The lipodepsipeptide empedopeptin inhibits cell wall biosynthesis through Ca\(^{2+}\)-dependent complex formation with peptidoglycan precursors.

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*Running title: Empedopeptin forms complexes with cell wall precursors

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**Background:** The mechanism of action of the potent antibiotic empedopeptin was not known.

**Results:** Empedopeptin forms calcium-dependent complexes with peptidoglycan precursors, particularly lipid II.

**Conclusions:** Bacterial cell wall synthesis is blocked by sequestration of the bactoprenol carrier.

**Significance:** This mechanism is proposed for a wider class of structurally related antibiotics including empedopeptin, the tripropeptins and plusbacins.

**SUMMARY**

Empedopeptin is a natural lipodepsipeptide antibiotic with potent antibacterial activity against multi-resistant Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* and penicillin-resistant *Streptococcus pneumoniae in vitro* and in animal models of bacterial infection. Here, we describe its so far elusive mechanism of antibacterial action. Empedopeptin selectively interferes with late stages of cell wall biosynthesis in intact bacterial cells as demonstrated by inhibition of N-acetyl-glucosamine incorporation into polymeric cell wall and the accumulation of the ultimate soluble peptidoglycan precursor UDP-N-acetyl-muramic acid-pentapeptide in the cytoplasm. Using membrane preparations and the complete cascade of purified, recombinant late-stage peptidoglycan biosynthetic enzymes and their respective purified substrates, we show that empedopeptin forms complexes with undecaprenyl pyrophosphate containing peptidoglycan precursors. The primary physiological target of empedopeptin is undecaprenyl-pyrophosphate-N-acetylmuramic acid-pentapeptide-N-acetyl-glucosamine (lipid II), which is readily accessible at the outside of the cell and which forms a complex with the antibiotic in a 1 : 2 molar stoichiometry. Lipid II is bound in a region that involves at least the pyrophosphate group, the first sugar, and the proximal parts of the stem peptide and undecaprenyl chain. Undecaprenyl pyrophosphate and also teichoic acid precursors are bound with lower affinity and constitute additional targets. Calcium ions are crucial for the antibacterial activity of empedopeptin, as they promote stronger interaction with its targets and with negatively charged phospholipids in the membrane. Based on the high structural similarity of empedopeptin to the tripropeptins and plusbacins, we propose this mechanism of action for the whole compound class.
INTRODUCTION

Empedopeptin is an amphoteric, cyclic lipodepsipeptide produced by the Gram-negative soil bacterium *Empedobacter haloabium* ATCC 31962. Two accompanying publications in 1984 described its isolation, structure determination and antibacterial activity *in vitro* and *in vivo* (1,2). Empedopeptin showed potent antibacterial activity against a broad range of aerobic and anaerobic Gram-positive bacteria, including the important pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Clostridium difficile*. Minimal inhibitory concentrations (MIC) in the low µg/ml range for susceptible as well as antibiotic-resistant isolates, substantial therapeutic efficacy in lethal blood-stream infections in mice, good pharmacokinetic parameters and low acute toxicity form a promising profile for the compound (1), especially in the light of rising vancomycin-resistance among staphylococci and enterococci. Therefore, we revisited empedopeptin and elucidated its molecular mechanism of action.

The overall negatively charged, water-soluble empedopeptin consists of an octapeptide core, cyclized by an ester bond, and a C14-myristic acid tail (2) (Fig. 1A). The macrolactone core comprises altering stretches of D- and L-configured amino acids (arginine, proline and serine) as well as the non-proteinogenic amino acids hydroxyaspartic acid and hydroxyproline. Empedopeptin has considerable structural similarity to the lipodepsipeptides of the triplopeptin and plusbacins group (3-7). The lower portion of the octapeptide core, which contains the three hydroxy amino acids plus arginine is actually identical in empedopeptin, the triplopeptins (A-E, Z) and plusbacins (A1-B4) (4), whereas moderate variations occur in the amino acid composition of the upper ring segment as well as the fatty acid side chain (Fig. 1B). The lipid tails occur branched or unbranched and are variable in length, the latter directly influencing antimicrobial activity. Although produced by different organisms, these lipodepsipeptides can basically be regarded as variants of the same structural scaffold.

It has been previously demonstrated that plusbacin A3 interferes with bacterial peptidoglycan biosynthesis (8), although the specific enzymatic reaction or target structure had remained elusive. While work on this publication was in progress it was reported that triplopeptin C can bind to undecaprenyl-pyrophosphate (C55-PP), thereby inhibiting the activity of undecaprenyl-pyrophosphate phosphatase (UppP) (9). Here, we show that empedopeptin exerts its antibacterial action by inhibition of peptidoglycan synthesis. However, using a comprehensive set of purified late-stage peptidoglycan synthetic enzymes and the corresponding purified substrates we demonstrate that this class of lipopeptide antibiotics sequesters further peptidoglycan precursors besides C55-PP and that the primary target of empedopeptin is lipid II, which is bound in a Ca²⁺-dependent fashion.

