# Studies on Two Compound Classes from Actinobacteria Exhibiting New Antibacterial Mechanisms of Action:

# Chelocardins and Telomycins

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meinen Großvater Walter

# ZUSAMMENFASSUNG

Diese Arbeit enthält Studien zweier bekannter, aber noch wenig untersuchter mikrobieller Naturstoffklassen hinsichtlich ihres Wirk- und Resistenzmechanismus, sowie ihrer Struktur-Aktivitäts-Beziehungen.

Chelocardin und sein amidiertes Derivat wurden als bakterizide Substanzen mit Wirksamkeit gegen ein weites Spektrum von Bakterienspezies und Resistenz-brechenden („resistance-breaking“) Eigenschaften charakterisiert. Es wurde gezeigt, dass Chelocardine einen Einfluss auf bakterielle Membranen ausüben und dass der Resistenzmechanismus gegenüber Chelocardin auf Efflux-Mechanismen beruht, die durch Mutationen im Repressorprotein RamR vermittelt werden.

Das Antibiotikum Telomycin wurde als stark bakterizid und Calcium-abhängig mit Wirksamkeit gegen Gram-positive Bakterien charakterisiert. Zwei semisynthetische Derivate zeigten eine verbesserte und Calcium-unabhängige antibakterielle Aktivität. Weiter wurde gezeigt, dass die Verbindungen in erster Linie mit der Zellmembran interagieren und dass Cardiolipin insbesondere für Telomycin einen wichtigen Bindepartner darstellt. Die Aktivität beider Derivate beruht nicht ausschließlich auf einer Bindung an Cardiolipin, was auf eine Interaktion mit weiteren Targetmolekülen hindeutet und einen Wirkmechanismus jenseits der Membranaktivität – auch für Telomycin – denkbar macht. Zudem wurden potentielle Proteintargets untersucht, jedoch konnte keine eindeutige Schlussfolgerung auf den Wirkmechanismus gezogen werden.

# ABSTRACT

Within this thesis, two known but rather underexploited microbial natural product classes were characterized in terms of their mechanisms of action, their resistance mechanisms and their structure-activity-relationships.

Chelocardin and its amidated analogue were shown to act bactericidal on a broad-spectrum of bacterial species including resistant isolates. Both molecules were shown to exert a cell membrane-based mechanism and the resistance mechanism of chelocardin was identified and characterized to rely on efflux mediated by mutations of the repressor protein RamR.

Telomycin was characterised to act strongly bactericidal on Gram-positive bacteria in a calcium-dependent manner. Two acylated derivatives were shown to exhibit an improved and calcium-independent activity pattern compared to the parent molecule. Studies as part of this thesis led to the assumption that the compounds interfere mainly with the cellular membrane and cardiolipin was characterised as main interaction partner, particularly for telomycin. However, the antibacterial activity of both acylated derivatives does not exclusively rely on a binding to this phospholipid. This hints towards interaction with additional target molecules and a mechanism of action beyond membrane activity, which might also hold true for telomycin itself. Potential protein targets were studied but the mechanism of action of telomycins could not be elucidated in detail.

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# 1 INTRODUCTION

## 1.1 EMERGENCE OF ANTIMICROBIAL RESISTANCE

The introduction of antibacterial agents to treat bacterial infections goes back to the early 1900s. A milestone was the discovery of penicillin from the fungus *Penicillium notatum* by Alexander Fleming in 1929.[1,2] The introduction of penicillin in the clinics is an integral part of the "Golden Era" of antibiotic discovery (1930s-1960s), which resulted in the development of more than 150 different types of antibiotic compounds[1]. However, the vast advent of new antibiotics implicated a concurrent emergence of antibiotic resistance (Figure 1-1).[1] Soon after the introduction of penicillin as antibiotic drug in the 1940s, first cases of antibiotic resistance were observed[1,3,4] – still until today a severe problem that accompanies not only the use of antibacterial agents but antimicrobial drugs in general.[5] In this context it is worth mentioning that already in 1945, Alexander Fleming declared a warning that resistant bacteria will appear if antibiotics are misused in terms of incorrect dosage.[6]

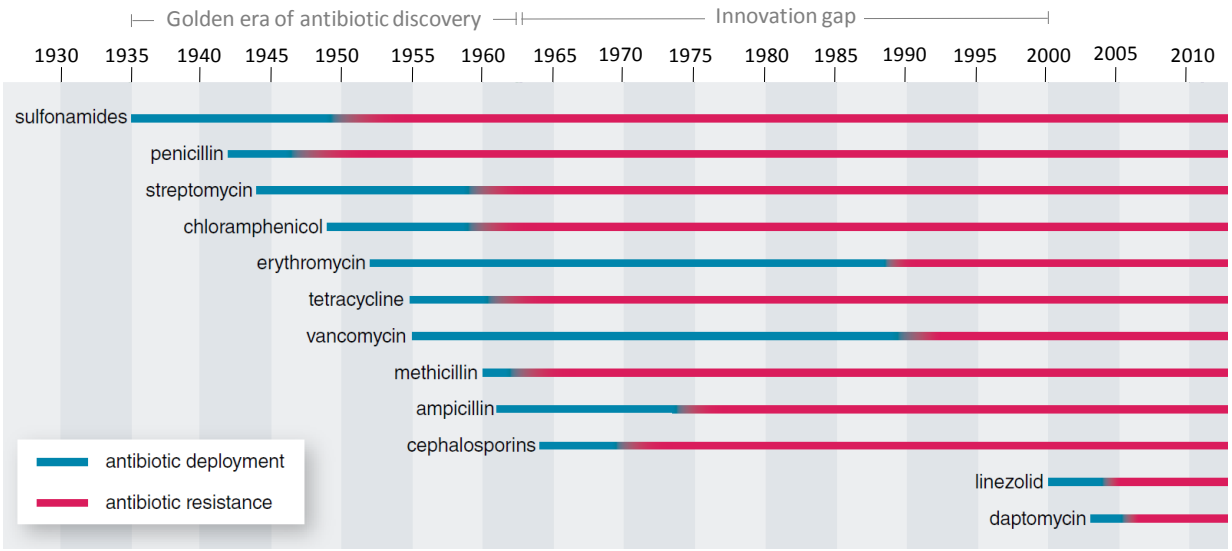



**Figure 1-1:** Timeline of the deployment of antibiotic agent and the development of resistance towards it. Adapted from Dantas and Sommer[1]

The development of antibiotic resistance has been described among all clinically important compound classes (e.g.: tetracyclines, glycopeptides)[1,7–9] and is spread among Gram-positive and Gram-negative bacteria. The most severe and hard-to-treat pathogens are grouped as so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.).[10–12] They depict a tremendous threat for the human population, since

they cause serious nosocomial infections and their treatment is hampered due to their ability of "escaping" the effects of clinical used antibiotics.[13–15] Moreover, an ongoing decline in the development of effective drugs contributes severely to the failure in the treatment of resistant pathogens.[16] This drop was already loomed in the mid-sixties by the "innovation gap" in the development of new effective drugs, a gap which lasted until the start of the new millennium (Figure 1-1) and we are now facing a "pre-antibiotic" era.[17–19] Until today, the development and approval of new antibacterial agents is only slowly progressing and it is accompanied by a permanent increase of the number and types of resistant bacteria (Figure 1-2)[5,8,20]. This problem is accompanied by the prevalence of multidrug-resistant (MDR) pathogens, which are described among Gram-positive and Gram-negative bacteria belonging to the ESAKPE panel. The most severe pathogens are methicillin-resistant *Staphyloccocus aureus* (MRSA), vancomycin-resistant Enterococci (VRE), Enterobacteriaceae (e.g. *Klebsiella pneumoniae*) and *Pseudomonas aeruginosa*.[8,21]

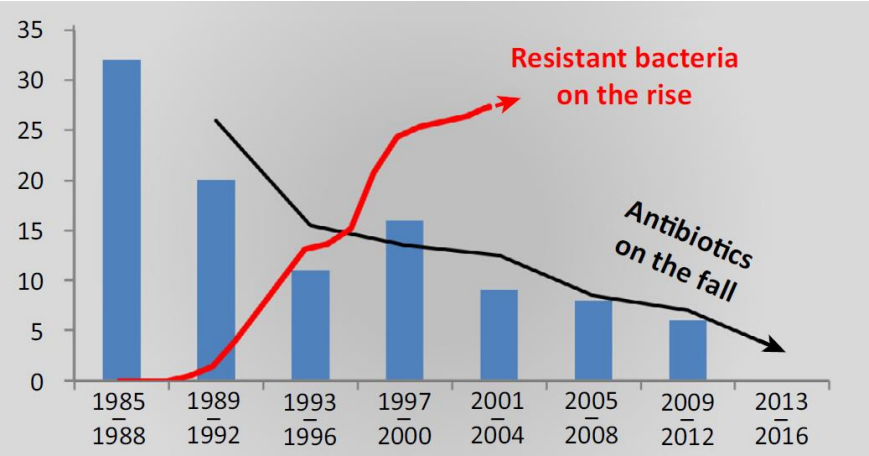



**Figure 1-2:** Representation of the permanent decrease in the development of new antibiotics (black arrow) and concurrent increase of resistant bacteria (red arrow; vancomycin-resistant bacteria as example). Adapted from Schäberle et al.[20]

The development of antibacterial resistance relies on several factors that emanate from humans, the bacteria and the antibiotic itself.[22–24] The rise of new MDR pathogens and the spread within health care units and into the community depicts a global problem and needs to be kept down by better hygiene standards and safety precautions for caretakers and patients.[5,25–27] Additionally, the overuse and incongruous prescription of antibiotics is a serious problem that contributes to the development of resistance.[28–30] Numerous studies point towards a direct connection of the application of antibiotics and the emergence of resistant pathogens in hospitals.[30,31] Moreover, the outstanding misuse of antibiotics in

agriculture (preventative or to promote growth) is as alarming. Resistant bacteria can be spread *via* direct contact of humans and animals (*via* farmers) or through the food chain during food preparation.[32] In general, antibiotic resistance refers to different protection mechanisms which are carried out by bacteria to adapt towards external stress.[33] It can rely on intrinsic factors such as the presence of resistance genes[34] within the bacteria's genome or resistance can be acquired. For instance, the spread of resistance determinants by horizontal gene transfer (HGT; e.g. transformation, conjugation or transduction) between different bacterial species contributes to acquired resistance properties.[29,33] This way of resistance spread is particularly problematic since it also occurs between unrelated bacteria genera like for instance between Gram-positive and Gram-negative bacteria.[35] One example is the appearance of *ermC* in Enterobacteriaceae (e.g. *E. coli*), a gene involved in resistance to macrolide, lincosamide and streptogramin (MLS) antibiotics (e.g. erythromycin) which was originally described in *Staphylococcus* species.[35,36] Moreover, bacteria can produce detoxifying enzymes that lead to an inactivation of the molecule and thus, loss of its antibacterial function. Those enzymes include β-lactamases or extended-spectrum β-lactamases (ESBL), which are mostly produced by Gram-negative bacteria and which mediate the hydrolysis of the β-lactam ring of β-lactam antibiotics, such as penicillins or carbapenemes.[37,38] Moreover, transferases are able to modify the molecule's structure (e.g. addition of acyl groups) to prevent its binding to the target molecule.[39,40] These enzymes include for instance phosphotransferases or acetyltransferases which are involved in modifying aminoglycosides such as streptomycin.[39] Another way of bacteria to protect themselves against antibacterial agents relies on modifications of the target molecule. Structural alterations contribute to resistance in a way, that the antibiotic is unable to bind to its target and to perform its antibacterial mechanism.[33,41] For instance, changes of the cell wall composition such as alterations of the peptidoglycan or mutations within penicillin-binding proteins (PBPs) lead to resistance of cell wall targeting compounds (e.g. glycopeptides).[33,40] Since target mutations involve modifications of important cellular processes, which often introduces a fitness cost for the bacterium, sometimes additional cellular changes are required to compensate for the acquired modifications and thereof relying altered functions.[40] For instance, the β-lactam antibiotic methicillin inhibits cell wall synthesis by binding to PBPs and thus, inhibiting their function. To compensate the loss in functional PBPs, MRSA bears the resistant determinant *mecA* which encode PBP2a, an alternative PBP which has only low affinity to β-lactams.[42–44] Due to additional alterations,

PBP2a is able to maintain the cell wall synthesis and resist the antibacterial effect of methicillin.[40,42,45]

The most frequently occurring resistance mechanisms are mediated by efflux of the compounds or by alterations of the composition of the outer membrane (OM) of Gram-negative bacteria.[22,23] Resistance through efflux relies on the (over-)expression of efflux systems, which are distributed among all clinically relevant bacteria including for instance *S. aureus* (e.g. MepA), *P. aeruginosa*:(e.g. MexAB-OprM) and *E. coli*: (e.g. AcrAB-TolC).[33,46,47] On the one hand, efflux pumps may act drug-specific (e.g. tetracycline resistance through pumps encoded by *tet* genes[48,49]). However, on the other hand classes of efflux pumps exist [e.g. major facilitator superfamily (MFS), family, multidrug and toxic compound extrusion (MATE) family, resistance-nodulation-division (RND) family] which are able to export a broad variety of structurally unrelated antimicrobial compounds, which contributes severely to an MDR phenotype.[40,46,50,51] Additionally, the OM of Gram-negative bacteria acts as an additional barrier to many commonly used antibacterial drugs (e.g. hydrophobic antibiotics) and contributes to impaired effectiveness due to hindered uptake.[52–54] However, small molecules (molecular weight < 600 Da[55]) are able to enter the cell through porins, by diffusing through the lipid bilayer or by binding to lipopolysaccharide (LPS; self-mediated uptake). Nonetheless, a change or re-arrangement of structural components of the OM hampers the uptake of these compounds.[22,56] In addition, even if a molecule is able to traverse the OM (e.g. through porins), efflux pumps counteract this process by pumping out the compounds before it is able to proceed its antibacterial action in many cases [40]; a process that severely adds to an MDR phenotype as well. Moreover, different resistance mechanisms are often mediated simultaneously[56] (e.g. increased efflux and altered OM) which complicates the treatment and underlines the urgent need of new antibacterial agents with new mechanisms of action an even new target molecules.[21] In general, the treatment of Gram-negative bacteria is *per se* more challenging due to the presence of the OM. In contrast, the OM is absent in Gram-positive bacteria what implicates a somewhat easier treatment approach for this type of bacteria. Nevertheless, the appearance of MDR Gram-positive bacteria is as alarming, especially since *S. aureus* (incl. MRSA) depicts on of the most threating pathogens among those causing hospital- and community-acquired infections.[15,57] Vancomycin (VAN) is used as first-line treatment against infections caused by MRSA and also against other Gram-positive bacteria including *Enterococcus* spp.. However, the emergence of reduced susceptibility towards vancomycin in staphylococcal and enterococcal pathogens [VRE, vancomycin-resistant *S. aureus* (VRSA) and vancomycin

intermediate *S. aureus* (VISA)][8,57,58] depicts a major threat in the treatment of nosocomial infections. Resistance of VISA and VRSA relies mainly on the presence of the resistance gene *vanA*, a gene that was originally described in VRE.[59–62] This furthermore underlines the ongoing risk of HGT and its severe contribution to MDR. Over the last years, only limited alternatives have been provided, among which the lipopeptide antibiotic daptomycin (DAP) depicts the last-resort treatment against MDR Gram-positive pathogens.[63] However, as suspected, the appearance of DAP-resistant bacteria was described shortly after its introduction into the clinics (Figure 1-1) and resistance is spread among different species of Gram-positive bacteria (*S. aureus*, *Enterococcus* spp.).[64,65] Regarding infections caused by MDR Gram-negative bacteria, effective drugs are likewise limited, especially due to the appearance of resistance against last-resort antibiotics which are characterized by them causing severe side-effects (e.g. colistin). Nevertheless, the treatment of infections caused by carbapenemase-producing MDR Gram-negative species relies mainly on the application of the polypeptide antibiotic colistin.[66] However, colistin-resistance has already been described and the risk of a spread among different species is worrisome due to recent studies describing plasmid-mediated colistin-resistance (*mcr-1* gene).[67,68]

These facts, with a special consideration of the severe increase of antimicrobial resistance (AMR), emphasize on the urgent need of alternative treatment schemes.[5] Only recently, the World Health Organization (WHO) published a priority list of pathogens, to guide the research and development (R&D) of new effective antibacterial compounds.[69] The list comprises 12 pathogens for which new antibacterial agents are urgently needed and aims to globally prioritize R&D of new treatments against those pathogens. Based on the species and type of resistance, the pathogens are grouped into critical, high and medium priority, and comprise all clinical relevant pathogens. The primarily focus (critical prioritized pathogens, Table 1-1) lies on Gram-negative pathogens (e.g. carbapenem-resistant Enterobacteriaceae), followed by MDR Gram-positive bacteria (highly prioritized; e.g. MRSA) and less severe Gram-negative species such as *Helicobacter* spp. or *Salmonella* spp.[69] In conclusion, due to the wide spread of AMR among all bacterial species, antibacterial agents with a novel mechanism of action and new target molecules need to be provided.[5,21]

