incorporation differs within the six lipopeptides and can be related to their structure, as discussed in detail.

the outer membrane and suggested to be a possible mechanism of self-resistance for the likewise Gram-negative LP-producing Pseudomonads [38]. The antibacterial activity observed in agar-diffusion assays confirmed in general the antibacterial potential of *Pseudomonas*-LPs towards enterococci [10], micrococci [15], staphylococci [10,16], bacilli [12–16] and mycobacteria [9,16,11]. However, the activities against *Listeria*, corynebacteria and an *Arthrobacter* species observed in this study have not been reported so far. Surprisingly, syringomycin E, known to be active towards *B. megaterium* (MIC = 5 µM, [12]) and *Mycobacterium smegmatis* (MIC = 1.5 µg/mL, [11]), and massetolide A, reported to be active against *M. tuberculosis* (MIC = 5–10 µg/mL, [9]) and *Mycobacterium avium-intracellulare* (MIC = 2.5–5 µg/mL, [9]), showed no activity towards comparable indicator strains. These results are not necessarily in contradiction, since others also demonstrated the inactivity of syringomycin E towards *B. megaterium* (ITM100) [15] and, in the case of massetolide A, another *Mycobacterium* species was used as test strain (*M. smegmatis* instead of *M. tuberculosis* or *M. avium-intracellulare*). The absence of activity of syringomycin E against *M. smegmatis* might be attributed to acquired resistance or species-specific variations of this strain regarding the cell-surface or cell wall composition [39,40].

Despite the literature reports, the observed antibacterial potential deduced from the agar-diffusion assay, the overall activity of most of the tested *Pseudomonas*-LPs have to be judged, according to their MIC values, as weak to moderate. Solely, the LP SB-253514, most unusual in structure, shows significant MICs towards several Gram-positives.

4.2. LPs display high membrane affinity

The different modes of peptide interaction with membranes, e.g. peptide attachment to or incorporation into the membranes, are mainly reflected by binding affinity data. The QCM binding assay provided data that confirm a strong binding affinity of all six LPs of this study to the differently composed model membranes. The k_D values in the low micromolar or high nanomolar range are comparable to that of target-mediated membrane interaction of other antibiotic active peptides. For instance, the cyclic LP friulimicin, which consists of a cyclic decapeptide N-terminally linked to an unsaturated branched fatty acid was recently shown to bind membranes in presence of the bactoprenol target C_{55}-P with a k_D value close to that of the LPs in this study (0.21 ± 0.08 µM) [41]. The membrane association rate of LPs, which even exceeds that of friulimicin ($3736 \pm 1983\ M^{-1}\ s^{-1}$), appears to be driven by two facts: the polar amino acid residues initially allow for interaction with the polar head groups of the phospholipids, followed by an incorporation of the fatty acids into the membrane core. Regarding the time frame of QCM detection, one can postulate that QCM data mainly reflect the peptide membrane attachment since the process of membrane insertion and reorganization is too slow to be fully considered under the dynamic flow conditions. The dominance of the polar moiety for strong binding affinities could explain the lower affinity of the dipeptide SB-253514 compared to the other derivatives possessing more polar regions. Compared to all other investigated LPs in this study, the most lipophilic SB-253514 (logP value of +5.21) is not able to associate to the membrane in a similar amount. The predominant role of polar interaction for peptide binding affinity also accounts for the minor impact of the different membrane compositions, which possesses similar surface regions despite of differences in fluidity.

Summarizing, QCM data showed a high membrane affinity for all tested LPs. However, there is no indication for differences in the binding affinity that might explain the differences in antibacterial activities of the tested LPs.

4.3. LP membrane insertion depends on the LP structure but is not related to antibacterial activity

Peptide incorporation into the membrane appears to be timely subordinated to the peptide attachment and thus cannot completely be detected by QCM. However, peptide insertion might be a serious contribution to bactericidal activity, since membrane-inserted peptides can possess a strong impact on bacterial membrane order and transport processes, even without changing the general barrier function.

The monolayer subphase assay allows investigating the incorporation process under equilibrium conditions. Regarding the extent of peptide incorporation given by the change in lateral surface pressure, the concentration of 0.5 µM appears to be near saturation since the increases in pressure were strongly diminished with each further injection. According to the absolute changes in pressure Δp, the *Pseudomonas*-LPs can be subdivided into three groups in relation to their structure.

Syringomycin E (SRE) appears unique with the lowest intensity for membrane incorporation. To structurally explain this finding, the peptide cycle first) possesses three charged amino acids leading to a distinct hydrophilic structure (logP value of −9.24) (Fig. 1) and is therefore repulsed by the lipid portion of the bilayer membrane and second) is directly linked to the fatty acid and thus could impede the fatty acid to fully insert into the membrane, even though SRE attaches to the membranes with high affinity due to its polar amino acids.

These postulations give further evidence that QCM data mainly reflect primarily the attachment of LPs to the membrane surface.

Entolysin B and orfamide A as a second group display the strongest incorporation into the membranes. Although the fatty acid chain of entolysin B is shorter than that of SRE, entolysin B has nine exocyclic amino acids, which can act as a spacer between the cycle and the fatty acid. Thus, the fatty acid is freely accessible for a membrane insertion without steric restrictions by the ring which appears to be also relevant for orfamide A.