EXPERIMENTAL PROCEDURES

Production and purification of empedopeptin. - Production of empedopeptin by *E. haloabium* ATCC 31962 on a 3 liter scale and extraction of the broth with butanol were performed as described (1). The crude butanol extract was subjected to reversed-phase (RP) vacuum liquid chromatography, using stepwise gradient elution from H₂O/MeOH (70:30) containing increasing proportions of MeOH followed by dichloromethane. LC-MS profiling indicated the pseudomolecular ion [M+H]⁺ and [M-H]⁻ peaks at 1126.6 and 1124.6 m/z indicative of empedopeptin in the 100% MeOH fraction, which was further purified by RP-HPLC using a linear gradient of 62:38-75:25 MeOH-H₂O (2 mM NH₄OAc) over a period of 30 min (Macherey-Nagel Nucleodur 100-5 C18, 250 x 8 mm, 5 µm; 1.8 ml/min flow rate; UV monitoring at 210 nm). Rechromatography of semipure empedopeptin by RP-HPLC employing two linear gradients of 60:40-65:35 MeOH-H₂O (2 mM NH₄OAc) over a period of 15 min and 65:35-90:10 over a 5-min period, followed by isocratic elution at 90:10 for an additional 10 min (Macherey-Nagel Nucleosil 120-5 C₁₈, 250 x 4 mm, 5 µm; 0.8 ml/min flow rate; UV monitoring at 210 nm) afforded 6.2 mg of pure empedopeptin.

Antibiotic susceptibility testing. - MICs were determined by standard broth microdilution, according to the CLSI guidelines (10), in polypropylene microtiter plates (Nunc brand) using cation-adjusted Mueller-Hinton broth (Oxoid). Experiments were conducted in the presence and absence of 1.25 mM Ca²⁺, which
equates to the concentration of ionized calcium in human serum (11).

**Precursor incorporation studies.** - To study the effect of empedopeptin on the synthesis of the major bacterial biopolymers, *Bacillus subtilis* was grown in Belitzky defined minimal medium (12) and the incorporation of tritium-labeled precursors (uridine, thymidine, phenylalanine and N-acetyl-glucosamine (GlcNAc); 0.02 MBq/ml) into the acid-precipitable cell fraction was measured as described previously (13). Cultures were incubated with empedopeptin at a concentration of 8 µg/ml and with vancomycin at 0.25 µg/ml, both corresponding to 8 x MIC in this medium. Belitzky medium contains 2 mM Ca²⁺.

**Determination of the membrane potential.** - The membrane potential was determined by a previously described method (14) using the lipophilic cation tetrathenylphosphonium (TPP⁺) that diffuses across the bacterial membrane in response to a trans-negative Δψ. 1 µCi/ml of [3H]TPP⁺ (26 Ci/mmol) was added to a culture of *S. aureus* SG511 (OD 600 0.7) in half-concentrated Mueller-Hinton broth at 37°C supplemented with 1.25 mM Ca²⁺ and cell-bound radioactivity was determined after filtration. For calculation of the membrane potential, cell-associated versus free TPP⁺ concentrations were applied to the Nernst equation. Culture aliquots were treated with empedopeptin (10 µg/ml, corresponding to 10-fold MIC) and the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP, 500 µM) as a control.

**Quantification of intracellular UDP-N-acetylmuramic acid pentapeptide (UDP-MurNAc-pp).** - We followed the protocol of Kohlrausch and Hölting to analyze the cytoplasmic nucleotide precursor pool (15). *Staphylococcus aureus* ATCC 29213 was grown in 20 ml of half-concentrated Mueller-Hinton broth with 1.25 mM Ca²⁺ to an OD₆₀₀ of 0.6 and incubated with 130 µg/ml of chloramphenicol for 15 min. Chloramphenicol is necessary to prevent, under the impact of the antibiotic under investigation, induction of autolytic processes and *de novo* synthesis of enzymes hydrolyzing the nucleotide-activated sugars thereby interfering with determination of the soluble precursor (16). Empedopeptin was added at 40 µg/ml and vancomycin at 5 µg/ml (both 10 x MIC) and incubated for another 45 min. Extraction of UDP-linked peptidoglycan precursors with boiling water and their analysis by HPLC was performed as described previously (17). Mass spectrometric confirmation of UDP-MurNAc-pp was conducted using a MALDI-TOF mass spectrometer (Bruker Biflex, Bruker Daltronics, Bremen, Germany) working in a linear negative mode. An aliquot of the HPLC fraction containing UDP-MurNAc-pp was mixed with 6-aza-2-thiothymine in 50 % (v/v) ethanol/20 mM ammonium citrate as matrix material and spotted onto a ground steel MALDI target plate. Mass spectra were recorded in the range of 1000 to 1500 Da and analyzed by Flexanalysis (Bruker Daltronics).

**In vitro lipid II synthesis with membrane preparations of Micrococcus luteus.** - In vitro lipid II synthesis was performed using membranes of *M. luteus* as described (18,19). In short, for analytical assays membrane preparations (200 µg protein) were incubated in the presence of purified substrates (5 nmol undecaprenyl-phosphate (C₅₅-P), 50 nmol UDP-MurNAc-pp and 50 nmol UDP-GlcNAc) in 60 mM Tris-HCl, 5 mM MgCl₂, pH 7.5, 1.25 mM Ca²⁺ and 0.5% (w/v) Triton X-100 in a total volume of 50 µl for 1 h at 30°C. Incorporation of [¹⁴C]-UDP-GlcNAc was used to quantify lipid II synthesized. Bactoprenol containing products were extracted with an equal volume (50 µl) of butanol/pyridine acetate (2:1; vol:vol; pH 4.2) and analyzed by thin layer chromatography (TLC) using chloroform:methanol:water:ammonia (88:48:10:1; vol:vol:vol:vol) as the solvent (20) and phosphomolybdic acid staining (21). Quantification was carried out by phosphoimaging in a Storm™ imaging system (GE Healthcare). Empedopeptin (molecular weight 1126.2 Da) was added in molar ratios of 0.5-2 with respect to C₅₅-P. Synthesis and purification of lipid II on a preparative scale was performed as described (21,22).