**Table 1-1:** Extract of the global priority list of pathogens published by the WHO.[69]

| Priority 1: Critical | Type of resistance |
|---|---|
| *Acinetobacter baumannii* | Carbapenem-resistant |
| *Pseudomonas aeruginosa* | Carbapenem-resistant |
| *Enterobacteriaceae** | Carbapenem-resistant, 3rd generation cephalosporin-resistant |

**K. pneumonia, E. coli, Enterobacter* spp., *Serratia* spp., *Proteus* spp., *Providencia* spp., *Morganella* spp.

## 1.2 SOURCES OF NEW ANTIBIOTICS

Not only since the "Golden Era" of antibiotic discovery, but already since centuries, major sources for the discovery and development of new pharmaceuticals are natural products (NPs). They comprise compounds exhibiting various biological activities derived from natural sources such as plants and microorganisms, including bacteria and fungi.[70–72] Ancient records of the medical use of herbal extracts demonstrate the importance of NPs as sources for active compounds since the advent of medicine.[70,73,74] The multiplicity of today's drugs derived from NPs and their use in a wide range of medical applications, such as anticancer, antitumor or antidiabetic therapy, underpins the importance of NPs in disease control.[72] Accounting not only NP and NP-derived drugs (26 %), but also NP-inspired drugs (synthetic compounds mimicking NPs or with NP-pharmacophore), more than 50 % of all new chemical entities (NCEs; 1562) approved by the Food and Drug Administration (FDA) between 1981 to 2014 are NP-like.[72] Additionally, one of the most important properties of NPs is their antibacterial effect. Until today, the percentage of antibacterial effective small molecules derived from nature constitute around 74 % of all approved antibacterial small molecules.[72] Interestingly, those compounds are mostly derived from soil living bacteria.[70,72] The beneficial effects of these secondary metabolites produced by bacteria have already been discovered long before the existence of the actual active metabolites has been proven. One example depicts ancient reports of using red soil from the Hashemite Kingdom of Jordan to treat skin infections.[75] Those applications already pointed towards the importance of soil-dwelling bacteria producing potent antibacterial compounds. Indeed, recent studies confirmed the effectiveness of red soil samples against *S. aureus* and *Micrococcus luteus* and identified several known antibiotic producer strains within the soil samples.[75] They comprise *Lysobacter* spp., *Bacillus* spp. and actinomycetes, with the latter one being the most studied and successful order of bacteria producing potent biologically active NPs.[76,77] Actinomycetes are Gram-positive bacteria and the order comprises - amongst others - species of the genera *Amycolatopsis* and *Streptomyces*, whereas *Streptomyces* is the most important genus with regards to antibiotic production.[78,79] Already 70 years ago, the first

antibiotic from *Streptomyces* (streptothricin) was described and the breakthrough of this genus as important source for new antibiotics was impelled by the isolation of streptomycin from *Streptomyces griseus* in 1944.[78,80] The aminoglycoside streptomycin was the first antibiotic effective against tuberculosis and is still used as second-line anti-tuberculosis drug.[81] Up to 80 % of contemporary used clinically relevant drugs are derived from *Streptomyces*.[77,78] Not only this, but also the wide diversity of structural and mechanistic features[77] - including all typical antibiotic mechanisms (inhibition of RNA, DNA, protein or cell wall synthesis; Figure 1-3) - illustrates the importance of this genus in the development of antibiotics. Examples for clinically used NPs and NP-derived drugs are DAP produced by *S. roseosporus*[82], VAN produced by *S. orientalis*[83] or tetracycline (TET) produced by *S. rimosus* and *S. aureofacines*.[49]

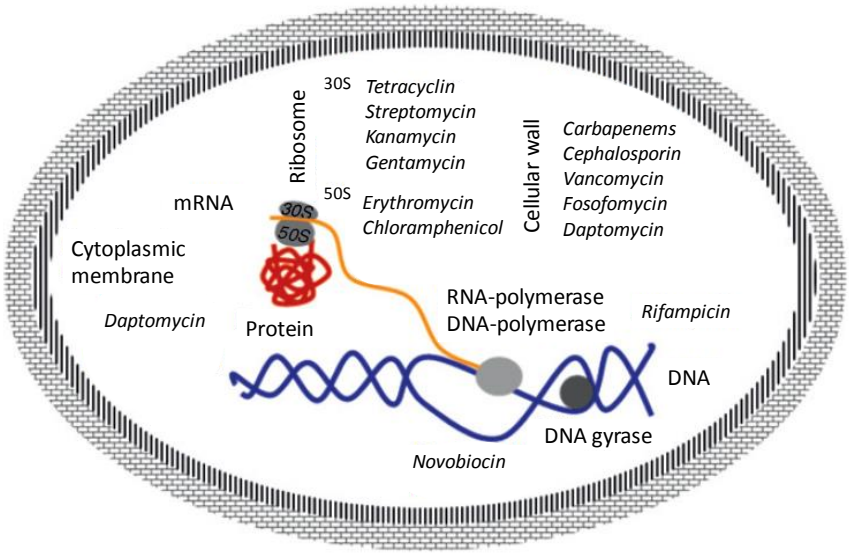



**Figure 1-3:** Schematic representation of the mechanism of action and target-site of several antibiotic compounds derived from actinobacteria. Adapted from Procopio et al.[77]

Nevertheless, the isolation of new compounds with potent antibacterial activity from former promising sources (e.g. *Streptomyces*) declines.[78] At first glance, this seems to rely on the complete exploitation of the microbial sources as producer of antimicrobial compounds, but studies estimate that there are still many unknown compounds to be discovered.[84] Nevertheless, the frequency of identifying novel antibiotics is dropping. This is due to the fact, that a further exploitation of promising sources is hampered since already known antibiotics appear in much higher frequencies what clouds the identification of so far unknown antibiotics.[84,85] Moreover, alternative approaches such as high-throughput

screening of natural product libraries on new potential targets or total chemical synthesis were rather unsuccessful in developing new potential lead structures.[86] Even though total synthesis seems to be a viable alternative to provide larger amounts of compounds, this route is accompanied by drawbacks. The structurally complex NPs often require a multiplicity of synthetic steps to finally yield the desired molecule.[87,88] Thus, new innovative strategies and novel sources for the development of new antimicrobial drugs are needed. One promising approach is the examination of so far unexplored or under-exploited genera (found for instance among rare actinobacteria, myxobacteria, marine sponges) and under-explored habitats (e.g. deep seas).[89–93] This approach is however often accompanied by major drawbacks such as difficulties regarding the cultivation of bacteria in laboratory environments and low yields of produced NPs.[94,95] Metagenomic studies estimated that of all microbial species, approximately 99 % are uncultured (no growth conditions reported under laboratory conditions), but at the same time they are promising sources to produce novel and potent compounds.[96,97] Thus, alternative and innovative strategies to increase biodiversity of strain collection and at the same time chemical diversity of isolated NPs are needed.[79,98,99] For instance, the development of an isolation chip[100] (iChip) led to the discovery of potent new antibiotics from previously uncultured bacteria, such as the cell wall inhibitor teixobactin.[101] Nonetheless, microbial fermentations, production optimization and the development of an efficient downstream process are highly challenging as well.[102,103] This implies to limitations in the biological and chemical characterization of potential new agents.

Considering these issues in NP discovery, an alternative way for drug development is the re-assessment of already known and effective, but neglected NP scaffolds. Such antibiotics might have shown suboptimal pharmacological properties, which could not be addressed at the time upon their discovery due to minor chemical or genetic engineering knowledge or other antibiotics with similar activity profile entered the market at the time of their discovery and development into a drug was not further pursued (e.g. octapeptins[104]). Already known NP scaffolds can serve as basis for further structural modifications to generate new derivatives through different chemical approaches (e.g. semi-synthesis). Moreover, biosynthetic studies such as the investigations of biosynthesis gene clusters and genetic engineering paves the way towards the development of new NP derivatives with improved pharmaceutical properties. Improvements of existing compound classes, based on these techniques, have already been shown to be successful for e.g. ceftaroline a cephalosporin class derivative.[105] In summary, this so-called rekindling of old antibiotics can provide

potent drug candidates, which can be forwarded into (pre-)clinical development[106] In antibiotic discovery and hit to lead programs the overall aim is to provide derivatives with optimized pharmaceutical properties (ADMET: absorption, distribution, metabolism, excretion, toxicity), improved activity, and resistance-breaking properties and without toxic liabilities.

Chelocardins and telomycins depict two examples of such under-exploited compound classes derived from actinobacteria, which will be further described in the following paragraphs (1.3 and 1.4).

## 1.3 CHELOCARDINS

Chelocardin (CHD; formerly known as M-319 or cetocycline; Figure 1-4) belongs to the family of tetracyclines (TCs; Figure 1-4) and is produced by the actinomycete *Amycolatopsis sulphurea*.[107] It was already described in the early 1960s and CHD exhibits – like other members of tetracycline family – broad-spectrum antibacterial activity.[107–109] TCs inhibit the protein biosynthesis by binding to 30S subunit of the ribosome and are used as first-line treatment against infectious diseases such as respiratory (e.g. atypical pneumonia) or genital infection (e.g. cervicitis) and additionally applied as alternative therapy for syphilis or community-acquired pneumonia.[48,110] Moreover, several non-antibacterial properties are described, such as anti-inflammatory or anti-apoptotic effects or the inhibition of matrix metalloproteinases.[110,111] Nevertheless, the main area of application of TCs comprises the treatment of bacterial infections. Their era as antibacterial agents began in 1948 with the isolation of chlortetracycline (aureomycin) from *S. aureofaciens*, followed by the discovery of oxytetracycline (OTC, terramycin) from *S. rimosus*.[112,113] Chemical modifications of chlortetracycline (catalytic hydrogenation) led to a C-7-deschloro derivative which was named tetracycline (TET), the structurally simplest member of the first generation of TCs. Shortly after, TET was also found to be naturally produced by *S. aureofaciens* and *S. rimosus* as it serves as precursor molecule in the generation of chlortetracycline.[114–116] Importantly, TET depicts one of the first semi-synthetically generated and FDA approved antibiotics used in the clinics.[49,117] Between the 1960s and the end of the 20$^{th}$ century Pfizer and Lederle Laboratories developed a second generation of tetracyclines. The overall aim was to improve pharmaceutical properties, to enhance TCs antibiotic activity and decrease toxic effects. The most potent TC derivative of that time was minocycline (Figure 1-4), which exhibits improved pharmacokinetic properties compared to previously applied TCs.[112,118] However,

due to increasing reports of resistance against TCs, a third generation – also known as glycylcyclines – was developed by using minocycline as precursor molecule.[119,120] The most prominent molecule among this series is tigecycline, which was the first member of the family of glycylcyclines and it was approved by the FDA in 2005.[121–123] Even though tigecycline shares the common mechanism of action of TCs through inhibiting the bacterial protein synthesis, this molecule is able to inhibit bacteria which carry TET-resistance genes (e.g. *tetB* coding for an efflux pump).[48,124–126] TCs act by binding to the 30S subunit of the ribosome, and block the binding site of aminoacyl-tRNA, which hinders the transfer of amino acid residues to peptide chains and consequently the elongation of the growing peptide chain is inhibited.

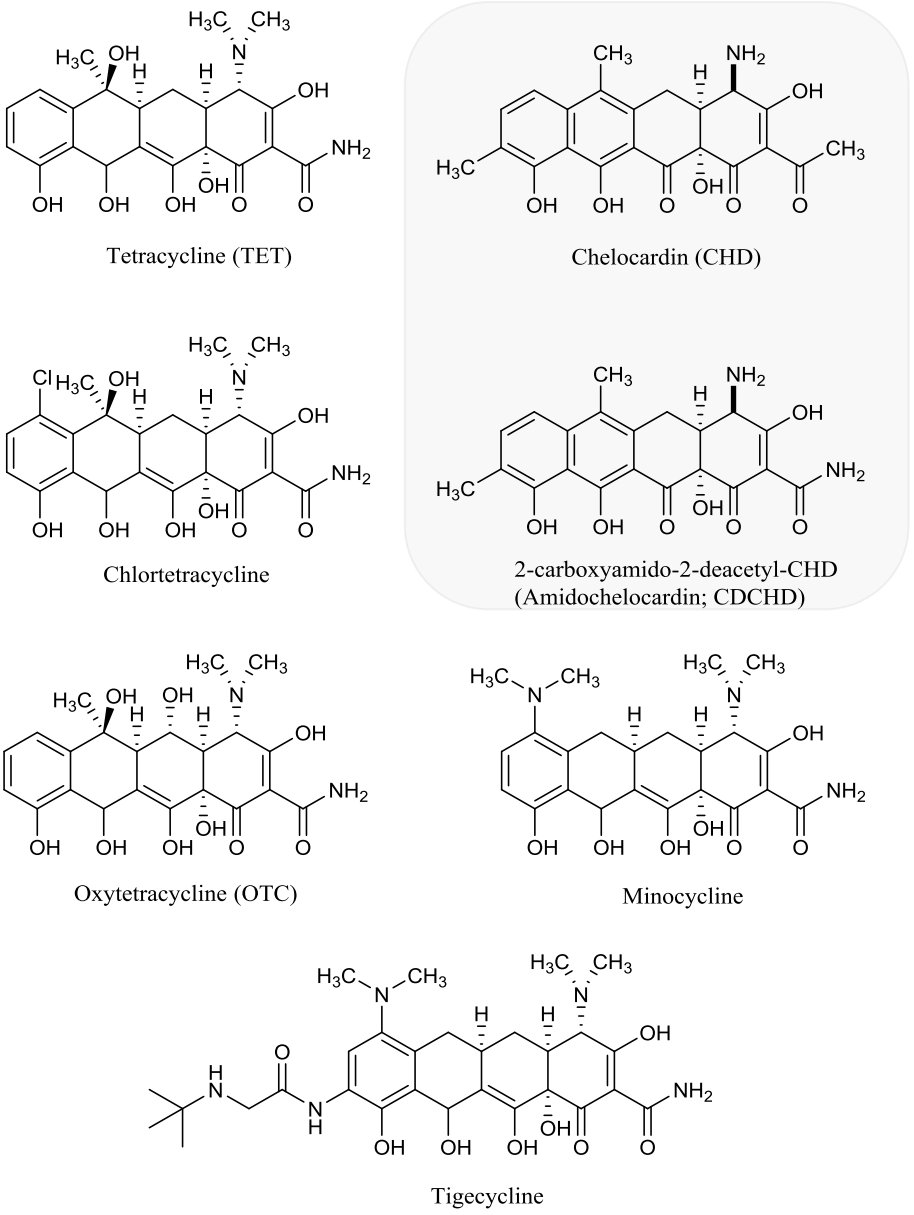



**Figure 1-4:** Structures of typical and atypical (light-grey box) tetracyclines and derivatives.

Importantly, CHD belongs to a group of TCs for which a different mechanism of action was described. This group includes anhydrotetracycline, anhydrochlortetracycline, 4-epi-anhydrochlortetracycline, 6-thiatetracycline, and CHD, all of which do not seem to interact with the ribosome, but rather interfere with the cytoplasmic bacterial membrane.[127–129] Moreover, this group of molecules act bactericidal, whereas all other TCs exhibit a bacteriostatic effect.[48,127] Based on these observations, the family of tetracycline antibiotics was divided into two classes: typical and atypical TCs.[128] Typical TCs act by inhibiting protein biosynthesis, whereas atypical TCs, such as CHD, seem to act on bacterial membranes. Previous studies indicated that the differences regarding the target site rely on

different structural features. All TCs (typical and atypical) share a common basic structure: a DCBA naphthacene core (Figure 1-5). However, the main difference of the two classes of TCs relies on a different aromatic pattern of the C ring. This seems to leads to a more planar structure in the case of atypical TCs (aromatic C ring), and an increased lipophilicity in comparison to typical TCs. The more lipophilic form of this TC sub-class of TC is thought to be the main reason why atypical TCs interfere with the membrane and do not primarily interact with the ribosome.[127,128] In the case of CHD, additional structural differences are the primary amine at C-4 (tertiary amine in typical TCs) and CHD bears an additional methyl-group at C-9. Moreover, typical TCs carry a carboxamido group at C-2, which is replaced by an acetyl group in CHD. Importantly, this structural feature at C-2 is found among all typical tetracyclines and is crucial for these molecules to perform their antibacterial action as observed in extensive SAR studies.[130–133]

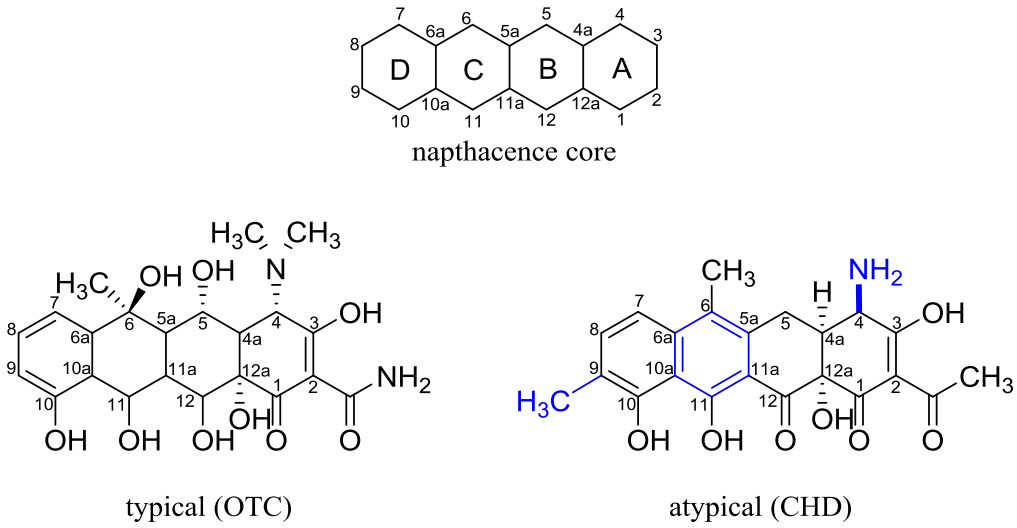



**Figure 1-5:** Structural differences of typical and atypical tetracyclines.

Besides the differences in the mechanism of action, the most important characteristic of CHD is its ability to inhibit tetracycline-resistant bacteria.[129,134,135] Resistance against TCs can rely on innate mechanisms, such as export by small molecule transporters (drug export) or a decreased permeability (OM). However, TET-specific resistance determinants contribute to acquired resistance mechanisms (Table 1-2). These mechanisms mainly rely on efflux mediated through 28 individual classes of pumps. Moreover, 12 resistance determinants are involved in ribosomal protection, two in compound degradation and five mutations are related to an impaired binding affinity to the ribosome.[112] Resistance through ribosomal protection is mediated by specific proteins (ribosomal protection proteins), which

hinder the compound to perform its action on the ribosome.[48] The frequent occurrence of resistance against TCs is primarily caused by resistance determinants on mobile genetic elements, such as plasmids or conjugative chromosomal elements. Those facilitate the horizontal gene transfer among different bacterial species.[136,137] Based on this, it is important to find alternative antibacterial agents, which are able to inhibit the multiplicity of bacteria bearing TC resistance determinants within their genome.