The third group comprises SB-253514, arthrofactin and massetolide A, which displayed a median incorporation tendency. Compared to orfamide A, arthrofactin also possesses a two amino acid residue spacer which separates the relative large cycle and the fatty acid. However, the shorter fatty acid chain of arthrofactin (C_{10}) compared to orfamide A (C_{14}) refers to a changed balance, which is also true for massetolide A (C_{10}).

Despite the different structure of SB-253514, the Δp value is comparable to that of arthrofactin and massetolide A. SB-253514 bears a C_{14} fatty acid and possesses the smallest possible cycle with two amino acid residues. One can speculate that such a peptide would excellently integrate into the membrane even without a spacer part. However, SB-253514 did not reach the Δp values of entolysin B and orfamide A. It is conceivable that the sugar moiety linked to the peptide impedes the complete incorporation of the fatty acid into the membrane. However, although these structural discussions allow interpreting the membrane insertion capacities of the LPs, they do not suggest those non-targeted mechanisms as relevant contribution for bactericidal activity.

In general, the amphipathic nature of LPs favors a certain impact on the membrane barrier function. This is further supported by studies showing syringomycin E [15,37,42] and other *Pseudomonas* LPs including massetolide A [43], syringopeptins [15,37,42], syringotoxin [15,37,44], tolaasin I [19], entolysins A and B [7] and WLIP [19] to be able to lyse red blood cells and partly to permeabilize model membranes. Further SRE data suggested that membrane lysis and pore formation occured at SRE concentrations > 0.5 μM, partly even at concentrations above 40 μM [36,42,45–49]. Referring to Hutchison et al. [36] syringomycin has to exceed a threshold concentration for micelle formation to induce permanent pore formation, whereas SRE in lower concentrations reversibly inserts into the membrane. Indeed, we could also detect the inability of SRE at the low concentration of 0.5 μM to change the membrane barrier function, as all the other LPs failed to show this efficiency, but in the MIC range SRE permeabilized the membrane presumably by micelle formation.

In contrast, arthrofactin, orfamide A, and massetolide A were not able to disrupt the POPC/DOPG/ergosterol membrane at MIC relevant ranges (50 μg mL^{-1}). In the case of massetolide A it was somewhat surprising, since massetolide A is known for hemolytic activities. The cause for this discrepancy should be related to the test systems. Erythrocyte membranes are asymmetrically composed with respect to charge and possess high intrabilayer exchange dynamics [50]. In contrast, the model membranes used in this study are composed of a fixed first layer and a well-regulated second layer representing a much less dynamic system than e.g. the erythrocyte membrane that is consequently not easily to permeabilize. However, it raises the question whether SRE is able to permeabilize the POPC/DOPG/ergosterol membrane at MIC concentrations whereas the other lipopeptides did not. Regarding the SRE structure, this peptide contains a certain fraction of positively charged amino acid residues (Fig. 1) which can induce a stronger interaction with the negatively charged membrane and membrane permeabilization although this can neither be correlated to the binding kinetics nor the subphase data.

However, despite the peculiarities of the permeability assays, unspecific membrane permeabilization appears not be a dominant reason for the bactericidal activity, considering the activity spectrum in Table 2 and focusing on the dipeptide SB-253514. The dipeptide displayed the best antibiotic activities in this study, but this activity can also not be explained by a membrane permeabilization, even at high and MIC exceeding concentrations. Other lipopeptides with antibiotic activities comparable to SB-253514, e.g. MSI-843, displays significant membrane permeabilization at peptide concentration clearly beneath the MIC [51]. Consequently, other mechanisms have to be involved in the SB-253514 activity. To date, for three groups of LPs two major targets in Gram-positive microbes have been identified: the LPs plusbacin A_3 [52] and the calcium dependent friulimicin [21] interfere with the cell wall biosynthesis by inhibition of peptidoglycan-synthesis or complexation of the bactoprenol phosphate carrier C_{55}-P, respectively. In contrast to these extracellular targets, LPs of the ADEP class act intracellularly by binding to the target enzyme ClpP [53] which leads to dysregulation of the bacterial proteolytic machinery. Subtilosin A is another example for a antimicrobial peptide, for which defined target structures are postulated to participate in activity additionally to non-specific peptide-membrane interactions [54]. Whether SB-253514 acts by one of the above mentioned mechanisms or by a novel mode of action is the subject of future studies.

5. Conclusion

This study provides for the first time a wide screening of antibiotic activity for *Pseudomonas* lipopeptides. Probing the antibacterial potential of six structurally different LPs produced by Pseudomonads revealed the glycosylated lipodipeptide SB-253514 as a promising antibacterial compound, while the other tested LPs showed surprisingly no or poor antibacterial activities. Subsequent membrane model studies gave further insights and a clear indication for strong membrane activity of a series of LPs. However, these investigations could not explain the antibiotic activity of SB-253514 and the lack of activity of the other *Pseudomonas*-LPs tested. Particularly intriguing is the finding that the commonly observed unspecific cytoplasmic membrane permeabilization capabilities of LPs, which usually affect membrane integrity and cause leakage of ions from the cell, can be ruled out as antibacterial mode of action of SB-253514. This study demonstrated that SB-253514 possesses most likely a specific mode of action, acting presumably via defined target structures and provided therefore the basis for further mode of action studies.

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