**Synthesis of [¹⁴C]-UDP-MurNAc-peptides by *S. aureus* MurA-F and DdlA enzymes.** - [¹⁴C]-UDP-MurNAc-pp was synthesized as described (23), with modifications (24). UDP-GlcNAc (100 nmol) was incubated with 15 µg of each of the recombinant histidine-tagged muropeptide synthetases MurA to MurF and D-ala-D-ala ligase DdlA in 50 mM bis-tris-propane, pH 8, 25 mM (NH₄)₂SO₄, 5 mM MgCl₂, 5 mM KCl, 0.5 mM DTT, 2 mM ATP, 2 mM phosphoenolpyruvate, 2 mM NADPH, 1 mM of
each amino acid ([\(14\text{C}\)]-L-Ala, D-Glu, L-Lys, D-Ala) and 10% DMSO in a total volume of 125 µl for 60 min at 30°C. 31.25 µl of the reaction mixture, corresponding to 25 nmol [\(14\text{C}\)]-UDP-MurNAc-pp were used in the MraY synthesis assay without further purification. UDP-MurNAc-peptide variants with shortened stem peptide, i.e. UDP-MurNAc-dipeptide and -tripeptide, were synthesized in the presence of the corresponding subset of muropeptide synthetases and were employed for the synthesis of lipid I(dipeptide) or lipid I(tripeptide), respectively.

In vitro peptidoglycan synthesis reactions using purified proteins and substrates. - The MurG (UDP-N-acetylglucosamine: N-acetylmuramic acid(pentapeptide)-undecaprenyl-pyrophosphate N-acetylglucosamine transferase) assay was performed in a final volume of 30 µl containing 2.5 nmol purified lipid I (25) and 25 nmol [\(14\text{C}\)]-UDP-GlcNAc in 200 mM Tris-HCl, 5.7 mM MgCl₂, pH 7.5, and 0.8% Triton X-100 in the presence of 0.45 µg of purified MurG-His₆ enzyme. The reaction mixture was incubated for 60 min at 30°C. The assay for synthesis of lipid II-Gly₁ catalyzed by FemX was performed as described previously (21). In vitro transglycosylation was determined by incubating 2 nmol [\(14\text{C}\)]-lipid II in 100 mM MES, 10 mM MgCl₂, pH 5.5 in a total volume of 50 µl. The reaction was initiated by the addition of 7.5 µg PBP2-His₆ and incubated for 1.5 h at 30°C (24). In all in vitro assays empedopeptin was added in molar ratios ranging from 0.5 to 2 with respect to the amount of C₅₅-P, lipid I and lipid II, respectively. 1.25 mM Ca²⁺ was added as indicated. Synthesized lipid intermediates were extracted from the reaction mixtures with n-butanol/pyridine acetate, pH 4.2 (2:1; vol/vol), analyzed by TLC as described above and quantified by phosphoimaging. In the PBP2 assay reaction mixtures were applied directly onto the thin layer plates without extraction and were developed in solvent B (butanol-acetic acid-water-pyridine (15:3:12:10, vol/vol/vol/vol)). Lipid containing peptidoglycan precursors were detected by phosphomolybdic acid staining.

Antagonization assays. - Antagonization of the antibiotic activity of empedopeptin by potential target molecules was performed by a MIC-type assay setup in microtiter plates. Empedopeptin (8 µg/ml corresponding to 8 x MIC) in Mueller-Hinton broth with 1.25 mM Ca²⁺ was mixed with potential antagonists (UDP-GlcNAc, UDP-MurNAc-pp, C₅₅-P, farnesyl-PP, C₅₆-PP, lipid I, lipid II, lipid III and lipid I variants with shortened stem peptides) in 0.156- to 10-fold molar excess with respect to the antibiotic. B. subtilis 168 (5 x 10⁵ cfu/ml) was added and visible bacterial growth was recorded after overnight incubation. The concentration of purified peptidoglycan precursors was quantified on the basis of their phosphate content as described (26).

Quartz crystal microbalance. - Quartz crystals were prepared and cleaned as described previously (27). Supported bilayers were completed by transferring a monolayer consisting of 90 mol% POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) / 10 mol% DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol)) onto a covalently fixed C₁₆H₃₃SH-monolayer using the Langmuir-Blodgett technique. Fitting the quartz crystals into the flow chamber was performed under water to guarantee the integrity of the bilayer. Empedopeptin was investigated at 1 µM in the presence and absence of 1.25 mM CaCl₂. A LiquiLab21 quartz crystal microbalance (ifake.V., Barleben, Germany) was employed, which enables the simultaneous detection of frequency and damping changes in real time. Measurements and calculation of the kinetic binding constants were performed as described (28,29).

RESULTS

Antimicrobial activity of empedopeptin. - Empedopeptin exhibited potent antibacterial activity against multi-resistant staphylococci and...
streptococci including methicillin-resistant *S. aureus* (MRSA) and penicillin-resistant *S. pneumoniae* (PRSP) (Tab. 1). The activity against enterococci was moderate in general, but notably included vancomycin-resistant isolates. *Escherichia coli* was only marginally affected, in accordance with the lack of activity reported earlier for other Gram-negative species (1), which probably reflects insufficient penetration of the rather large (1126.2 Da), net-negatively charged compound through the outer membrane. Minimal inhibitory concentrations of empedopeptin were strongly influenced by the concentration of calcium ions in the culture medium. Addition of 1.25 mM Ca²⁺, equating the Ca²⁺-concentration in human serum (11), to standard Mueller Hinton broth reduced the MIC values two- to sixteen-fold depending on the test strain. It is important to note in this context that the calcium levels recommended by the Clinical Laboratory Standards Institute for cation adjusted Mueller-Hinton broth (20-25 mg/l Ca²⁺) (10) were not sufficient to achieve full empedopeptin activity.