**Table 1-2:** Genes and mutations involved in tetracycline resistance. Adapted from Nguyen et al.[112]

| Efflux | | | Ribosomal protection | Degradation | rRNA mutations |
|---|---|---|---|---|---|
| *tetA* | *tetY* | *tetAB(46)* | *tetM* | *tetX* | G1058C |
| *tetB* | *tetZ* | *tcr3* | *tetO* | *tet37* | A926T |
| *tetC* | *tet30* | *otrC* | *tetQ* | | G927T |
| *tetD* | *tet31* | *otrB* | *tetS* | | A928C |
| *tetE* | *tet33* | | *tetT* | | ΔG942 |
| *tetG* | *tet35* | | *tetW* | | |
| *tetH* | *tet38* | | *tetB(P)* | | |
| *tetJ* | *tet39* | | *tet32* | | |
| *tetK* | *tet40* | | *tetB36* | | |
| *tetL* | *tet41* | | *tet44* | | |
| *tetA(P)* | *tet42* | | *ortA* | | |
| *tetV* | *tet45* | | *tet* | | |

*tet*: tetracycline resistance gene; *ort*: oxytetracycline resistance gene; Δ: deletion

Due to its broad-spectrum activity and putative lack of cross-resistance with TCs, CHD depicts a promising candidate to be used as alternative to typical TCs. Moreover, in the late 1970s, it was already shown that CHD displays potent *in vivo* efficacy. In a small phase II clinical study, twelve patients suffering from urinary tract infections (pyelonephritis) caused by Gram-negative bacteria were treated with CHD and infection was cured in all cases.[138]

Even though the effectiveness of CHD comprises the inhibition of a wide range of bacteria, one bacterial species belonging to the ESKAPE group of pathogens is not susceptible. Natural CHD is not able to inhibit the growth of *P. aeruginosa*, a species that is also found among TC-resistant pathogens.[48] To improve CHD's pharmaceutical properties and to widen its antibacterial spectrum, structural modifications were introduced. Based on the knowledge that the carboxamido group onC-2 of typical TCs is crucial for their activity, this moiety was installed on CHD through biosynthetic engineering. 2-carboxamindo-2-deacetyl-CHD (amidochelocardin, CDCHD, Figure 1-4) depicts a CHD derivative, which comprises all structural important features from typical and atypical TCs.[139] Activity studies revealed

CDCHD to exhibit an improved antibacterial activity in comparison to the parent molecule CHD, including significant inhibitory effects against clinical isolates of *Pseudomonas* species.[139]

Despite the fact that chelocardins depict potent molecules with resistance-breaking properties, their mechanism of action is still far from understood and it is only controversially discussed. On the one hand, reports describe CHD as inhibitor of protein synthesis[140] and on the other hand, CHD was shown to induce cell lysis by interfering with the cell membrane and no interaction with the ribosome was found.[127–129,135] Interestingly, a recent study, which was based on a global proteome analysis, predicated a dual mechanism of action of CHD through inhibition of the protein biosynthesis and interference with the cell membrane in a concentration-dependent manner (low concentrations: protein biosynthesis; higher concentrations: membrane).[141] However, no specific hint towards the main target molecule exists and it still remains elusive.

To further optimize the compound class of chelocardins and to develop a potent antibiotic drug, which is able to overcome AMR, is important to identify the target molecule and to fully characterize the mechanism of action.

## 1.4 TELOMYCINS

Telomycin (TM; Figure 1-6) belongs to the family of cyclic depsipeptide antibiotic molecules and is composed of 11 amino acids, five of which are non-proteinogenic amino acids.[142–144] The natural product TM is already known since the 1950s and is produced by the actinomycete *Streptomyces canus.*[145–147] Besides reports of TM's effectiveness against Gram-positive bacteria[144,146,148] and its complete structural elucidation more than 20 years after the first description of TM[142–144], no extensive investigation on the natural product was documented until the characterisation of the biosynthesis gene cluster in 2015.[148] Within the latter study, natural lipopeptides were identified to serve as precursors for TM and intriguingly, these derivatives showed improved activity compared to TM itself.[148] Overall, lipopeptides are a promising class of NPs with potent antibacterial activity. Natural sources for lipopeptide antibiotics are mainly actinobacteria, but also other species such as *Bacillus* spp., *Enterobacter* spp. or *Citrobacter* spp. were described to produce potent lipopeptides.[149,150] Lipopeptides are structurally related as they are composed of a fatty acyl chain which is covalently bound to the *N*-terminus of a linear or cyclic peptide core.[19,151,152] However, due to variations in the composition of the lipid tail and the peptide chain, diverse

classes of lipopeptides exist (e.g. surfactin, fengycin, polymyxins).[149,153] Their lipid tails can differ in length ($C_6$-$C_{18}$) and composition (type of fatty acid moiety) and amino acids can vary in their type (e.g. acidic, basic, aromatic), in the number (2-25) or in their configuration (D, L).[154] Moreover, due to the fact that lipopeptides are generated via a non-ribosomal biosynthetic pathway, they are often composed of non-proteinogenic or unusually configured or modified proteinogenic amino acids.[19,154] Lipopeptides are active against MDR bacteria, but most do not exhibit broad-spectrum activity and rather act one type of bacteria (Gram-positive or Gram-negative).[19,151,155] In contrast to anionic lipopeptides – which solely act on Gram-positive bacteria (e.g. DAP, surfactin) – cationic lipopeptides are able to travers the OM (self-mediated uptake) and inhibit Gram-negative bacteria (e.g. polymyxin B).[19,155] The characterization of the first lipopeptides is already more than 60 years ago. Among the anionic lipopeptide class amphomycin was the first characterized member and isolated in the early 1950s[156], followed by the discovery of structurally related classes such as friulimicins and glycinocins.[154] Regarding cationic lipopeptides, polymyxins depict one of the oldest family among this type of lipopeptide antibiotics.[153,157] Over the past decades, only few new antibiotics were approved by the FDA, among which the cyclic lipopeptide DAP was successfully approved for the treatment of complicated skin and soft tissue infections caused by Gram-positive bacteria including MRSA.[63,158] Nevertheless, the exact mechanism of action of DAP and of most lipopeptides is not complete understood, but they seem interact with the bacterial membrane.[59,155] Moreover – regardless of their net charge – all lipopeptides are able to interact with the bacterial membrane via their lipid side chain. Certainly, a class of calcium-dependent-antibiotics (CDA) exists among lipopeptides comprising for instance DAP or tsushimycin, both of which require the presence of divalent ions ($Ca^{2+}$) to fulfil their complete activity.[151] Only recently, a novel class of CDA cyclic lipopeptides, the malacidins, were described. Malacidins were discovery within on a culture-independent screening approach and characterized to exhibit promising resistance-breaking properties including activities against MDR Gram-positive bacteria.[159] This supports not only the potency of lipopeptides, but also the importance of applying novel approaches to identify new compound classes. Regarding the class of telomycins (TMs) Fu et al. identified new natural telomycin derivatives, and described the semi-synthesis of several lipopeptide TM analogues with improved effectiveness. One semi-synthetic compound depicts TM-Dodec (Figure 1-6), an acyl-derivative bearing an additional dodecanoic acid side chain at the *iso*-$^{1}$Asp terminus of the natural molecule.

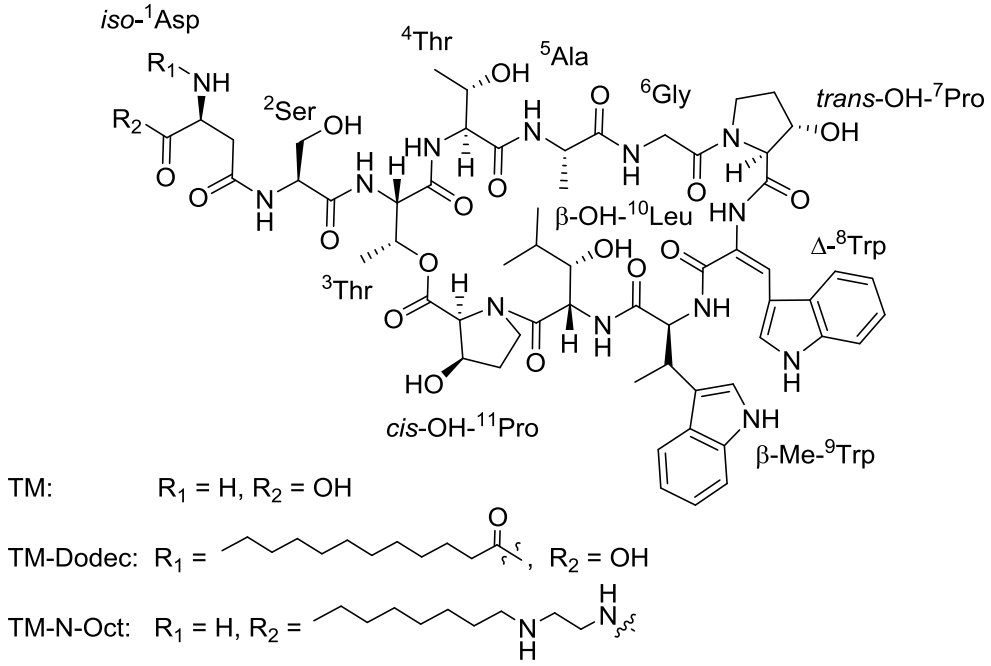



**Figure 1-6:** Structure of the natural product telomycin (TM) and semi-synthetic generated derivatives TM-Dodec and TM-*N*-Oct.

TM is described to act bactericidal against Gram-positive bacteria including MRSA, VISA and VRE.[146,148,160] Since the prevalence of MDR among Gram-positive bacteria is still increasing and alternative treatments are missing, TMs seems to be a viable alternative for the treatment of infections caused by such pathogens. This area of application might be especially favourable since TM is effective against DAP-resistant pathogens and the acylated derivative TM-Dodec exerts an even stronger bactericidal effect on *S. aureus* compared to the gold-standard antibiotic DAP.[148]

Only recently, Magarvey and co-workers described the identification of TM's putative target molecule. Within their study, a large collection of NPs (1908) was profiled by applying a retro-biosynthetic algorithm, aiming to identify antibiotic classes with unknown mechanisms of action or mechanisms of resistance. Thereby, TM was identified as promising candidate and a further investigation led to the characterization of the phospholipid cardiolipin as binding partner for TMs.[161] Besides this report and a study on a truncated version of TM, named LL-AO341$\beta_1$ (Figure 1-7), no reports on the mechanism of action exist. LL-AO341$\beta_1$ was described not to be involved in classical antibacterial mechanisms such as the inhibition of RNA, DNA, protein and peptidoglycan synthesis. However, this molecule was postulated to interact with the cytoplasmic membrane by a "de-energization" effect.[160] Nevertheless,

even though CL was identified as target molecule, solely the binding of TMs to CL is unlikely to cause the bactericidal effects, especially since a depletion of CL is not lethal.[162] Therefor it still needs to be further investigated if TMs act on a specific target protein and how exactly this compound class performs the bactericidal mechanism of action. Additionally, the impact of the addition of acyl chains to TM (TM-Dodec, TM-*N*-Oct; Figure 1-6) should be considered as well.

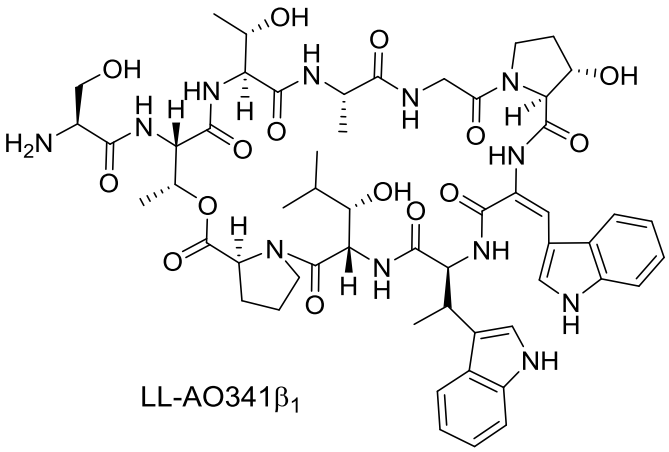



**Figure 1-7:** Structure of truncated TM analogue LL-AO341$\beta_1$.

## 1.5 OUTLINE OF THE DISSERTATION

The aim of the present work was the elucidation of the mechanism of action and resistance mechanism of chelocardins and telomycins and the identification of (additional) target molecules for both compound classes.

The first part of this study deals with the biological characterisation of chelocardin and its amidated analogue 2-carboxamindo-2-deacetyl-CHD (CDCHD). For both derivatives a comprehensive *in vitro* activity-based screen was performed and antibacterial as well as cytotoxic effects were investigated. Moreover, derivatives derived from semi-synthesis and genetic engineering were used to establish structure-activity relationships and to compare the *in vitro* potency of these compounds to both parent molecules, CHD and CDCHD, aiming to identify a derivative with improved overall profile.

Several *in vitro* studies were applied to reveal further insight into the mechanism of action of both compounds with a focus on cell viability and cellular processes such as the synthesis of macromolecules. Additionally, Gram-positive and Gram-negative wildtype species

served to generate CHD- and CDCHD-resistant mutants aiming to characterise the mechanism underlying resistance and preferably to identify the target molecule.

In the second part, telomycin and two semi-synthetic derived analogues, TM-Dodec and TM-*N*-Oct were biologically characterized, and screened for their antibacterial effectiveness against laboratory pathogens and clinical isolates. The *in vitro* generation of TM- and TM-Dodec-resistant bacteria and analysis of their genomes in comparison to the wildtype genome was performed to elucidate the resistance mechanism and to gain further hints towards the target molecule. Those results served as basis for the investigation of the mechanism of action of TM and both acyl derivatives. Studies included the characterization of the interaction of the compounds with potential binding partners and target fishing experiments with biotinylated tool compounds.

# 2 MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 COMPOUNDS

Chelocardin is produced by *Amycolatopsis sulphurea* NRRL2822 and derivatives were synthesized or isolated from the genetically engineered producer strain. Compounds were kindly provided by several co-workers from HZI and HIPS: Dr. Antoine Abou Fayad, Dr. Tadeja Lukežič, Chantal Bader, Dr. Rolf Jansen, Kerstin Schober, Dr. Suryanarayana Birudukota and Dr. Charlotte Grandclaudon.

Telomycin is produced by *Streptomyces canus* ATCC 12646 and derivatives were synthesized from the isolated natural product. All compounds were kindly provided by Dr. Armin Bauer (Sanofi) and Dr. Antoine Abou Fayad (HIPS-MINS).

### 2.1.2 CHEMICALS

All chemicals were of reagent grade quality and were obtained from commercial sources.