**Metabolic pathway targeted by empedopeptin.**

To study the impact of the antibiotic on the biosynthetic capacity of intact bacterial cells, we followed the incorporation of radiolabeled metabolic precursors into bacterial macromolecules. Empedopeptin at 8-fold the MIC rapidly and strongly interfered with the incorporation of [³H]-GlcNAc into the cell wall of *B. subtilis*, whereas DNA and protein biosynthesis remained unaffected (Fig. 2). Incorporation of [³H]-uridine into RNA was also diminished after prolonged exposure of the cells to the antibiotic (30 min). This can be explained by the fact that empedopeptin triggered a substantial accumulation of a soluble UDP-activated peptidoglycan precursor (UDP-N-acetyl-muramic acid pentapeptide) in the bacterial cytoplasm as outlined below, thereby depleted the uridine pool for RNA synthesis. Vancomycin, an established and specific inhibitor of peptidoglycan synthesis, induced a comparable effect at 8 x MIC (Fig. 2).

Peptidoglycan synthesis takes place in two different cellular compartments. At first, several cytoplasmic enzymes assemble the soluble, activated UDP- N-acetyl-muramic acid pentapeptide (UDP-MurNAc-pp). The sugar-peptide moiety is then transferred to the membrane anchor undecaprenyl phosphate (C₅₅-P) to yield a membrane- standing monomer, which is further decorated, flipped across the membrane to the exterior and incorporated into the 3-dimensional peptidoglycan network. In order to distinguish, whether empedopeptin interferes with peptidoglycan synthesis in the cytoplasmic versus the membrane-associated phase, we determined the cytoplasmic levels of UDP-MurNAc-pp. Antibiotics that interfere with the late stages of peptidoglycan synthesis, such as vancomycin, are known to trigger an accumulation of this ultimate soluble peptidoglycan precursor in the cytoplasm. Treatment of *S. aureus* with empedopeptin (10 x MIC) led to a significant accumulation of UDP- MurNAc-pp, similar to vancomycin-treated controls at corresponding multiples of the MIC (Fig. 3 A).

The results with whole cells indicated peptidoglycan synthesis as the target pathway of empedopeptin. However, as the antibiotic structure contains a lipophilic tail of considerable length, we also investigated, whether the compound might cause additional damage to the cytoplasmic membrane. Disruption of membrane integrity results in a strong decrease of the membrane potential ΔΨ. To determine ΔΨ in the presence of empedopeptin we quantified the intracellular versus extracellular concentration of the tritium-labeled lipophilic cation tetraphenylphosphonium (TPP⁺), which diffuses freely across the bacterial membrane in response to a trans-negative ΔΨ. When growing cells of *S. aureus* were treated with the antibiotic, only a slight drop of membrane potential by 20 mV was observed followed by rapid recovery (Fig. 3B). In contrast, the addition of the uncoupling ionophor carbonyl cyanide m-chlorophenylhydrazone resulted in a substantial ΔΨ decrease of about 70 mV (Fig. 3B).

**Impact of empedopeptin on in vitro peptidoglycan biosynthesis.** The biosynthesis of the bacterial cell wall requires the coordinated interplay of a number of enzymes and their respective substrates. (For an overview on the reactions involved see Fig. 6 in the discussion section.) After completion of UDP-MurNAc-pp synthesis in the cytoplasm the membrane-bound enzyme MraY couples the sugar-peptide precursor to the lipid carrier C₅₅-P yielding lipid I (30). The transferase MurG subsequently adds GlcNAc to the muramic acid moiety of lipid I yielding lipid II.
(31). This central cell wall building block is characteristically modified in different species. In staphylococci, the peptidyltransferases FemXAB catalyze the attachment of a specific pentaglycine interpeptide bridge to the stem peptide (21,32), before the precursor is translocated across the cytoplasmic membrane (33) and incorporated into the peptidoglycan network. As our studies with intact bacterial cells had suggested an inhibitory activity of empedopeptin on membrane-associated stages of peptidoglycan synthesis, we next investigated the effect of the antibiotic in various in vitro systems capable of the respective reactions.

Membrane preparations of M. luteus catalyze the membrane-associated synthesis of lipid II by MraY and MurG in vitro, in the presence of defined amounts of the soluble precursors UDP-MurNAc-pp and UDP-GlcNAc as well as the bactoprenol carrier C55-P. In the control reaction without antibiotic C55-P was completely converted to lipid II by the sequential activity of MraY and MurG, whereas addition of empedopeptin reduced the amount of lipid II (Fig. 4A). Quantitative analysis using [14C]-UDP-GlcNAc to specifically label lipid II revealed a concentration dependent reduction of the lipid II amount. Addition of equimolar concentrations of empedopeptin with respect to C55-P resulted in a 50% decrease in extracted lipid II and a two-fold molar excess of antibiotic led to an almost complete absence of lipid II (Fig. 4B).

Target of empedopeptin and impact of Ca2+ on target interaction. - To narrow down the molecular target of empedopeptin and to elucidate the role of Ca2+ in the inhibitory process, individual peptidoglycan biosynthesis reactions were analyzed in vitro using the purified, recombinant proteins MraY, MurG, FemX and PBP2 together with their respective purified substrates in presence and absence of empedopeptin. Testing the MurG catalyzed conversion of lipid I to lipid II as well as the incorporation of the first glycine residue into lipid II by recombinant FemX and the transglycosylation reaction by PBP2, we found all three reactions to be inhibited in a concentration-dependent manner (Fig. 4, C-F). The addition of Ca2+ considerably enhanced the effect of empedopeptin and led to a complete inhibition of the reactions at a 2:1 stoichiometry of empedopeptin to lipid precursor. Approximately physiological concentrations of calcium (1 to 1.25 mM) were required for empedopeptin to exert full inhibition, as exemplified for the FemX catalyzed glycine addition (Fig. 4F).

To investigate, if the stimulatory effect was specific for calcium or would likewise be triggered by other divalent cations, the influence of Mg2+, Mn2+, Fe2+ and Zn2+ was explored in several of the in vitro systems described above. For Mg2+ we can only draw limited conclusions, since all in vitro systems depend on the presence of Mg2+ and omitting it from the assay mixtures prevented enzyme activity. Moreover, the presence of Zn2+ inhibited some enzymes, while others were more tolerant to the ion. Due to these experimental constraints we measured all enzymes in the presence of 10 mM Mg2+ and determined the empedopeptin efficacy after further addition of 1.25 mM of the particular cation under investigation. Under these conditions the cations stimulated the activity of empedopeptin in the following order: Ca2+ > Fe2+ > Zn2+ > Mn2+ > Mg2+. Calcium takes up a special position among all cations tested, because it substantially increased the efficacy of empedopeptin even in the presence of 10 mM Mg2+. Taking into account the physiological concentrations of the cations in human blood, where Fe2+, Zn2+ and Mn2+ are only available in traces, it is reasonable to presume that calcium has an important and prominent role in promoting the activity of empedopeptin against bacterial infections in the host.

It is important to note that empedopeptin inhibited the MurG, FemX and PBP2 reactions at comparable concentrations, which made a specific interference with one of the enzymes highly unlikely and pointed to an interaction with the peptidoglycan precursors instead. The three enzymes discussed so far utilize undecaprenylpyrophosphate containing peptidoglycan precursors that include also a sugar moiety. In contrast, two enzymes that use the structurally simpler C55-P as substrate were unaffected by empedopeptin, when applied in the same molar ratio, namely MraY catalyzing the synthesis of lipid I (Fig. S1, supplementary material) and TagO catalyzing the formation of undecaprenol-PP-GlcNAc (lipid III), the first membrane bound lipid intermediate of teichoic acid biosynthesis (34,35) (Fig. S2, supplementary material). Moreover,
dephosphorylation of C55-PP by UppP (36) was also not markedly inhibited at a twofold molar excess of empedopeptin over C55-PP (Fig. S3, supplementary material).

**Binding site of empedopeptin at the peptidoglycan precursors.** - The interaction of empedopeptin with different undecaprenol-bound peptidoglycan precursors was directly demonstrated by incubating each lipid precursor with the antibiotic in buffer in various molar ratios followed by analysis via thin layer chromatography (Fig. 5). Free lipid I and II were clearly visible at defined positions in the chromatogram, while free empedopeptin was not detectable by the phosphomolybdic acid staining method applied. In complex with empedopeptin, lipid I and II migrated considerably slower and remained close to the application spot. In accordance with the results obtained in the enzyme reaction assays described above, a twofold molar excess of empedopeptin was sufficient for full sequestration of the peptidoglycan precursors lipid I and II in a stable complex that even withstood solvent treatment during separation by thin layer chromatography. In contrast, even a fourfold molar excess of the antibiotic could not trap undecaprenylpyrophosphate in a stable complex under these conditions (Fig. 5). Notwithstanding, empedopeptin had some influence on the migration behavior of undecaprenylpyrophosphate during thin layer chromatography, because the lipid moved as a more focused band in the presence of the antibiotic (compare Fig. 5C lane 1 to lane 2 to 4).

In order to narrow down the region of the lipid precursors that interacts with empedopeptin, we performed antagonization assays with diverse cell wall precursors and truncated variants thereof (Tab. 2). A concentration of empedopeptin corresponding to 8 x MIC was pre-incubated with a 0.156 to 10-fold molar excess of the potentially antagonizing agents before being tested for growth inhibition of *B. subtilis* in Ca2+-supplemented (1.25 mM) culture broth. Lipid II and lipid I showed the strongest antagonizing effect on the antimicrobial activity of empedopeptin, confirming that the antibiotic is effectively trapped in a complex with the purified peptidoglycan precursors. Hence, it is no longer available for interaction with its membrane-
indicated a 2:1 binding stoichiometry of antibiotic to lipid precursors and the complex was remarkably stable even in the presence of the butanol/pyridine acetate used to develop the thin layer plates depicted in figure 5.

The presence of Ca$^{2+}$ ions, in a concentration as they occur in human serum (11), was required for full inhibitory activity of empedopeptin in our in vitro peptidoglycan synthesis assays. As several of these assay systems contained purified enzymes and substrates, it can be excluded that this stimulating effect is merely due to facilitated interaction of the antibiotic with the bacterial membrane. Rather, our data suggest that calcium markedly promotes the direct interaction of empedopeptin with its target. Empedopeptin is an amphipathic molecule with a hydrophobic region containing the myristoyl side chain and two proline residues opposite to a decidedly hydrophilic, net-negative region that includes the arginine as well as two aspartic acid residues. It is reasonable to conclude that positively charged calcium ions play a crucial role in bridging the antibiotic to the pyrophosphate region of the lipid precursors. Considering the 2:1 stoichiometry of the complex, Ca$^{2+}$ ions might also facilitate the formation of empedopeptin dimers by bridging two aspartic acid moieties. In addition, our study with target-free artificial phospholipids layers (Tab. 3) demonstrates that the interaction with negatively charged membranes is also enhanced in the presence of calcium. The strong decrease of the MIC values in media containing physiological calcium concentrations (Tab. 1) indicates that these ionic effects are critical for the antibacterial activity of empedopeptin.