### 2.1.3 BACTERIAL GROWTH MEDIUM

All media were prepared in Milli-Q-water (MQ) and sterilized by autoclaving. Agar-based media were prepared by the addition of 1.6 % agar (Sigma Aldrich) to the medium.

**Table 2-1**: Bacterial growth medium.

| Medium | Ingredients | Concentration |
|---|---|---|
| CASO agar (Carl Roth); pH 7.3 | casein peptone | 1.5 % |
| | soya peptone | 5 % |
| | NaCl | 5 % |
| | Agar | 1.5 % |
| EBS; pH 7 | peptone from casein | 0.5 % |
| | peptone from beef | 0.1 % |
| | peptone from protease | 0.5 % |
| | yeast extract | 0.1 % |
| Middlebrook 7H9 medium (M7H9) | Sigma-Aldrich® Middlebrook 7H9 | 19.7 % |
| | albumin-dextrose-catalase enrichment | 10 % |
| | Glycerol | 2 mL/L |

| | | |
|---|---|---|
| Müller Hinton broth (MHB, BD Difco); pH 7.4 | casein hydrolysate | 1.75 % |
| | beef infusion solids | 0.2 % |
| | Starch | 0.15 % |
| Müller Hinton broth II (MHBII, BD Difco); cation adjusted; pH 7.3 | casein acid hydrolysate | 1.75 % |
| | beef extract | 0.3 % |
| | starch | 0.15 % |
| | $CaCl_2$ | 1.25 mM |
| | $MgCl_2$ | 0.8 mM |
| LB; pH 8.0 | NaCl | 1 % |
| | tryptone | 1 % |
| | yeast extract | 0.5 % |
| $LB_{low}$-Hyg; pH 8.0 | NaCl | 0.5 % |
| | tryptone | 1 % |
| | yeast extract | 0.5 % |
| | Hygromycin B | 100 µg/mL |
| LB-Apr; pH 8.0 | NaCl | 1 % |
| | tryptone | 1 % |
| | yeast extract | 0.5 % |
| | Apramycin | 50 µg/mL |
| SOC | NaCl | 0.05 % |
| | tryptone | 2 % |
| | yeast extract | 0.5 % |
| | KCl | 2.5 mM |
| | $MgCl_2$ | 10 mM |
| | $MgSO_4$ | 10 mM |
| | glucose | 20 mM |
| Tryptic Soy Broth (TSB); pH 7.3 | peptone from casein | 1.7 % |
| | peptone from soya | 0.3 % |
| | NaCl | 0.5 % |
| | $K_2HPO_4$ | 0.25 % |
| | glucose | 0.15 % |
| | yeast extract | 0.25 % |
| TSB-G, pH 7.3 | peptone from casein | 1.7 % |
| | peptone from soya | 0.3 % |
| | NaCl | 0.5 % |
| | $K_2HPO_4$ | 0.25 % |
| | glucose | 0.9 % |
| | yeast extract | 0.25 % |

### 2.1.4 MICROORGANISMS

**Table 2-2:** Microorganisms.

| Classification | Strain | Source |
|---|---|---|
| Gram-negative | *Acinetobacter baumannii* DSM-30008 | DSMZ |
| | *Burkholderia cenocepacia* DSM-16553 | DSMZ |
| | *Citrobacter freundii* DSM-30039 | DSMZ |
| | *Enterobacter aerogenes* DSM-30053 | DSMZ |
| | *Escherichia coli* DSM-1116 | DSMZ |
| | *Escherichia coli* ATCC-25922 | ATCC |
| | *Escherichia coli* TolC | internal strain collection |
| | *Escherichia coli* WT | Prof. Dr. Peter Heisig[a] |
| | *Escherichia coli* WT-III | Prof. Dr. Peter Heisig[a] |
| | *Escherichia coli* WT-3 | Prof. Dr. Peter Heisig[a] |
| | *Haemophilus influenzae* DSM-11970 | DSMZ |
| | *Klebsiella pneumoniae* DSM-30104 | DSMZ |
| | *Pseudomonas aeruginosa* DSM-11128 | DSMZ |
| | *Pseudomonas aeruginosa* PA14 | internal strain collection |
| | *Pseudomonas aeruginosa* PA14 Δ*mexAB* | Prof. Dr. Susanne Häußle[b] |
| | *Pseudomonas aeruginosa* PA14 Δ*mexCD* | Prof. Dr. Susanne Häußle[b] |
| | *Pseudomonas aeruginosa* PA14 Δ*mexEF* | Prof. Dr. Susanne Häußle[b] |
| | *Pseudomonas aeruginosa* PA14 Δ*mexXY* | Prof. Dr. Susanne Häußle[b] |
| | *Pseudomonas aeruginosa* DSM-24599 | DSMZ |
| | *Proteus vulgaris* DSM-2140 | DSMZ |
| | *Proteus mirabilis* DSM-4479 | DSMZ |
| | *Serratia marcescens* DSM-30121 | DSMZ |
| Gram-positive | *Bacillus subtilis* DSM-10 | DSMZ |
| | *Bacillus megaterium* DSM-32 | DSMZ |
| | *Corynebacterium glutamicum* DSM-20300 | DSMZ |
| | *Enterococcus faecalis* DSM-20478 | DSMZ |
| | *Enterococcus faecium* DSM-20477 | ATCC |
| | *Streptococcus pneumoniae* DSM-11865 | DSMZ |
| | *Micrococcus luteus* DSM-1790 | DSMZ |
| | *Micrococcus luteus* DSM-20030 | DSMZ |
| | *Mycobacterium smegmatis* $mc^2155$ | ATCC |
| | *Mycobacterium bovis* BCG DSM-43990 | DSMZ |
| | *Nocardia asteroides* DSM-43757 | DSMZ |
| | *Staphylococcus epidermidis* DSM-28765 | DSMZ |
| | *Staphylococcus carnosus* DSM-20501 | DSMZ |
| | *Staphylococcus aureus* DSM-346 | DSMZ |
| | *Staphylococcus aureus* DSM-11822 | DSMZ |
| | *Staphylococcus aureus* ATCC-29213 | ATCC |
| | *Staphylococcus aureus* Cowan1 | Prof. Dr. Markus Bischoff[c] |
| | *Staphylococcus aureus* Newman | Prof. Dr. Markus Bischoff[c] |
| | *Staphylococcus aureus* N315 | Prof. Dr. Markus Bischoff[c] |
| | *Staphylococcus aureus* Mu50 | Prof. Dr. Markus Bischoff[c] |

[a] University Hamburg, Department Biochemistry and Molecular Biology, Hamburg, Germany ; [b] Helmholtz Centre for Infection Research, Braunschweig/Twincore, Hannover, Germany; [c] Saarland University Hospital, Homburg, Germany

### 2.1.5 CELL LINES

**Table 2-3:** Cell lines.

| Cell line | Species | Type | Medium | Supplements |
|---|---|---|---|---|
| HCT-116 | human | colon carcinoma | McCoy's 5A | 10 % FBS |
| HepG2 | human | hepatocellular carcinoma | DMEM | 10 % FBS |
| L929 | murine | connective tissue fibroblast | RPMI-1640 | 10 % FBS |
| RAW 264.7 | murine | macrophage-like (leukemia virus-induced tumour) | DMEM | 10 % FBS |
| THP-1 | human | acute monocytic leukemia | RPMI-1640 | 10 % FBS |
| U-2 OS | human | bone osteosarcoma | McCoy's 5A | 10 % FBS |

### 2.1.6 CELL CULTURE MEDIUM AND REAGENTS

**Table 2-4:** Cell culture reagents.

| Reagent | Description | Manufacturer |
|---|---|---|
| Dulbecco's modified Eagle's Medium (DMEM) | 4500 mg/L glucose<br>L-glutamine<br>$NaHCO_3$ | Sigma-Aldrich, Steinheim, Germany |
| McCoy's 5A | L-glutamine<br>$NaHCO_3$ | Invitrogen, Karlsruhe, Germany |
| Rosewell Park Memorial Institute Medium (RPMI-1640) | L-glutamine<br>$NaHCO_3$ | Sigma-Aldrich, Taufkirchen, Germany |
| Foetal bovine serum (FBS) | heat inactivated, FBS Good | PAN Biotech GmbH, Aidenbach, Deutschland |

### 2.1.7 BUFFER

<u>HBS-P (pH 7.4)</u>

| | |
|---|---|
| HEPES | 0.01 M |
| NaCl | 0.15 M |
| Tween-20 | 0.005 % (*v*/*v*) |

<u>HBS-EP (pH 7.4)</u>

| | |
|---|---|
| HEPES | 0.01 M |
| NaCl | 0.15 M |
| EDTA | 3 mM |
| Tween-20 | 0.005 % (*v*/*v*) |

HEPES buffer (pH 7.2, adjusted with NaCl)

| | |
|---|---|
| HEPES | 5 mM |

NAE buffer

| | |
|---|---|
| Na-acetate (3M, pH 5.1) | 50 mM |
| EDTA | 10 mM |

NAES buffer

NAE buffer + 1 % (*w*/*v*) SDS

NAE-phenol

50 mL NAE buffer per 500 mL aqua-phenol

PBS (pH 7.45)

| | |
|---|---|
| sodium phosphates | 10 mM |
| KCl | 2.68 mM |
| NaCl | 140 nM |

One solid PBS tablet (Gibco™, Thermo Fisher Scientific) was dissolved in 500 mL distilled water.

PBS-T

PBS + 0.05 % (*v*/*v*) Tween-20

SPR coupling buffer

| | |
|---|---|
| Acetic acid | 1 mM |

pH was adjusted to 4, 4.5, 5 or 5.5 with sodium hydroxide

| | |
|---|---|
| Maleic acid | 1 mM |

pH was adjusted to 6, 6.5 or 7 with sodium hydroxide

TBS-T (pH 7.0, adjusted with HCl)

| | |
|---|---|
| Tris | 0.1 M |
| NaCl | 0.15 M |
| Tween-20 | 0.1 % (*v*/*v*) |

TCA (10 %)

| | |
|---|---|
| Trichloroacetic acid | 10 % (*w*/*v*) |
| NaCl | 1 M |

TCA (2.5 %)

| | |
|---|---|
| Trichloroacetic acid | 2.5 % (*w*/*v*) |
| NaCl | 1 M |

TMRM assay buffer (pH 7.35, adjusted with 10 N NaOH)

| | |
|---|---|
| NaCl | 135 mM |
| KCl | 5.4 mM |
| $MgCl_2$ | 2 mM |
| Glucose | 10 mM |
| $CaCl_2$ | 2 mM |
| HEPES | 10 mM |

**SDS-PAGE**

SDS-PAGE loading buffer (4 x)

| | |
|---|---|
| Tris/HCl (1 M, pH 6.8) | 500 mM |
| Glycerol (87 %) | 30% (*v*/*v*) |
| Bromophenol blue | 0.01% (w/*v*) |
| SDS | 10% (w/*v*) |
| DTT | 4 mM |

SDS-PAGE running buffer (4x; pH 8.6, adjusted with HCl)

| | |
|---|---|
| Tris | 250 mM |
| Glycine | 2 M |

SDS-PAGE separating buffer (4x; pH 8.8, adjusted with HCl)

| | |
|---|---|
| Tris | 1.5 M |
| 10 % SDS | 4 % (*w*/*v*) |

SDS-PAGE stacking buffer (4x; pH 6.7, adjusted with HCl)

| | |
|---|---|
| Tris | 0.5 M |
| 10 % SDS | 4 % (*w*/*v*) |

Separating gel (12.5 %)

| | |
|---|---|
| Bis-Acrylamide (30 % *v*/*v*) | 5 mL |
| 4x separating buffer (pH 8.8) | 2.5 mL |
| $dH_20$ | 2.5 mL |
| TEMED | 10 µL |
| APS (10 %) | 100 µL |

Stacking gel (5 %)

| | |
|---|---|
| Bis-Acrylamide (30 % *v*/*v*) | 830 µL |
| 4x stacking buffer (pH 6.8) | 1275 µL |
| $dH_20$ | 3 mL |
| TEMED | 5 µL |
| APS (10 % *w*/*v*) | 50 µL |

### 2.1.8 OTHER REAGENTS

**Table 2-5:** Reagents.

| Reagent | Description | Manufacturer |
|---|---|---|
| Aqua-Phenol | Water saturated phenol | Carl Roth GmbH, Karlsruhe, Germany |
| Bis-acrylamide | 30 % (*v*/*v*) | Carl Roth GmbH, Karlsruhe, Germany |
| DECP water | Ambion® DEPC-treated water nuclease-free | Thermo Fisher Scientific, Schwerte, Germany |
| DMSO | | Sigma-Aldrich, Taufkirchen, Germany |
| DNase I | RNase-free, 2 U/mL | New England Biolabs GmbH, Frankfurt a. Main, Germany |
| Hoechst33342 | | Invitrogen, Karlsruhe, Germany |
| Lysozyme | 20,000 U/mg | Carl Roth GmbH, Karlsruhe, Germany |
| MTT | Thiazolyl blue tetrazolium bromide | Sigma-Aldrich, Taufkirchen, Germany |
| NAO | Acridine orange 10-nonyl bromide | Sigma-Aldrich, Taufkirchen, Germany |
| NPN | 1-*N*-phenylnaphtylamine | Sigma-Aldrich, Taufkirchen, Germany |
| PBS | pH 7.4, without $Ca^{2+}$/$Mg^{2+}$ | Sigma-Aldrich, Taufkirchen, Germany |
| Ribonuclease A | ~ 90 U/mg | Carl Roth GmbH, Karlsruhe, Germany |
| Phenol:chloroform:isoamyl alcohol | 25:24:1 | Carl Roth GmbH, Karlsruhe, Germany |
| Polymerase | GoTag Green Mastermix | Promega, Madison, WI, USA |
| Proteinase K from *Tritirachium album* | ≥ 30 U/mg | Sigma-Aldrich, Taufkirchen, Germany |
| Proteinase inhibitor | cOmplete™ EDTA-free proteinase inhibitor cocktail | Sigma-Aldrich, Taufkirchen, Germany |
| Random Primer | 3 ug/µL | Thermo Fisher Scientific, Schwerte, Germany |
| RevertAid Premium Reverse Transcriptase | 200 U/µL | Thermo Fisher Scientific, Schwerte, Germany |

| | | |
|---|---|---|
| RiboLock RNase Inhibitor | 40 U/µL | Thermo Fisher Scientific, Schwerte, Germany |
| Restriction enzymes | | Thermo Fisher Scientific, Schwerte, Germany |
| Tetramethyl rhodamine methyl ester (TMRM) | | Invitrogen, Karlsruhe, Germany |
| Tetramethyl ethylenediamine (TEMED) | | Carl Roth GmbH, Karlsruhe, Germany |

### 2.1.9 CONSUMABLES AND TECHNICAL EQUIPMENT

**Table 2-6:** Consumables.

| **Description** | **Type** | **Manufacturer** |
|---|---|---|
| 6-well plates | Costar® 6 well, sterile, clear, flat bottom | Coring Life Sciences, Amsterdam, Netherlands |
| 24-well plates | Corning® CellBIND® 24 well, sterile, clear, flat bottom | Coring Life Sciences, Amsterdam, Netherlands |
| 96-well plates | Corning® CellBIND® 96 well, sterile, clear, flat bottom | Coring Life Sciences, Amsterdam, Netherlands |
| | 96 well imaging plates, black, clear, flat bottom | BD Biosciences, Heidelberg, Germany |
| | 96 well, sterile, clear, flat bottom | Sarstedt AG & Co., Nümbrecht, Germany |
| | 96 well, white, flat bottom | Coring Life Sciences, Amsterdam, Netherlands |
| | 96 well, black, flat bottom | Greiner Bio-One, Frinckhausen, Germany |
| Culture flasks | 25 $cm^2$ (T-25), filter cap | Sarstedt AG & Co., Nümbrecht, Germany |
| | 75 $cm^2$ (T-75), filter cap | |
| Cyro beads | Chemically treated beads covered with special cryogenetic preservative solution within a cryovial | Mast Diagnostica GmbH, Reinfeld, Germany |
| Filter | Glass microfiber binder free filter, 25 mm diameter | Whatman™, GE Healthcare, Germany |
| | Whatman™ cellulose filter grade 1 | |
| Petri dishes | 60 x 15 mm, sterile | Greiner Bio-One, Frinckhausen, Germany |

| | | |
|---|---|---|
| Kits | miRNAse mini Kit | Qiagen GmbH, Hilden, Germany |
| | BacLight™ Bacterial Membrane Potential Kit (B34950) | Invitrogen, Karlsruhe, Germany |
| | LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy and quantitative assays | Invitrogen, Karlsruhe, Germany |
| | SPR amine coupling kit | Biacore, GE Healthcare, Germany |
| | NucleoSpin® Gel and PCR Clean-up Kit | Macherey-Nagel, Düren, Germany |
| Magnetic Beads | M-280 Dynabeads; Streptavidin Magentic beads | Invitrogen, Karlsruhe, Germany |
| Serological pipettes | Corning®Costar® Stripette (2 mL, 5 mL, 10 mL, 25 mL, 50 mL) | Coring Life Sciences, Amsterdam, Netherlands |
| SPR vials | Plastic Vials and Caps, Ø 11 mm Plastic Vials, Ø 15 mm | Biacore, GE Healthcare, Germany |
| SPR chips | CM5 | Biacore, GE Healthcare, Germany |
| TLC plates | Silica gel, 0.2 mm, 8x4 cm aluminium cards | Sigma-Aldrich, Taufkirchen, Germany |
| Tubes | 15 mL PhaseLockGel™ tubes | Eppendorf, Hamburg, Germany |