Calcium ions are also involved in the interaction of other antibiotics with peptidoglycan precursors. Recently it was demonstrated that the antibiotic activity of friulimicin B is dependent on the Ca$^{2+}$-mediated complex formation with C$_{55}$-P (38). Calcium was also shown to promote the interaction of particular antibiotics carrying the mersacidin-type binding motif with their target lipid II, although the antibiotic activity was only slightly enhanced in these cases (39).

Our phospholipid binding study demonstrated high affinity of empedopeptin for negatively charged membranes per se. The electric currents detected by cyclic voltammetry on model membranes even pointed to membrane perturbations upon empedopeptin binding. The effects detected in this model membrane system correlated with a slight drop of the membrane potential of bacterial cells after empedopeptin addition. However, the effect on $\Delta \Psi$ was only temporary and there was no indication for a significant disturbance of membrane integrity. Rather, this effect suggests an intimate interaction of the lipophilic antibiotic with the cytoplasmic membrane in accordance with its membrane standing targets. Along the same line, the continuation of DNA, RNA and protein synthesis in the presence of empedopeptin precludes a gross depolarization of the cytoplasmic membrane. These biosynthetic processes are highly energy consuming and require constant ATP replenishment by oxidative phosphorylation. Also, the fact that UDP-MurNAc-pp accumulated in the cytoplasm indicates a functional membrane barrier, because the precursor was retained inside the cells.

In antagonization assays very low amounts of externally added lipid I or lipid II were sufficient to fully complex free empedopeptin and to prevent its interaction with bacterial cells (Tab. 2). Among the different peptidoglycan precursors tested, lipid II and lipid I were equally well bound by the antibiotic indicating that the second sugar GlcNAc is not crucial for the interaction. The C-terminal region of the stem peptide containing the L-lysyl-D-alanyl-D-alanine moiety does also not significantly contribute to binding, as obvious from the comparison of lipid I with lipid I(dipeptide). This result highlights that the target site of empedopeptin differs from that of the glycopeptide antibiotics (40) and is in accordance with the activity of empedopeptin against vancomycin-resistant bacterial isolates. However, if the cell wall precursor lacks the whole stem peptide and the lactyl moiety of MurNAc, as it occurs in lipid III, its potential for complex formation is fourfold lower than that of lipid I or lipid II. Undecaprenyl pyrophosphate as such is also recognized by empedopeptin, but the absence of the GlcNAc moiety further reduced the affinity by a factor of two. It is also noteworthy that in a similar antagonization study with tripropeptin C$_{20}$-PP showed stronger antagonizing potential than C$_{15}$-PP (9), suggesting that the undecaprenyl chain does also take part in the interaction. In summary, the interaction of empedopeptin with lipid II
appears to involve the pyrophosphate group, the first sugar, and the first one or two amino acids of the stem peptide as well as the proximal part of the undecaprenyl chain (Fig. 6).

Due to its comparably large size empedopeptin will probably not enter the bacterial cell. Whether it can flip across the membrane to interact with lipid I that resides within the inner phospholipid leaflet is questionable and remains to be studied. Therefore, the surface exposed lipid II appears as the likely physiological target of empedopeptin. Since lipid II is bound with high affinity, its sequestration at low antibiotic concentrations and concomitant inhibition of peptidoglycan biosynthesis is probably the primary killing event. As shown for a variety of other antibacterial agents among them lipopeptides, lantibiotics and defensins (25, 41), lipid II binding is sufficient to prevent the synthesis of novel peptidoglycan followed by bacterial cell lysis and death. Bacterial cells exposed to a lipid II binder do not merely suffer from inhibition of the transglycosylation step in peptidoglycan synthesis, but from interruption of the lipid cycle and general depletion of the undecaprenyl pool. Depletion of free bactoprenol inhibits several reactions in peptidoglycan synthesis and also the membrane-associated stages of teichoic acid and capsule biosynthesis, which also rely on C_{55}-P as carrier.

However, empedopeptin differs from several other lipid II binders described to date (e.g. mersacidin and plectasin) (18, 25)) by its affinity for additional bactoprenol-containing precursors that appear outside the cell. C_{55}-PP can be sequestered, as probably can be the lipid III structural motif within extracellular teichoic acid precursors (e.g. within C_{55}-PP-GlcNAc-N-acetylmannosamine-(glycerophosphate)$_{12}$- (ribitolphosphate)$_{10-40}$ in S. aureus) (42). Among these structures empedopeptin has the highest affinity for lipid II, which we consider its primary and main interaction site. However, if bacterial cells are exposed to sufficiently high empedopeptin concentrations, the antibiotic will probably recognize the other structures as well, thereby trapping the bactoprenol carrier at more than one stage in parallel. As interaction with multiple targets is beneficial in the light of reduced resistance development (43, 44) this characteristic is an advantage of empedopeptin.

While this work was in progress a report on the mechanism of action of tripropeptin C was published, proposing C_{55}-PP as the primary target and its dephosphorylation as the target reaction (9). The authors based their conclusion on biosynthesis reactions with bacterial membrane fractions and a MIC-type antagonization assay with externally added C_{55}-PP. Similar to our results they found that the activity of tripropeptin C was antagonized by a comparably high molar excess of C_{55}-PP. However, neither the purified lipids I, II, or III nor purified peptidoglycan biosynthetic enzymes were employed in this study. As a consequence, the assay systems applied did not allow for measuring the affinity of tripropeptin C for the full spectrum of peptidoglycan precursors or to investigate individual enzyme reactions separately in the absence of other interfering precursors and enzymes. On the basis of the clear results obtained in our defined systems and in the light of the strong structural similarity between empedopeptin, the tripropeptins and the plusbacins (Fig. 1) we propose that complex formation with lipid II as primary target and with C_{55}-PP and the lipid III moiety as secondary targets represents the mechanism of antibacterial action for this entire class of compounds.

ACKNOWLEDGEMENTS

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Reference List


ABBREVIATIONS:
The abbreviations used are: CCCP, carbonyl cyanide m-chlorophenylhydrazone; C55-P, undecaprenyl-phosphate; C55-PP, undecaprenyl-pyrophosphate; DOPG, 1,2-dioleoyl-sn-glycerol-3-phospho-(1′-rac-glycerol; EMP, empedopeptin; GlcNAc, N-acetyl-glucosamine; lipid I, undecaprenyl-pyrophosphate-N-acetyl-muramic acid(pentapeptide); lipid II, undecaprenyl-pyrophosphate-N-acetyl-muramic acid (pentapeptide)-N-acetyl-glucosamine; lipid II-Gly1, undecaprenyl-pyrophosphate-N-acetyl-muramic acid (pentapeptide-glycine)-N-acetyl-glucosamine; lipid III, undecaprenyl-PP-GlcNAc; MIC, minimal inhibitory concentration; MRSA, methicillin-resistant Staphylococcus aureus; OD, optical density; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; PRSP, penicillin-resistant Streptococcus pneumoniae; QCM, quartz crystal microbalance; RP, reversed-phase; TLC, thin layer chromatography; TPP+, tetraphenylphosphonium; UDP-MurNAc-pp, UDP-N-acetyl-muramic acid pentapeptide; VRE, vancomycin-resistant Enterococcus.

FIGURE LEGENDS

Figure 1: Structure of the lipodepsipeptide antibiotic empedopeptin (A) and comparison with the tripropeptins and plusbacins (B). Hy-Pro, hydroxy-proline; Hy-Asp, hydroxyl aspartic acid.

Figure 2: Impact of empedopeptin on macromolecular biosyntheses of B. subtilis 168. Untreated (triangle), empedopeptin-treated (squares) or vancomycin-treated (open circles) cells (both 8 x MIC) were incubated with tritium-labeled precursors to monitor their incorporation into (A) proteins, (B) DNA, (C) RNA and (D) cell wall. [3H]-phenylalanine was used to quantify protein synthesis, [3H]-thymidine for DNA synthesis, [3H]-uridine for RNA-synthesis, and [3H]-GlcNAc for cell wall synthesis.

Figure 3: Intracellular accumulation of the soluble cell wall precursor UDP-MurNAc-pentapeptide in S. aureus ATCC 29213 (A). Cells were treated with empedopeptin (solid line), vancomycin (dotted line), both at 10-fold MIC, or left untreated (dashed line). The intracellular nucleotide pool was analyzed after extraction. HPLC analysis and subsequent mass spectrometry (inserted small panel) confirmed the identity of UDP-MurNAc-pp. Its monoisotopic mass is 1149.35. In addition to the singly charged ion, the mono- and disodium salts were detected. Influence of empedopeptin on the membrane potential of S. aureus SG511 (B). ΔΨ was calculated from the distribution of the lipophilic cation tetraphenylphosphonium (TPP+) inside and outside the cells. The arrow indicates the time of antibiotic or CCCP addition, respectively. Empedopeptin (10 x MIC, triangle); CCCP (500 µM, squares); control (diamonds).

Figure 4: Effect of empedopeptin on membrane-associated stages of peptidoglycan synthesis in vitro. In vitro lipid II synthesis catalyzed by M. luteus membrane preparations (A, B). (A) Thin layer chromatography of n-butanol/pyridine acetate extracts and detection by phosphomolybdic acid staining. (B) Quantification of synthesized lipid II in n-butanol/pyridine acetate extracts of the membrane preparation via incorporated [14C]-UDP-GlcNAc and phosphoimaging. (C, D, E, F) Reactions catalyzed by purified enzymes from purified substrates. (C) Lipid II synthesis from lipid I by MurG quantified via incorporated [14C]-UDP-GlcNAc and (D, F) lipid II-Gly1 synthesis from lipid II by FemX quantified via incorporated [14C]-glycine. (E) The transglycosylation reaction catalyzed by PBP2 was quantified by the reduction of free [14C]-lipid II. (C, D, E) Inhibitory activity of empedopeptin in the absence (gray bars) or presence (white bars) of 1.25 mM Ca2+ or (F) in the presence of a calcium gradient. The FemX reaction was chosen as an exemplary system to probe the effect of a calcium concentration range. For all reactions the amount of products synthesized by control reactions in the absence of antibiotic was set to 100%. Empedopeptin was added at molar ratios of 0.5:1, 1:1, and 2:1 with respect to the lipid substrates as indicated. In panel F the molar ratio of lipid II:empedopeptin was 1:2. The lantibiotic nisin in a twofold
molar excess served as a control. Mean values and standard deviations from three independent experiments are shown. EMP, empedopeptin; LI, lipid I; LII, lipid II.

Figure 5: Complex formation of empedopeptin with the peptidoglycan precursors lipid I, lipid II and C₅₅-PP and estimation of binding stoichiometry. Lipid I (A), lipid II (B) and C₅₅-PP (C) were incubated with empedopeptin (EMP) at indicated molar ratios in the presence of 1.25 mM calcium. The stable complex of empedopeptin with lipid I or II remains close to the application spot, whereas free lipids migrate to the indicated positions. C₅₅-PP was not trapped in a complex up to a fourfold molar excess of the antibiotic. Rf(lipid I) 0.33, Rf(lipid I/EMP complex) 0.17, Rf(lipid II) 0.3, Rf(lipid II/EMP complex) 0.16, Rf(C₅₅-PP) 0.37; EMP, empedopeptin.