**Table 2-7:** Technical equipment.

| **Device** | **Type** | **Manufacturer** |
|---|---|---|
| Balances | Acculab Vicon | Sartorius, Göttingen, Germany |
| | Precisa XB200A | Grevimetrics AG, Dietikon, Switzerland |
| Centrifuges | 5180R | Eppendorf, Hamburg, Germany |
| | CT15RE | VWR, Leuven, Belgium |
| | Ultracentrifuge, Avanti J-26 S | Beckman Coulter, Krefeld, Germany |
| Concentrator | SpeedVac Concentrator Plus | Eppendorf, Hamburg, Germany |
| Freeze dryer | Vacuum freeze dryer | Christ, Osterode am Harz, Germany |
| French press | Fresh press | Thermo Fisher Scientific, Schwerte, Germany |
| Incubator | 37 °C | Memmert, Büchenbach, Germany |
| | $CO_2$, Innova CO-170 | New Brunswick Scientific, Edison, USA |

| | | |
|---|---|---|
| Microscope (automated) | Pathway 855 | BD, Biosciences, Heidelberg, Germany |
| Microscope (optical) | ECLIPSE TS100 | Niko, Düsseldorf, Germany |
| PCR cycler | Mastercycler | Eppendorf, Hamburg, Germany |
| Photometer (UV/Vis) | NanoDrop 2000c (spectral) | Thermo Fisher Scientific, Schwerte, Germany |
| | BioPhotometer Plus | Eppendorf, Hamburg, Germany |
| Pipettors | Accu-jet pro | Brand, Wertheim, Germany |
| Pipettes | Transferpette® S, (0.1-2.5 µL, 0.5-10 µL, 2-20 µL, 20-200 µL, 100-1000 µL) | Brand, Wertheim, Germany |
| | Transferpette® S-8 (20-200 µL) | |
| | Transferpette® S-12 (20-200 µL) | |
| Plate reader | Infinite 200 M Pro | Tecan Group Ltd., Männedorf, Switzerland |
| qPCR cycler | Peqstar 96Q | Peqlab Biotechnology GmbH, Erlangen, Germany |
| Safety Cabinets | Hera Safe, KS-18 | Kendro, Langenselbold, Germany |
| Scintillation counter | MicroBeta TriLux 1450 LSC & Luminescence | Perkin Elmer, Waltham, MA, USA |
| Shaker | Infors HT Minitron Shaker | Infors HAT, Basel, Switzerland |
| Surface Plasmon resonance (SPR) | X100 | Biacore, GE Healthcare, Germany |

### 2.1.10 PLASMIDS

**Table 2-8:** Plasmids.

| Plasmid | Description | Selection marker | Source |
|---|---|---|---|
| pIJ773 | template for apramycin resistance cassette | apramycin | Huang et al.[163] |
| pACBSR-Hyg | p15A replicon plasmid; arabinose-inducible λ-Red recombinase | hygromycin | Huang et al.[163] |
| pFLP-Hyg | heat-shock inducible FLP recombinase | hygromycin | Huang et al.[163] |

### 2.1.11 PRIMER

**Table 2-9:** Primer for *in vitro* translation, heterologous expression and gene knockout.

| Primer | Sequence (5' → 3' direction) |
|---|---|
| ***In vitro* translation** | |
| Fluc_fwd | CGAGATCTCGATCCCGC |
| Fluc_rev | TTATTAATGATGATGATGATGATG |
| **Heterologous expression** | |
| YcdA_fwd | TCTACTTCCAGGGAGGCGGCCCTGCGGAGAAGAAAGAATCAGAAACA |
| YcdA_rev | CAGACTCGAGGCATGCAAGCTTTATTCTTTGATGAATGCTTCGACT |
| QoxA_fwd | TCTACTTCCAGGGAGGCGGCCCTTATTCGTTAGAAAAAGCTCCTGAAGC |
| QoxA_rev | CAGACTCGAGGCATGCAAGCTTCATTCTTCTGTATCATCAGACTTCT |
| **Gene knockout** | |
| RamRKO_fwd | CCTGGTCAGACGTGCCAAGATCGGCGGTTTGTTTAAACCTGCGTGAGGAAAAAAGTAGTGATTCCGGGGATCCGTCGACC |
| RamRKO_rev | CGATACGGTGAGCGCAGGGATGCAGCATCTCAGGGGTCATTTGGCGTCCGCCTCATGCAGTGTAGGCTGGAGCTGCTTC |
| RamRconf_fwd | GATATAACTTGATTATGAGT |
| RamRconf_rev | GCCCGCGAATAGTCATGGT |

**Table 2-10:** Primer for qPCR

| Primer | Sequence (5' → 3' direction) | gene |
|---|---|---|
| RamA_fwd | GGCATCTGCAACGGCTG | *ramA* |
| RamA_rev | GCAGCAGCTTCCTTTCGC | |
| AcrA_fwd | ACCAAAGTCACCTCGCCG | *acrA* |
| AcrA_rev | CCTGCTGGTACCGCAACA | |
| AcrB_fwd | GGACGGTTCCCAGGTTCG | *acrB* |
| AcrB_rev | TTTTCCTCACCCGGACGC | |
| 16S_fwd | ACGGGCGGTGTGTACAAG | 16S rRNA |
| 16S_rev | GGCCCCCTGGACAAAGAC | |

### 2.1.12 ISOTOPES

**Table 2-11:** Isotopes.

| Isotope | Manufacturer |
|---|---|
| Deoxythymidine 5'-Triphosphate, [Methyl-$^{3}$H], Tetrasodium Salt | Perkin Elmer, Waltham, MA, USA |
| Uridine 5'Triphosphate, Tetrasodium Salt [5,6-$^{3}$H] | Perkin Elmer, Waltham, MA, USA |
| L-[Methyl-$^{3}$H]-Methionine | Perkin Elmer, Waltham, MA, USA |
| Glucosamine D-[1-3H] hydrochloride | American Radiolabeled Chemicals, Saint Louis, MO, USA |

## 2.2 METHODS

*Methods marked with an asterisk (*) were applied by collaborators and are described in brief. Methods marked with a double asterisk (**) were partly applied by collaborators and partly by the author.*

### 2.2.1 BACTERIAL CULTIVATION

Bacterial strains were either conserved as glycerol stocks (50 % (*v/v*) glycerol) or in cryovials, containing 'cryo beads' (MAST CRYOBANK™) and stored at -80°C. To obtain a liquid culture one 'cryo bead' or a few microliters of the glycerol stock were inoculated in the appropriate growth medium and incubated overnight at 30°C or 37°C at 210 rpm or at 37°C/5% $CO_2$. Cultures at a specific growth phase were obtained by re-inoculation of the overnight cultures followed by cultivation under appropriate conditions up to a respective optical density of 600 nm ($OD_{600}$). Bacterial cultures on agar-based growth medium were prepared by streaking a few microliters of a liquid culture on an agar plate followed by an overnight incubation at the appropriate growth conditions.

### 2.2.2 MIC DETERMINATION

** All microorganisms were handled according to standard procedures and were obtained from the German Collection of Microorganisms and Cell Cultures (*Deutsche Sammlung für Mikroorganismen und Zellkulturen*, DSMZ) and the American Type Culture Collection (ATCC) or were part of our internal strain collection. Minimal inhibitory concentrations (MIC) for *M. bovis* were determined in 5 mL cultures (three to four weeks incubation) and all other values were determined by micro-dilution assays.[164] Overnight cultures were diluted in modified tryptic soy broth (TSB, *Enterococcus* spp.), in Middlebrook 7H9 (M7H9;

Sigma-Aldrich®; mycobacteria), Mueller-Hinton broth (MHB, or all other listed bacteria for chelocardin-related studies) or cation-adjusted Mueller-Hinton broth II (MHBII, for all other listed bacteria for telomycin-related studies) to achieve a final inoculum of approximately 1 x $10^6$ CFU/mL. Serial dilutions of compounds were prepared in sterile clear 96-well plates (Sarstedt AG & Co). The cell suspension was added and microorganisms were grown for 16-18 h at either 30°C or 37°C (*Enterococcus* spp. were grown under microaerophilic conditions). Growth inhibition was assessed by visual inspection and MIC values were determined as the lowest concentration of antibiotic at which no visible growth was observed.

**MIC determination for *M. tuberculosis***

* MIC determination for *M. tuberculosis* (strain H37Rv) was performed by Alamar Blue assay[165] as described previously.[166,167] In brief, 2-fold serial dilutions of compounds were prepared in M7H9 broth (Sigma-Aldrich) in round-bottom 96-well plates and 100 µL *M. tuberculosis* suspension ($10^5$ CFU/mL in M7H9 broth) was added per well. Plates were stored in plastic bags and incubated at 37°C for seven days. At day seven, 10 µL Alamar Blue solution (Invitrogen) was added per well and incubated for additional 24 h at 37°C. Fluorescence was measured (excitation: 544 nm; emission: 590 nm) by using a microplate reader (FLUOstar OPTIMA, MG LABTECH). MIC was defined based on relative fluorescence as lowest drug concentration leading to an inhibition of ≥ 90 % compared to the control. $MIC_{vis}$ values were determined visually by determination of a colour change from blue to pink ($MIC_{vis}$: lowest drug concentration preventing colour change).

**MIC/MBC determination of clinical uropathogens**

* MIC determinations of clinical bacterial uropathogens were performed by micro-dilution assays according to guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST; DIN EN ISO 20776-1).[168] MIC values were determined as lowest concentration leading to no visible growth. Minimum bactericidal concentrations (MBC) were determined by inoculation of 3 µL/well of the 96-well plates (used for MIC determination) to rectangular blood-agar plates (Iso-Sensitest + 5 % sheep blood) and incubated overnight at 37°C. MBC values were determined as the lowest concentration which leads to killing of 99.9 % (> log3) of each inoculum.

### 2.2.3 CELL VIABILITY BASED ON ATP QUANTIFICATION

To determine cell viability based on the amount of ATP present in bacterial cells, the luminescence-based BacTiter-Glo™ Microbial Cell Viability Kit (Promega) was used. 1 mL overnight culture of *S. aureus* Newman wildtype and *cls2* disrupted mutant was washed with MHB medium and 100 µL cell solution was added per well to a white, flat bottom 96-well plate (Corning). Cells were treated with 2-fold MIC per compound for 1 h at 37°C and 800 rpm. 100 µL of the BacTiter-Glo reagent was added per well and mixed briefly (900 rpm). Luminescence was recorded by using a microplate reader (Tecan Infinite M200 Pro).

### 2.2.4 BIOFILM FORMATION

Overnight cultures of *S. aureus* Newman and TM-Dodec-resistant mutants were adjusted to a final cell concentration of $10^6$ CFU/mL in TSB-G. 200 µL cells were added per well to 96-well plates and incubated at 37°C for 24 and 48 h. To determine biofilm formation, cells were washed twice with 200 µL MQ and 50 µL of 0.5 % (*w*/*v*) crystal violet was added. After 10 min of staining at room temperature, cells were washed twice with MQ and 200 µL EtOH were added per well. Optical density at 590 nm was measured using a microplate reader plate (Tecan Infinite M200 Pro).

### 2.2.5 TIME-KILL CURVES

Overnight cultures of *S. carnosus* DSM-20501 or *K. pneumoniae* DSM-30104 were diluted in MHB medium to achieve a start inoculum of $10^6$ CFU/mL. Compounds were added at the assigned concentrations and cell viability was assessed at several time points (*S. aureus*: 0, 0.5, 1, 2, 4, 6, 9, 12, 24 h; *K. pneumoniae*: 0, 0.5, 1, 1.5, 2, 3, 4, 6, 9, 12, 24 h) by CFU counts of samples (in 0.9 % (*w*/*v*) NaCl) in serial dilution after overnight incubation at 37°C on solid medium (CASO agar). Time-kill curves were obtained by plotting CFU counts ($log_{10}$ scale) versus time.

### 2.2.6 LIVE DEAD ASSAY

Bactericidal effects were determined by using the BacLight™ LIVE/DEAD Bacterial Viability Kit (Invitrogen) following the manufacturer protocol with minor modifications. An overnight culture of *E. coli* DSM-1116 was sub-cultivated in MHB medium to reach a start inoculum of approximately 2.5 x $10^8$ CFU/mL ($OD_{600}$: 0.5). Untreated control cells were centrifuged for 20 min at 4,000 rpm (5180R Eppendorf), the supernatant was discarded and the pellets were re-suspended in 2 mL 0.85 % (*w*/*v*) NaCl. Live and dead cells were prepared

by adding 1 mL cells to either 7 mL 0.85 % (*w*/*v*) NaCl (live, negative control) or to 7 mL 70 % (*v*/*v*) isopropyl (dead, positive control) and incubated for 1 h at room temperature. Antibacterial activities were examined by exposing cells for 4 h to compounds at 5-fold MIC. All samples were centrifuged for 20 min at 4,000 rpm (5180R Eppendorf), washed with 5 mL 0.85 % (*w*/*v*) NaCl and centrifuged again. The pellets were re-suspended in 10 mL 0.85 % (*w*/*v*) NaCl and the $OD_{600}$ values were measured (BioPhotometer Plus, Eppendorf). All samples were adjusted to the lowest $OD_{600}$ and a standard curve was prepared by mixing five different portions of live:dead control cells (0, 10, 50, 90, 100 % live cells). A 2-fold staining solution was prepared (6 µL SYTO9, 6 µL propidium iodide (PI) added to 2 mL MQ) and all samples were stained (1:1) for 15 min at room temperature, protected from light. Fluorescence was measured using a microplate reader (Tecan Infinite M200 Pro) at excitation was recorded at a fixed wavelength (485 nm) and emission at 530 nm (green) or 630 nm (red). The ratio of green and red fluorescence was calculated and the percentage of live cells was determined.

### 2.2.7 NPN UPTAKE

Uptake of 1-*N*-phenylnaphtylamine (NPN) was performed as described by Masschelein *et al.*[169]. In brief, an overnight culture of *E. coli* DSM-1116 was sub-cultivated (1:100) in fresh MHB medium at 37°C and 210 rpm to obtain cells in exponential growth phase ($OD_{600}$ of 0.6). Cells were centrifuged for 20 min at 4,000 rpm (5180R Eppendorf) and washed with 5 mM HEPES (pH 7.2). Compounds were diluted in 50 µL 5 mM HEPES (pH 7.2) at concentrations 3-fold higher than the intended final concentration. 100 µL cells were added per well and mixed with the previously diluted compounds (50 µL). Control samples were prepared as listed in Table 2-12 and all samples were incubation for 2 h at 37°C. 50 µL NPN staining solution (40 µM NPN in 5mM HEPES, pH 7.2) were added and fluorescence was measured within 3 min using a microplate reader plate (Tecan Infinite M200 Pro). After excitation at 355 nm fluorescence was detected at an emission wavelength of 405 nm, followed by measuring optical densities of each sample at 600 nm (BioPhotometer Plus, Eppendorf). The relative NPN uptake factor was calculated in the following way:

$$\text{realtive NPN uptake factor} = \frac{x}{\text{fluorescene value of control E}}$$

$$\text{with } x = \frac{\text{fluorescence value sample with NPN}}{\text{OD 600 of sample with NPN}} - \frac{\text{fluorescence value sample without NPN}}{\text{OD 600 of sample without NPN}}$$

**Table 2-12:** Control samples used for NPN uptake assay

| Control | Bacterial suspension [µL] | 5 mM HEPES [µL] | Compound [µL] | NPN [µL] |
|---|---|---|---|---|
| A | 100 | 50 | - | 50 |
| B | 100 | 50 | 50 | - |
| C | 100 | 100 | - | - |
| D | - | 150 | - | 50 |
| E | - | 200 | - | - |

### 2.2.8 MEMBRANE POTENTIAL

Membrane potential was determined using the BacLight Membrane Potential Kit (Invitrogen) according to manufacturer's instructions with minor modifications. An overnight culture of *S. aureus* Newman was sub-cultivated in fresh MHB medium to obtain a start inoculum of $10^8$ CFU/mL. Compounds were added to 1 mL cells at the assigned concentration. Cells exposed to 15 µM carbonyl cyanide 3- chlorophenylhydrazone (CCCP) served as positive control [exposure during staining with $DiOC_2(3)$]. After 0.5 and 2 h incubation, samples were washed once with PBS (pH 7.4) and re-suspended in 100 µL PBS (pH 7.4). 30 µM 3,3'-diethyloxacarbocyanin iodide ($DiOC_2(3)$ was added per sample and after 30 min staining at room temperature, samples were transferred to black 96-well plates (Greiner Bio One) and fluorescence was measured using a microplate reader (Tecan Infinite M200 Pro). After excitation at 488 nm, red fluorescence was detected at an emission wavelength of 675 nm and green fluorescence at 525 nm. To determine changes of the membrane potential, the ratio of red and green fluorescence was calculated relative to untreated control cells.