Figure 6: Overview of peptidoglycan biosynthesis in S. aureus and schematic model for the mechanism of action of empedopeptin. Starting from UDP-GlcNAc the ultimate soluble precursor UDP-MurNAc is synthesized in the cytoplasm by the sequential action of MurA to MurF enzymes. Lipid I is formed in the first membrane-associated step at the inner face of the membrane by MraY, which transfers the soluble sugar-peptide moiety to the lipid carrier C₅₅-P. The transferase MurG subsequently links a GlcNAc moiety to the muramoyl portion of lipid I, yielding lipid II. In S. aureus lipid II is further decorated by the addition of five glycine residues, catalyzed by FemXAB enzymes. Finally, lipid II is translocated across the membrane and the peptidoglycan-monomer is incorporated into the growing peptidoglycan network by transglycosylation and transpeptidation. Once the lipid II monomer appears at the outside of the cytoplasmic membrane, empedopeptin binds to it in a Ca²⁺-dependent manner at a region involving the pyrophosphate group, the first sugar and the proximal parts of the stem peptide and the undecaprenyl chain. Dimerization of empedopeptin may occur in the course of this process. C₅₅-PP is also bound at the outer leaflet of the cytoplasmic membrane, but with lower affinity than lipid II. Size, shape and positioning of all components are schematic and not based on structural data. PBPs, penicillin binding proteins.
Table 1: Antibacterial activity of empedopeptin against representative bacterial strains in the absence and presence of 1.25 mM Ca$^{2+}$. MRSA, methicillin-resistant *Staphylococcus aureus*; PRSP, penicillin-resistant *Streptococcus pneumoniae*; VRE, vancomycin-resistant *Enterococcus*.

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>MIC (µg/ml) without Ca$^{2+}$</th>
<th>+ 1.25 mM Ca$^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus ATCC 29213</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Staphylococcus aureus N315 (MRSA)</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Staphylococcus aureus SG 511</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Staphylococcus simulans 22</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus pneumoniae DSM 11865 (PRSP)</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Streptococcus pyogenes ATCC 10389</td>
<td>1</td>
<td>0.25</td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Enterococcus faecium BM4147 (VRE)</td>
<td>32</td>
<td>16</td>
</tr>
<tr>
<td>Bacillus subtilis 168</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Micrococcus luteus DSM 1790</td>
<td>0.125</td>
<td>&lt; 0.062</td>
</tr>
<tr>
<td><em>Escherichia coli</em> W3110</td>
<td>&gt; 64</td>
<td>&gt; 64</td>
</tr>
</tbody>
</table>
Table 2: Antagonistic effect of peptidoglycan and wall teichoic acid precursors and truncated variants thereof on the antimicrobial activity of empedopeptin. Empedopeptin at 8 x MIC was exposed to the potentially antagonizing agents in the indicated molar ratios prior to mixture with *B. subtilis* in culture broth containing 1.25 mM Ca$^{2+}$. Results of two independent experiments are summarized. +, antagonization; -, no antagonization.

<table>
<thead>
<tr>
<th>lipid intermediate</th>
<th>molar ratio of precursor to empedopeptin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 x</td>
</tr>
<tr>
<td>UDP-GlcNAc</td>
<td>-</td>
</tr>
<tr>
<td>UDP-MurNAc-pp</td>
<td>-</td>
</tr>
<tr>
<td>C$_{55}$-P</td>
<td>-</td>
</tr>
<tr>
<td>C$_{15}$-PP</td>
<td>+</td>
</tr>
<tr>
<td>C$_{55}$-PP</td>
<td>+</td>
</tr>
<tr>
<td>lipid I- (dipeptide)</td>
<td>+</td>
</tr>
<tr>
<td>lipid I- (tripeptide)</td>
<td>+</td>
</tr>
<tr>
<td>lipid I- (pentapeptide)</td>
<td>+</td>
</tr>
<tr>
<td>lipid II</td>
<td>+</td>
</tr>
<tr>
<td>lipid III</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 3: Binding affinity of empedopeptin to phospholipid model membranes. Association rate ($k_{ass}$), dissociation rate ($k_{diss}$), and overall binding affinity ($k_D$) of empedopeptin as determined by the QCM technique either in the absence or presence of 1.25 mM CaCl$_2$.

<table>
<thead>
<tr>
<th></th>
<th>$k_{ass}$ [M$^{-1}$ s$^{-1}$]</th>
<th>$k_{diss}$ [s$^{-1}$]</th>
<th>$k_D$ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>without Ca$^{2+}$</td>
<td>2835 ± 507</td>
<td>2.10 x 10$^{-3}$ ± 0.86 x 10$^{-3}$</td>
<td>0.77 ± 0.41</td>
</tr>
<tr>
<td>1.25 mM Ca$^{2+}$</td>
<td>4736 ± 1262</td>
<td>1.47 x 10$^{-3}$ ± 0.70 x 10$^{-3}$</td>
<td>0.30 ± 0.11</td>
</tr>
</tbody>
</table>
Fig. 2

A. Protein

B. DNA

C. RNA

D. Cell wall

- ▲ untreated control
- ■ empedopeptin-treated (8 x MIC)
- ○ vancomycin-treated (8 x MIC)
Fig. 5

A

lipid I

lipid I: empedopeptin complex

lipid II

lipid II: empedopeptin complex

B

lipid II

lipid II: empedopeptin complex

C

C_{55}-PP

C_{55}-PP: EMP

C_{55}-PP

C_{55}-PP: EMP
The lipodepsipeptide empedopeptin inhibits cell wall biosynthesis through Ca2+-dependent complex formation with peptidoglycan precursors. Anna Müller, Daniela Münch, Yvonne Schmidt, Katrin Reder-Christ, Guido Schiffer, Gerd Bendas, Harald Gross, Hans-Georg Sahl, Tanja Schneider and Heike Brötz-Oesterhelt

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