### 2.2.9 MACROMOLECULE BIOSYNTHESIS

To examine the inhibition of macromolecule biosynthesis, an overnight culture of *S. carnosus* DSM-20501 was sub-cultivated (1:100) in TSB medium at 37°C to obtain a start inoculum of approximately $10^8$ CFU/mL ($OD_{600}$: 0.2-0.4). The respective isotope [RNA: $^3$H-uridine (Perkin Elmer); protein: $^3$H-thymidine (Perkin Elmer); DNA: $^3$H-methionine (Perkin Elmer); cell wall: $^3$H-glucosamine (American Radiolabeled Chemicals)] was added at a final concentration of 1 µCi/µL and compounds were added at 0.5-fold MIC. Samples were incubated for 180 min at 37°C under permanent shaking (approx. 200 rpm) and sampling took place at several time points (0, 10, 20, 30, 45, 60, 90, 120, 180 min). 200 µL per sample was added to scintillation tubes (Perkin Elmer) containing 2 mL pre-cooled 10 % (*w*/*v*) TCA/1M NaCl, precipitated on ice for at least 45 min and filtered through 25 mm glass

microfiber (PALL Corporation). Filters were washed with 4 mL 2.5 % (*w*/*v*) TCA/1M NaCl and transferred into new scintillation tubes. 4 mL scintillation liquid (Perkin Elmer) was added per tube and radioactivity was measured by a scintillation counter (MicroBeta TriLux 1450 LSC & Luminescence; Perkin Elmer) for 5 min per sample. Radioactivity was plotted in counts per minute (cpm) against time.

### 2.2.10 *IN VITRO* TRANSLATION ASSAY

* *In vitro* translation assays were performed using the RTS 100 *E. coli* HY Kit (5PRIME) according to manufacturer's instructions. T7-bacteriophage DNA dependent RNA polymerase was cloned in front of a 130 pmol DNA product of Firefly luciferase [generated in a previous step by PCR using primers Fluc_fwd and Fluc_rev (listed in Table 2-9); reduction of reaction size to 5 µL)]. 1 µL per compound per test condition was added at the assigned concentration and samples were incubated for 1 h at 30°C and 750 rpm. 0.5 µL of each sample was added per well to a 96-well plate containing 7.5 µL kanamycin (50 mg/mL) and 40 µL Fluc Substrate (PROMEGA, Cat. No. E655207). Luminescence was recorded using a microplate reader (Tecan Infinite M1000).

### 2.2.11 GENERATION OF CHELOCARDIN-RESISTANT MUTANTS

** Resistant chelocardin mutants were generated by exposing wildtype strains *E. coli* DSM-1116 and *K. pneumoniae* DSM-30104 to stepwise increasing CHD concentrations (*E. coli*: 2.5, 5, 7.7, 12.5, 15, 20, 30 µg/mL; *K. pneumoniae*: 2, 4, 8 µg/mL) in a repetitive process of inoculation of single resistant colonies from agar plates into liquid medium with a concurrent increase of CHD concentration. To gain CDCHD resistant mutants, the above described approach was likewise used by exposing wildtype strain *K. pneumoniae* DSM-30104 and *S. aur*eus N315 to stepwise increasing CDCHD concentrations (*K. pneumoniae*: 2, 4, 8 µg/mL; *S. aureus*: 4, 8, 12 µg/mL).

### 2.2.12 GENERATION OF TELOMYCIN-RESISTANT MUTANTS

** Resistant telomycin mutants were generated by exposing wildtype strain *S. aureus* Newman to stepwise increasing concentrations of either TM (8, 30, 50, 100 µg/mL + 10 mM $CaCl_2$) or TM-Dodec (10, 20, 50, 100 µg/mL+ 1.25 mM $CaCl_2$) in a repetitive process of inoculation of single resistant colonies from agar plates into liquid medium with a concurrent increase of telomycin concentration. The frequency of spontaneous generated resistant telomycin mutants was determined as described by Butler et al.[170] and Evans et al.[171].

*S. aureus* Newman was incubated at a final concentration of $10^7$ (TM) or $10^8$ (TM-Dodec) CFU/plate overnight at 37°C on agar plates containing compounds at 4-fold MIC. In addition, control plates with no antibiotic were incubated with *S. aureus* Newman in several dilutions. The frequency of resistance was determined by dividing CFUs counted on antibiotic containing plate by the total CFU number of control plate.

### 2.2.13 ISOLATION OF BACTERIAL GENENOMIC DNA

DNA isolation was performed by standard phenol-chloroform extraction. Bacteria were cultivated overnight in 5 mL of their respective growth medium and centrifuged for 10 min at 4°C and 3,500 rpm (5180R Eppendorf) After washing with Milli-Q-water (MQ) cell pellets were re-suspended in 1.8 mL10 mM Tris-HCl (pH 8.0), incubated for 3.5 h at 37°C and inverted every 30 min. 200 µL proteinase K (20 mg/mL) and 20 µL 20 % (*w*/*v*) SDS were added and incubated for 2 h at 55°C. Subsequently, 100 µL RNase A (20 mg/mL) was added and incubated for additional 30 min. 2 mL phenol:chloroform:isoamyl alcohol (25:24:1) was added and after 1 h incubation at 5 rpm and room temperature, samples were centrifuged for 10 min at 3,500 rpm (5180R Eppendorf). The upper, aqueous phase was added to 2 mL phenol:chloroform:isoamyl alcohol (25:24:1) and incubated again at room temperature for 1 h at 5 rpm. After centrifuging for 10 min at 3,500 rpm (5180R Eppendorf), 1/10 volumes 3 M NaOAc (pH 4.8) and 2.5 volumes ice cold 100 % EtOH were added to the aqueous phase and precipitated DNA was transferred to 1 mL ice cold 70 % (*v*/*v*) EtOH. After centrifugation for 5 min at 4°C and 4,000 rpm (5180R Eppendorf), DNA was dried overnight at room temperature and dissolved in 100 µL MQ at 55°C and 300 rpm for 1 h. DNA concentration and purity was measured by using a spectrometer (NanoDrop 2000c Thermo Fisher Scientific).

### 2.2.14 WHOLE GENOMCE SEQUENCING AND ANALYSIS

* Whole genome sequencing of strains obtained by *in vitro* resistance development, as described in section 2.2.11 and 2.2.12 were sequenced by using Illumina sequencing technology on a MiSeq platform at the Helmholtz Centre for Infection Research (Braunschweig, Germany). Sequencing was performed in paired-end fashion. Raw data were analysed by an alignment against the reference genome sequences of *E. coli* DSM-1116 (NCBI GenBank accession code: NC_017635), *S. aureus* Newman (NCBI GenBank accession code: NC_009641) and *K. pneumoniae* DSM-30104. The genome sequence of *K. pneumoniae* DSM-30104 was not readily available from the public databases, therefore,

to obtain its complete reference sequence, high-molecular-weight DNA was submitted for sequencing with PacBio technology at the German Collection of Microorganisms and Cell Cultures (*Deutsche Sammlung für Mikroorganismen und Zellkulturen*, DSMZ). After *de novo* sequence assembly of raw data with SMRT portal provided by Pacific Biosciences (Menlo Park, CA, USA), a single contig for each chromosome and three extrachromosomal replicating elements were obtained (chromosome: 5,295,933 bp; pKPD1: 105,910 bp; pKPD2: 96,086 bp; pKPD3: 44,026 bp). The reference-guided whole genome assembly of Illumina raw sequencing data for all mutant samples was carried out in Geneious software[172] (Biomatters Ltd., Auckland, New Zealand) with "low sensitivity" parameters, otherwise the parameters were left default. The consensus calling for mutant and control strains was performed in Geneious software by executing the "generate consensus sequence" command and using "highest quality" as consensus calling algorithm, while other parameters were left default. Comparison of consensus sequences generated in the previous step was performed in Geneious software with the help of "MAUVE" whole-genome alignment plugin. Apart from using default settings, the "assume collinear genomes" option was selected to be on. The final step of the analysis comprised of manual verification of consensus inconsistencies between samples and comparing those to the reference and control sample sequences.

### 2.2.15 GENE KNOCKOUT IN *KLEBSIELLA PNEUMONIAE*

The generation of knockout mutants was performed as described by Huang et al.[163] with minor modifications. For preparing electrocompetent *K. pneumoniae* (wildtype: DSM-30104) cells, an overnight culture was sub-cultivated in 5 mL LB medium at 37°C and 210 rpm to obtain an $OD_{600}$ of 0.4-0.6. Cells were centrifuged at 15,000 rpm and 4°C (CT15RE VWR) for 15 min. The supernatant was discarded and the cells were washed with 2 mL ice-cold MQ water, followed by centrifugations as indicated above. The washing-centrifugation-step was repeated for additional two times and cells were finally diluted in 100 µL ice-cold MQ. 400 ng pACBSR-Hyg was added to 50 µL cells, transferred to ice cold electroporation cuvettes (0.2 cm gap) and electroporated at 2,500 V. Subsequently 250 µL pre-warmed SOC medium was added and cells were incubated for 60 min at 30 °C. 100 µL culture was spread onto $LB_{low}$-Hyg agar plates and incubated overnight at 30°C. A single colony (*K. pneumoniae* + pACBSR-Hyg) was inoculated in $LB_{low}$-Hyg medium overnight at 30° and 210 rpm. 50 µL overnight culture were added to medium containing 4.5 mL $LB_{low}$-Hyg medium, 450 µL 1 M L-arabinose and incubated until an $OD_{600}$ of 0.4-0.6 was reached. Electrocompetent cells were prepared as described above and cells were finally diluted in

100 µL ice-cold MQ. Approximately 1µg FRT-flanked $Apr^R$ knockout cassette [$Apr^R$; prepared by PCR: pIJ773 served as template to gain apramycin resistance gene (*aac(3)IV*) and flanking FRT sites; primers RamRKO_fwd and RamRKO_rev were used (listed in Table 2-9)] was added to 100 µL cells, transferred to ice cold electroporation cuvettes (0.2 cm gap) and electroporated at 2,500 V. Subsequently 500 µL pre-warmed SOC medium was added and cells were incubated for 90 min at 37 °C. The culture was spread onto two LB-Apr agar plates and incubated overnight at 37°C. Colonies were screened for the insertion of the knockout by PCR and correct transformants were spread on LB-Apr agar plates and incubated overnight at 37°C. Single colonies of the confirmed mutants were streaked on LB-Apr agar plates, incubated at 37°C overnight and this procedure was repeated for three days. Mutants that lost pACBSR-Hyg were detected by streaking colonies on LB-Apr and $LB_{low}$-Hyg agar plates following incubation at 37°C overnight. Hygromycin sensitive mutants were inoculated in 5 mL LB-Apr medium and incubated overnight at 37°C at 210 rpm. To remove the knockout cassette, 50 µL overnight culture was sub-cultivated at 37°C in 5 mL LB-Apr medium to reach an $OD_{600}$ of 0.4-0.6. Electrocompetent cells were prepared as described above and cells were finally diluted in 100 µL ice-cold MQ. 300 ng pFLP-Hyg was added to 50 µL cells, transferred to ice cold electroporation cuvettes (0.2 cm gap) and electroporated at 2,500 V. Subsequently 250 µL pre-warmed SOC medium was added and cells were incubated for 60 min at 30 °C. 100 µL culture was spread onto $LB_{low}$-Hyg agar plates and incubated overnight at 30°C. Mutants were heat-shocked by incubation at 43°C overnight and single colonies were streaked onto LB and LB-Apr agar plates and incubated overnight at 37°C. Apramycin-sensitive mutants were passed three times on LB agar plates and incubated each time overnight at 37°C. The loss of pFLP-Hyg was determined by streaking single colonies on $LB_{low}$-Hyg and LB agar plates. Hygromycin-sensitive mutants were prepared for sequencing by performing PCR (using primers RamRconf_fwd and RamRconf_rev; Table 2-9) and DNA was subsequently purified by using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel).

### 2.2.16 GENE KNOCKOUT IN *STAPHYLOCOCCUS AUREUS*

* Gene knockout with *S. aureus* Newman was performed by using the TargeTron®-Gene-Knockout-System Kit (Sigma Aldrich) with minor modifications. In brief, a PCR was performed to re-target the intron by primer-mediated mutations at several position within the target site. This reaction was followed by the purification of the PCR product by using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel). Afterwards, a restriction

digestion with the purified DNA and pNL9164 was performed. The reaction mixtures (Table 2-13) were incubated for 40 min at 37°C, following 20 min at 60°C and 10 min at 80°C. The digested templates were ligated and transformed into *E.coli* DH10b cells. After plasmid isolation, it was first transformed into *S. aureus* RN4220 following the transformation into *S. aureus* Newman (21 kV/cm, 100 Ω, and 25 µF). To induce gene disruption, *S. aureus* Newman cells (+ pNL9164) were incubated overnight at 32°C on BHI (Brain Heart Infusion) agar plates containing 10 µg/mL erythromycin. To confirm intron insertions, a colony PCR was performed by using primers designed with the TargeTron algorithm tool. The curing of the plasmid was performed by a heat shock reaction at 43°C overnight, followed by screening for erythromycin-sensitive colonies.

**Table 2-13:** Restriction digest reagent composition.

| Reagent | Template | |
|---|---|---|
| | pNL9164 | Purified DNA |
| Vector or PCR product (~200 ng) | 2 µL | 9.7 µL |
| 10X restriction enzyme buffer | 2 µL (Tango) | 2 µL |
| Hind III (20U/µL) | 1 µL | 1 µL |
| BsrG I (10U/µL) | 1 µL | 1 µL |
| Dpn I (20U/µL) | / | 1 µL |
| Water | 14 µL | 5.3 µL |

### 2.2.17 GENE EXPRESSION ANALYSIS

#### RNA isolation

150 mL of bacteria in mid-log phase was centrifuged for 10 min and 8,000 rpm (5180R Eppendorf) at 37°C. 4 mL pre-heated (60°C) NAE-Phenol was added to the pellet and incubated for 5 min at 60°C. 4 mL pre-heated (60°C) NAES buffer was added and incubated for additional 5 min at 60°C, followed by 5 min incubation on ice. Samples were centrifuged for 8 min at 8,000 rpm and 4°C (Avanti J-26 S Beckman) and the water phase was transferred to a PhaseLockGel-tube (Eppendorf). 4 mL phenol:chloroform (6:1) was added and mixed for 2 min, followed by centrifugation for 8 min at 8,000 rpm and 4°C (Avanti J-26 S Beckman). The above described transfer of the water phase and following centrifugation step was repeated two more times. The upper phase was mixed with 4 mL ice-cold isopropanol and 400 µL 3 M sodium acetate (pH 5.1). RNA was precipitated overnight at -20°C and samples were centrifuged for 40 min at 8,000 rpm and 4°C (Avanti J-26 S Beckman). Pellets were washed with 70 % (*v*/*v*) EtOH and dried for 30 min by using an exsiccator. RNA was diluted in 500 µL DECP water and purified by using miRNAeasy mini

kit (Qiagen). Concentration and purity was measured by using a spectrometer (NanoDrop 2000c Thermo Fisher Scientific).

**Reverse transcription**

RNA was transcribed into cDNA by using the RevertAid Premium Reverse Transcriptase (Thermo Fisher Scientific) following the manufacturer's instructions. The reaction mixture (5 µg RNA, 1 µL 100 pmol random primer, 0.5 mM dNTPs, 14.5 µL DECP water, 4 µL 5x buffer, 0.5 µL RiboLock, 20 µL RevertAid Premium Reverse Transcriptase) was incubated for 10 min at 20°C, following 30 min at 50°C and 5 min at 85°C.

**Quantification of gene expression**

Gene expression was measured by qPCR using the Peqstar 96Q (Peqlab) cycler. 10 µL reaction mixture was prepared by mixing 1 µL cDNA (diluted 1:10), 0.5 µL per primer (primer pairs listed in Table 2-10), 3 µL DECP water and 5 µL Gotag Mastermix (Promega). Gene expression was measured by qPCR as indicated in Table 2-14 and relative transcription levels compared to the wildtype were calculated by the $2^{-\Delta\Delta Ct}$ method[173] and normalized to 16S rRNA.

**Table 2-14:** qPCR cycler adjustments.

| Step | Temperature [°C] | Time [min] | Repeat [x-fold] |
|---|---|---|---|
| Denaturation | 95 | 7 | 1 |
| Denaturation | 95 | 10 | 40 |
| Annealing/elongation | X[a] | 1 | 40 |
| Melting curve 1.step | 95 | 0.25 | 1 |
| Melting curve 2.step | 60 | 1 | 1 |
| Melting curve 3.step | 95 | 0.25 | 1 |

[a] *ramA*: 54, *acrA*: 55, *acrB*: 55

## 2.2.18 LIPID BINDING

* Direct binding assays were performed as described by Makino et al.[174]. In brief, 50 µL lipid-mix (2.56 µM in EtOH) and lipoteichoic acid (LTA)-mix (2.65 µg/mL in EtOH) were transferred to surface treated 96-well plates (Nunc Polysorp). After the evaporation of EtOH (2 h) final concentrations of 128 pmol/well (lipids) and 128 ng/well (LTA) were achieved. Wells were blocked with 100 µL of 3 % BSA (Albumin Fraction V, biotin-free, Roth) in PBS by incubation overnight at 4°C. Plates were washed three times with 300 µL PBS-T per well and compounds were added at a final concentration of 0.7 µM/well (in 1 % BSA in

PBS). After 2 h incubation at room temperature, plates were washed six times with 300 µL PBS-T per well and a click reaction was performed by adding 100 µL click-mastermix (1 mL mastermix: 920 µL 1 % BSA in PBS, 20 µL $CuSO_4$, 50 µL sodium ascorbate, 10 µL azide-PEG3-biotin conjugate) per well for 2 h at room temperature. After six times of washing with PBS-T (300 µL per well), 100 µL streptavidin-coupled horseradish peroxidase (HRP; 1:1000 in 1 % BSA in PBS) was added per well for 1 h at room temperature. Plates were washed six times with PBST (300 µL per well) and 100 µL 1-fold TMB ELISA substrate solution (eBioscience) was added per well. After 15 min incubation at room temperature (protected from light), the colour reaction was stopped by adding 50 µL $H_3PO_4$ (1M) per well. Within 15 min optical densities were determined at 450 nm by using a microplate reader. To determine concentration dependent binding effects to cardiolipin, the procedure was performed as described above and additionally $CaCl_2$ and EDTA were added during the blocking step at final concentration of 10 mM. Cardiolipin was diluted in 1:2 serial dilutions to achieve final concentrations ranging from 4-128 pmol/well.

### 2.2.19 LIPID EXTRACTION

Lipid extraction was carried out as described by Bligh et al.[175] with minor modifications. 50 mL overnight culture of *S. aureus* Newman wildtype and *S. aureus* Newman *cls1* or *cls2* deficient mutants was centrifuged for 15 min at 4,000 rpm and 4°C (5180R Eppendorf). Cell pellets were lyophilized (freeze dryer; Christ) and re-suspended in PBS (pH 7.2) at 10 mL/10 mg cell pellet. 1 mg/mL lysozyme was added per sample and incubated for 30 min at 37°C. Lipids were extracted by chloroform-methanol-dd$H_2O$ (2:2:1) extraction. 10 mL chloroform and 20 mL MeOH were added to lysed cells, mixed briefly and additional 10 mL chloroform were added. Following brief mixing, 10 mL dd$H_2O$ was added and mixed again. The mixture was filtered (Whatman No.1; GE Healthcare Europe GmbH) and chloroform and alcohol layers were separated by using separating funnels. The chloroform layer was dried using a vacuum concentrator (SpeedVac Concentrator plus; Eppendorf) and re-dissolved 1:10 in chloroform.

### 2.2.20 THIN-LAYER CHROMATOGRAPHY

Lipids were extracted as described in section 2.2.19 and applied to silica thin-layer chromatography (TLC) plates (Sigma-Aldrich) by adding twice 1 µL per sample. TLC plates were developed with chloroform-methanol-acetic acid (8:2:0.1). The plates were sprayed with $CuSO_4$ and heated to detect the lipids

### 2.2.21 PROTEIN ISOLATION

Proteins isolation from *B. subtilis* DSM-10 was performed as described by Jin *et al*.[176] with minor modifications. Overnight cultures were sub-cultivated in 200 mL fresh medium at 37°C to obtain an inoculum at an $OD_{600}$ of 1.0. Cells were centrifuged for 10 min at 4,000 rpm (5180R Eppendorf) and washed twice with 10 mL TSB-T. EDTA-free cocktail protease inhibitor (Sigma-Aldrich) was added and cells were broken by French Press (Thermo Fisher Scientific), followed by centrifugation at 4000 rpm and 4°C (5180R Eppendorf) for 10 min to remove unbroken cells and membrane debris. Protein concentration was measured by Bradford protein assay.[177]

### 2.2.22 PROTEIN PULL DOWN AND ENZYMATIC DIGESTION

Proteins were isolated as described in section 2.2.21 and pull down was carried out as described by Jin *et al*.[176] with minor modifications. 50 µM compound was added to 900 µL cell lysate and incubated for 60 min at 4°C (gently rolling). 100 µL Streptavidin Magentic beads (M-280 Dynabeads Invitrogen) was added and incubated for 1 h at 4°C. Beads were washed three times with TBS-T and 1-fold SDS sample buffer was added. After boiling for 10 min at 95°C proteins were separated by SDS-PAGE[178] and desired protein bands were prepared for MALDI-TOF analysis as described by Shevchenko *et al*[179]. In brief, SDS-PAGE was washed in MQ overnight and desired protein bands were cut out and incubated with 500 µL acetonitrile (ACN) for 10 min. All liquid was removed and 50 µL DTT (10 mM in 100 mM $NH_4HCO_3$) was added for 30 min at 56°C. Samples were cooled down to room temperature and 500 µL ACN was added for 10 min. All liquid was removed and 30 µL iodoacetamide solution (55 mM IAA in 100 mM $NH_4HCO_3$) was added and samples were incubated for 10 min at room temperature (protected from light). Again, 500 µL ACN was added for 10 min and after removal of all liquid, gel bands were de-stained by washing twice with 100 mM $NH_4HCO_3$/ACN (1:1). 500 µL ACN was added for 10 min and after removal, 30 µL sequencing-grade trypsin (13 ng/µL in 10 mM $NH_4HCO_3$/10% (*v*/*v*) ACN) was added. After 3 h incubation at 4°C 15 µL $NH_4HCO$ (100 mM) was added and samples were incubated overnight at 37°C and further analysed by mass-spectrometry (see 2.2.23).

### 2.2.23 PROTEIN IDENTIFICATION BY MADLI-TOF/MS OR LC-MS/MS

* Proteins separated by SDS-PAGE, were prepared as described in section 2.2.22. The resulting peptides were extracted, desalted using ZipTip ($^{18}$C Millipore) and analysed by MALDI-TOF/MS using a Bruker Ultraflex TOF mass spectrometer (Bruker Daltonics,

Bremen) with alpha-cyano-4-hydroxycinnamic acid as matrix. Proteins were identified from the peptide mass fingerprints using the MASCOT search engine. Alternatively, LC-MS/MS analyses were performed on a DionexUltiMate 3000 n-RSLC system connected to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Peptides were loaded onto a C18 pre-column (3 µm, Acclaim, 75 µm x 20 mm, Dionex) and washed for 3 min (flow rate of 6 µL/min). Peptide separation was performed on an analytical column (2 µm, Acclaim PepMap RSLC, 75 µm x 25 cm, Dionex) at 350 µL/min via a linear 60 min gradient with HPLC buffer A (0.1 % FA in water) and 25 % HPLC buffer B (0.1 % formic acid in ACN), followed by a 30 min gradient from 25 to 50% of buffer B. MS/MS raw data files were searched against UniProtKB/Swiss-Prot protein database on a MASCOT server.

### 2.2.24 HETEROLOGOUS PROTEIN EXPRESSION AND PURIFICATION

* For biochemical experiments, truncated versions of QoxA and YcdA were used. Final constructs were generated by insertion of the respective gene sequences into a modified pCOLADuet-1 (EMD Millipore) plasmid using sequence and ligation independent cloning (SLIC) with primers listed in Table 2-9 (YcdA_fwd, YcdA_rev; QoxA_fwd; QoxA_rev). This resulted in fusion proteins composed of an N-terminal MBP tag followed by a StrepII-tag[180] and a TEV protease cleavage site. The fusion proteins were expressed in *E. coli* BL21 CodonPlus (DE3) RIL (8× 1 L auto-induction medium[181]) for 24 h at 20 °C. The cell pellets were re-suspended in lysis buffer (20 mM HEPES, 200 mM NaCl, 5 % (*v*/*v*) glycerol, pH 7.6 supplemented with DNaseII and lysozyme), homogenized and centrifuged. Subsequently, supernatants were applied separately onto columns packed with StrepTactin Sepharose High Performance resin on an ÄKTA protein purification system (both GE Healthcare Life Sciences). The proteins were eluted with 5 mM D-Desthiobiotin (Sigma-Aldrich) in the same buffer and the *N*-terminal tags were cleaved off using TEV protease during overnight dialysis against 20 mM HEPES, 200 mM NaCl, 5 % (*v*/*v*) glycerol (pH 7.6). To remove the tag, a second StrepTactin purification step was performed and the flow-through containing the respective proteins was concentrated using a Vivaspin 6 10 kDa cutoff concentrator (GE Healthcare Life Sciences). As a polishing step, each protein was passed through a Superdex 75 16/60 pg column (GE Healthcare Life Sciences) using the same buffer and fractions containing YcdA or QoxA were concentrated. Protein purity was assessed by SDS-PAGE and protein concentration was determined spectrophotometrically using extinction coefficients calculated via the ProtParam web server[182].

### 2.2.25 SURFACE PLASMON RESONANCE

Surface plasmon resonance (SPR) analysis was performed with a Biacore X100 device (GE Healthcare). Proteins were immobilized on a CM5 chip (Biacore) by standard amine coupling. YcdA and QoxA were diluted to 40 µg/mL and 20 µg/mL, respectively in sodium acetate buffer (pH 4.5) and immobilized for 100 to 200 sec. Compounds were injected at increasing concentrations in the presence or absence of 5 mM $CaCl_2$. Binding interactions were monitored at 25 °C with a flow rate of 30 µL/min in HBS-EP/1% (*v*/*v*) DMSO (± 5 mM $CaCl_2$) as running buffer. The theoretical maximal RU ($R_{max}$; RU: resonance units) was determined in the following way (assuming a 1:1 binding stoichiometry):

$$R_{max}(RU) = \frac{MW_{analyte}}{MW_{ligand}} \times \text{immobilized ligand level (RU)}$$

The equilibrium dissociation constants ($K_D$) were either determined by the ratio of association rate ($k_a$) to dissociation rate constant ($k_d$) or by steady-state affinity analysis using the Biacore X100 evaluation software 2.0.1.

### 2.2.26 CULTIVATION OF EUKARYOTIC CELL LINES

Cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (*Deutsche Sammlung für Mikroorganismen und Zellkulturen*, DSMZ) and the American Type Culture Collection (ATCC) and handled as recommended by the depositor. Cultivation was performed in the respective medium in a saturated water vapour atmosphere at 37°C and 5% $CO_2$. Cell splitting took place twice a week. Medium of adherent cell cultures was discarded and cells were washed with PBS (pH 7.4) followed by detaching the cells by the addition of trypsin. The reaction was stopped by the addition of fresh FBS-containing medium and splitting was performed at 1:5 to 1:20 ratios in fresh medium. Suspension cultures were split directly at 1:5 to 1:20 ratio into fresh medium.

### 2.2.27 $IC_{50}$ DETERMINATION (MTT)

Cells were seeded at a cell density of $5 \times 10^4$ cells per well in sterile 96-well plates (CellBIND® Corning) in 180 µL of the respective medium supplemented with 10 % FBS and incubated for 2 h at 37 °C and 5 % $CO_2$ followed by treatment with compounds in serial dilutions. After 5 d of incubation, 20 µL of 5 mg/mL stock MTT (thiazolyl blue tetrazolium bromide) in PBS (pH 7.4) were added per well and incubated for additional 2 h at 37°C and 5 % $CO_2$. The medium was discarded and 100 µL 2-propanol/10 N HCl (250:1) were added

to dissolve the formazan granules. The absorbance was measured at 570 nm using a microplate reader (Tecan Infinite Pro M200) and cell viability was determined relative to the respective solvent control. $IC_{50}$ values (half maximal inhibitory concentration) were determined by sigmoidal curve fitting using Origin6.1G software.

### 2.2.28 KILLING OF INTRACELLULAR BACTERIA

Murine macrophages (RAW264.7) were seeded at 2.5 x $10^5$ cells per well in 24-well plates (CellBIND® Corning) and incubated overnight at 37°C and 5 % $CO_2$. To infect the cells, an overnight culture of *S. aureus* N315 was diluted to 3 x $10^7$ CFU/mL and 100 µL cell suspension was added per well RAW264.7 (MOI of 10) and incubated for 2 h at 37°C and 5 % CO2. After three times of washing with PBS (pH 7.4), extracellular bacteria were killed by the addition of 50 µg/mL gentamicin for 90 min followed by three times washing with PBS. Compounds were added at the assigned concentration and cells were treated for 18 h at 37°C and 5 % $CO_2$. After three time washing with PBS (pH 7.4), 1 mL ice cold MQ was added per well and after 5-10 min, the cell suspension was diluted 1:100 and 1:1000 in 0.9 % (*w*/*v*) NaCl and plated on CASO agar. Intracellular *S. aureus* concentration was determined by CFU count after 24 h incubation at 37°C.

### 2.2.29 TMRM STAINING

Human bone osteosarcoma cells (U-2 OS) cells were seeded at a cell density of 5 x $10^3$ cells per well in 96-well imaging plates and incubated for 2 d at 37°C and 5 % $CO_2$. Compounds were added at the assigned concentration and cells were treated for 3 h at 37°C and 5 % $CO_2$. After two washing steps with assay buffer (100 µL/well), 100 µL staining solution (5 µg/mL Hoechst33342 and 20 nM TMRM in assay buffer) were added per well and cells were stained for 1 h at 37°C and 5% $CO_2$. Cells were washed (100 µL/well assay buffer) and examined on an automated microscope (BD Pathways 855) with appropriate filter sets for rhodamine and Hoechst.

# 3 CHELOCARDINS

## CONTRIBUTIONS

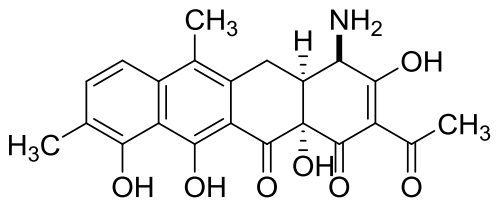
Several co-workers from HZI and HIPS provided natural products and derivatives: Kerstin Schober, Dr. Antoine Abou Fayad, Dr. Rolf Jansen, Dr. Tadeja Lukežič, Dr. Suryanarayana Birudukota, Dr. Charlotte Grandclaudon, Chantal Bader. Determination of MIC values of TET-resistant strains and plasma protein binding were partly performed at SANOFI. Dr. Maria Loose (JLU Gießen, Clinic for Urology, Prof. Dr. med. Florian Wagenlehner, DZIF-TTU9) determined MIC and MBC values of clinical isolated uropathogens. Fabian Nguyen (Gene Center, LMU, Munich, Dr. Daniel Wilson) performed *in vitro* translation assays. Dr. Jennifer Herrmann (HIPS-MINS) generated resistant *E.coli* mutants. Dr. Nestor Zaburannyi (HIPS-MINS) performed *in silico* genome analyses. All other presented experiments were performed by the author.

## 3.1 RESULTS

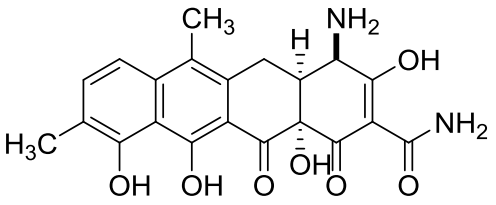
### 3.1.1 MECHANISM OF ACTION

#### 3.1.1.1 ANTIMICROBIAL SPECTRUM

The antibacterial activity of chelocardin (CHD) and its amidated derivative 2-carboxamindo-2-deacetyl-CHD (CDCHD, Figure 3-1) against Gram-positive and Gram-negative bacteria, as well as against Mycobacteria, was determined. Growth inhibition was assessed in standard micro-broth dilution assays by visual inspection and MIC (minimal inhibitory concentration) values were determined as the lowest concentration of antibiotic at which no visible growth was observed. For Gram-positive bacteria, both compounds showed inhibitory activities predominantly in the 1-digit µg/mL range (MIC = 2-8 µg/mL), including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *Staphylococcus aureus* (VISA) and multidrug-resistant (MDR) *Staphylococcus aureus* (Table 3-1). However, against *Bacillus subtilis*, *Enterococcus* spp. and *Micrococcus luteus* activity dropped 2- to 8-fold (MIC = 8-32 µg/mL) in comparison to all other tested Gram-positive pathogens. Compared to Gram-positive bacteria, susceptibility of Gram-negative bacteria was generally higher. For *Proteus* species, *Serratia marcescens*, *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae* and *Haemophilus influenza* MIC values between 0.25 and 4 µg/mL were achieved. Only *Enterobacter aerogenes* and *Pseudomonas aeruginosa* were less susceptible with MIC values ranging from 4 to 64 µg/mL depending on the strain. Against Mycobacteria, both compounds showed good activities with MIC values of 1-4 µg/mL. Throughout, CDCHD showed slightly improved inhibitory activities compared to the parent molecule CHD, in particular against *P. aeruginosa*.




chelocardin
(CHD)




2-carboxamido-2-deacetyl-chelocardin
(CDCHD)

**Figure 3-1:** Chemical structure of chelocardin (CHD) and the amidated derivative 2-carboxamindo-2-deacetyl-CHD (CDCHD).

**Table 3-1:** MIC values of CHD, CDCHD and tetracycline (TET) against Gram-positive and Gram-negative bacteria. [a]multidrug-resistant *S. aureus*; [b]methicillin-resistant *S. aureus*; [c]vancomycin-intermediate *S. aureus*; n.d.: not determined.

| Classification | Strain | MIC [µg/mL] | | |
|---|---|---|---|---|
| | | CHD | CDCHD | TET |
| Gram-positive | *Bacillus subtilis* DSM-10 | 16 | 8 | 1 |
| | *Enterococcus faecium* DSM-20477 | 8 | 4 | 1 |
| | *Enterococcus faecalis* DSM-20478 | 8 | 4 | 1 |
| | *Micrococcus luteus* DSM-20030 | 32 | 16 | 4 |
| | *Mycobacterium smegmatis* $mc^2155$ | 4 | 4 | n.d. |
| | *Mycobacterium bovis* BCG DSM-43990 | 1 | 4 | n.d. |
| | *Mycobacterium tuberculosis* H37RvMA | 1 | 4 | n.d. |
| | *Staphylococcus aureus* | | | |
| | DSM-346 | 4 | 2 | 0.125 |
| | DSM-11822[a] | 4 | 2 | n.d. |
| | ATCC-29213 | 4 | 8 | 0.125 |
| | Newman | 4 | 4 | 0.125 |
| | N315[b] | 4 | 4 | 0.125 |
| | Mu50[b,c] | 2 | 4 | 0.125 |
| | *Staphylococcus carnosus* DSM-20105 | 4 | 4 | 0.25 |
| Gram-negative | *Citrobacter freundii* DSM-30039 | 1 | 1 | 2 |
| | *Enterobacter aerogenes* DSM-30053 | 16 | 8 | 32 |
| | *Escherichia coli* | | | |
| | DSM-1116 | 1 | 0.5 | 0.5 |
| | ATCC-25922 | 2 | 2 | n.d. |
| | TolC-deficient | 0.5 | 0.25 | 0.5 |
| | *Haemophilus influenzae* DSM-11970 | 4 | 1 | 2 |
| | *Klebsiella pneumoniae* DSM-30104 | 1 | 0.5 | 0.5 |
| | *Proteus vulgaris* DSM-2140 | 0.25 | 0.25 | 1 |
| | *Proteus mirabilis* DSM-4479 | 0.5 | 1 | 4 |
| | *Pseudomonas aeruginosa* | | | |
| | DSM-11128 | 32 | 16 | 64 |
| | DSM-24599 | 64 | 16 | 32 |
| | PA14 | 32 | 4 | 32 |
| | *Serratia marcescens* DSM-30121 | 4 | 2 | 32 |

In comparison to tetracycline (TET), Gram-positive bacteria were less susceptible to CHD and CDCHD. Against Gram-negative bacteria, overall comparable activities were observed, except for *S. marcescens* against which both chelocardins showed 8- to 16-fold improved activities compared to TET. Additionally, activities of CHD and CDCHD against TET-resistant *E. coli*, *K. pneumoniae*, *Pseudomonas pseudoalcaligenes* and *Serratia liquefaciens* were determined, in which resistance is mediated via efflux (*tetA*, *tetB*), ribosomal protection

(*tetM*, *tetW*), or enzymatic inactivation (*tet34*). MIC values revealed the potency of CHD and CDCHD, as they inhibited growth of all tested tetracycline resistant bacteria (Table 3-2).

**Table 3-2:** MIC values of CHD, CDCHD and TET against tetracycline-resistant strains. n.d.: not determined. **values were determined by the authors, all other listed values were determined at SANOFI as part of a collaboration.*

| TET resistant strain | Resistance gene | Resistance mechanism | MIC [µg/mL] | | |
|---|---|---|---|---|---|
| | | | CHD | CDCHD | TET |
| **E. coli* XL-1 blue | *tetA* | Efflux | 2 | n.d. | > 64 |
| **E. coli* Hs996pTOTepoE | *tetA* | Efflux | 1 | n.d. | > 64 |
| *E. coli* | *tetB* | Efflux | n.d. | 4 | > 64 |
| *E. coli* | *tetM* | ribosomal protection | n.d. | 0.5 | 64 |
| *E. coli* | *tetW* | ribosomal protection | n.d. | 1 | > 64 |
| *E. coli* 49 | *tetB* | Efflux | n.d. | 4 | > 64 |
| *E. coli* 74 | *tetB* | Efflux | n.d. | 8 | > 64 |
| *S. liquefaciens* | *tetB*; *tet34* | efflux; enzymatic inactivation | n.d. | 2 | > 64 |
| *P. pseudoalcaligenes* | *tetB*; *tet34* | efflux; enzymatic inactivation | n.d. | 2 | > 64 |
| *K. pneumoniae* 3 | *tetA* | Efflux | n.d. | 8 | > 64 |
| *K. pneumoniae* 8 | *tetA* | Efflux | n.d. | 32 | > 64 |
| *K. pneumoniae* 24 | *tetA* | Efflux | n.d. | 8 | > 64 |

To investigate the drop in activity of CHD against *Pseudomonas* spp., CHD and CDCHD were tested against a panel of *P. aeruginosa* PA14 efflux pump deficient mutants (Δ*mexAB*, Δ*mexCD*, Δ*mexEF*, Δ*mexXY*). A deletion of the major efflux pump encoded by *mexAB* led to a 16-fold increase of CHD activity and 8-fold increase of CDCHD activity, as well as a 16-fold increase of TET activity (Table 3-3). Efflux pumps encoded by *mexCD*, *mexEF* and *mexXY*, which are usually expressed only under stress conditions, did not affect activities of CHD and CDCHD.

**Table 3-3:** Minimal inhibitory concentration (MIC) of CHD and CDCHD against *P. aeruginosa* PA14 wildtype and efflux pump deficient mutants (Δ*mexAB*, Δ*mexCD*, Δ*mexEF*, Δ*mexXY*).

| *P. aeruginosa* PA14 | MIC [µg/mL] | | |
|---|---|---|---|
| | CHD | CDCHD | TET |
| Wildtype | 32 | 4 | 32 |
| Δ*mexAB* | 2 | 0.5 | 2 |
| Δ*mexCD* | 32 | 8 | n.d. |
| Δ*mexEF* | 16 | 8 | n.d. |
| Δ*mexXY* | 32 | 4 | n.d. |

To determine if CHD exerts bactericidal or bacteriostatic effects, *S. carnosus* DSM-20501 was exposed to CHD at concentrations between 0.0625- and 64-fold MIC over 24 h and cell viability was assessed by CFU (colony forming units) count (Figure 3-2). At concentration of 0- to 0.125-fold MIC, an exponential cell growth and no influence of CHD on the growth behaviour was observed. An increase to 2-fold MIC led to delayed growth in the exponential phase and overall lower cell numbers in the stationary phase. Treatment with 16 to 256 µg/mL CHD (4- to 64-fold MIC) resulted in 2- to 3-$\log_{10}$ reduction of counted CFU after 24 h, representing a bactericidal effect.

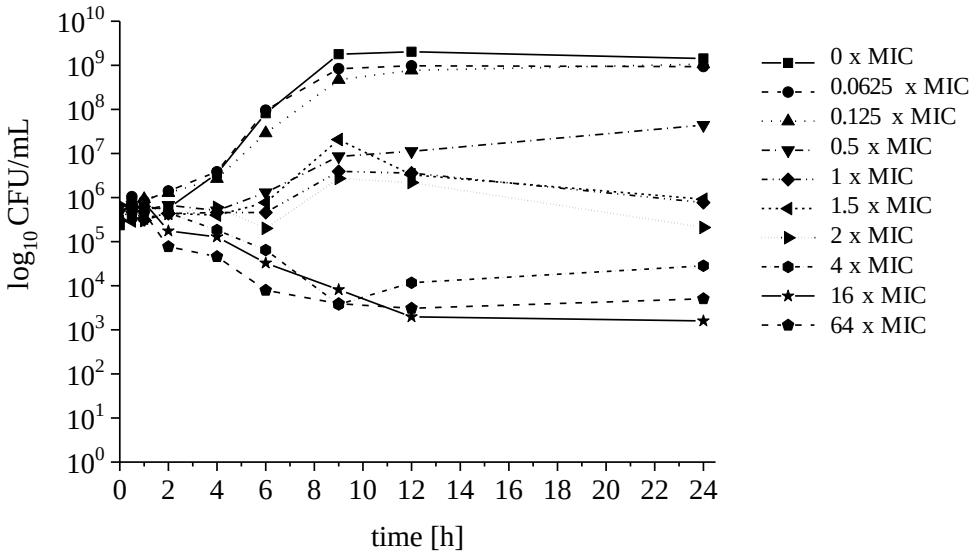



**Figure 3-2:** Time-kill curves of *S. carnosus* DSM-20105 exposed to CHD (MIC: 4 µg/mL) at concentration between 0.0625- and 64-fold MIC. Cell viability was determined by CFU (colony forming units) count over 24 h.

To compare the time-kill effects of CHD and CDCHD, *K. pneumoniae* DSM-30104 was exposed to CHD and CDCHD at 0.5- and 2-fold MIC over 24 h (Figure 3-3). As expected, bacteria exposed to 0.5-fold MIC of either CHD or CDCHD showed a retarded growth behaviour and both compounds used at 2-fold MIC caused a time-dependent decline of CFU by a 1-$\log_{10}$ (CHD) and 3-$\log_{10}$ (CDCHD) reduction of counted bacteria. In both cases, the effect of delayed growth or killing was more pronounced when cells were exposed to CDCHD.

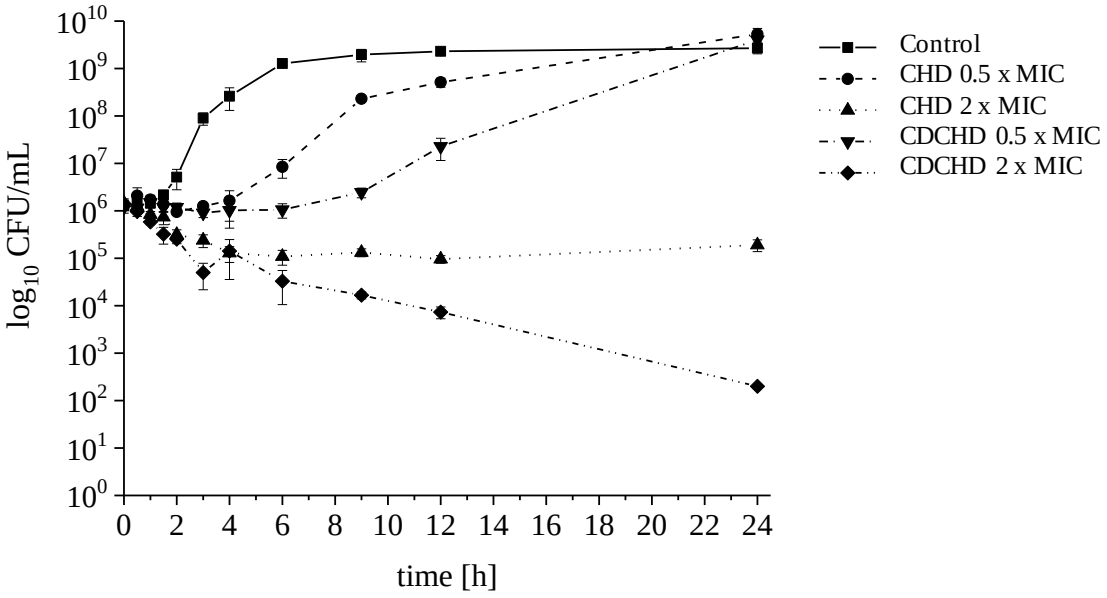



**Figure 3-3:** Time-kill curves of *K. pneumoniae* DSM-30104 exposed to CHD (MIC: 1µg/mL) and CDCHD (MIC: 0.5 µg/mL) at 0.5- and 2-fold MIC. Cell viability was determined over 24 h by three independent CFU (colony forming units) counts. Data are represented as mean ± standard deviation (SD).

The bactericidal effect of CHD was also compared to the effect of tetracycline by using the BacLight™ LIVE/DEAD Bacterial Viability Kit (Invitrogen). *E. coli* DSM-1116 was exposed to 5-fold MIC of either CHD or TET for 4 h. Cells were stained with nucleic acid binding dyes propidium iodide (PI) and SYTO9 and fluorescence was measured on a microplate reader [excitation: 485 nm; emission: 530 nm (green), 630 (red)]. The green fluorescent dye SYTO9 penetrates bacterial membranes and stains all nucleic acid containing cells, whereas the red fluorescent dye PI is membrane impermanent and only penetrates cells with damaged membranes. When both dyes are present within the cell (cells with destroyed membranes), SYTO9 fluorescence is displaced by PI fluorescence and its green emission is quenched due to fluorescence resonance energy transfer (FRET)[183,184]. The percentage of live cells was determined by the ratio of green and red fluorescence. Treatment with CHD led to a 40 % reduction of live cells, whereas TET only killed around 15 %, revealing a stronger bactericidal behaviour of CHD compared to TET (Table 3-4).

**Table 3-4:** Bactericidal effect of CHD and TET after exposure of *E. coli* DSM-1116 for 4 h at 5-fold MIC compared to untreated cells.

| | Untreated | CHD | TET |
|---|---|---|---|
| % live bacteria | 99.1 | 59.3 | 84.3 |

Since both compounds showed inhibitory activity against typical uropathogenic bacterial species (*P. aeruginosa*, *K. pneumoniae*, *E. aerogenes*, *Proteus* spp., *Enterococcus* spp. and *E. coli*) CHD and CDCHD were screened against uropathogenic clinical isolates, including multidrug-resistant isolates [resistance via extended-spectrum β-lactamases (ESBL) or TEM-β-lactamases; colistin-resistant isolates]. MIC and MBC (minimal bactericidal concentrations) values were determined and in contrast to all tested laboratory strains (Table 3-1), CHD showed by tendency better antibacterial activities compared to CDCHD (Table 3-5). No major differences in susceptibility were observed when multidrug-resistant strains were tested compared to sensitive strains. CDCHD showed higher bactericidal activities with a marginal shift of MIC *vs.* MBC compared to CHD. Clinical *P. aeruginosa* isolates were not susceptible to both compounds.

**Table 3-5:** Minimal inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of CHD and CDCHD against uropathogenic clinical isolates. Values denote median of MIC/MBC values per group and were determined in three independent measurements per isolate. ESBL: extended-spectrum β lactamases.

| Isolate (n=) | MIC [µg/mL] | | MBC [µg/mL] | |
|---|---|---|---|---|
| | CHD | CDCHD | CHD | CDCHD |
| *Escherichia coli* | | | | |
| sensitive (15) | 2 | 4 | 16 | 4 |
| TEM β-lactamase (8) | 2-4 | 8 | 16 | 8 |
| ESBL (7) | 4 | 4 | 32 | 8 |
| colistin-resistant (5) | 8 | 8 | 32 | 16 |
| *Enterococcus faecalis* (19) | 8 | 16 | > 64 | 16 |
| *Enterococcus faecium* (6) | 4 | 8 | 32 | 16 |
| *Enterobacter* spp. (8) | 4 | 4 | 16 | 8-16 |
| *Klebsiella* spp. | | | | |
| sensitive (7) | 4 | 4 | 16 | 8 |
| ESBL (2) | 4 | 4-8 | 32 | 8-16 |
| carbapenem-resistant (2) | 4 | 4-8 | 32-64 | 8 |
| *Proteus* spp. | | | | |
| sensitive (7) | 4 | 8 | 8 | 64 |
| ESBL (2) | 4 | 16-32 | 4-8 | 32-64 |
| *Pseudomonas aeruginosa* (10) | > 64 | 64 | > 64 | > 64 |

In initial studies, reported MIC values[139] were determined by using chelocardins prepared as HCl adducts in methanol. Due to later on discovered stability issues (e.g. spontaneous epimerization), a modified formulation for both chelocardins was provided and both compounds were further on used as sodium salts with citrate as stabilizer (in water). To exclude that the changed formulation caused reverted potency of CHD and CDCHD, a comparison of CHD and CDCHD in either MeOH (HCl adducts) or water (Na salts with Na-citrate) was performed on some exemplary pathogens (Table 3-6, Table 3-7). Throughout, MIC values were comparable and thus, it was confirmed that CDCHD is by tendency less active compared to CHD on uropathogenic clinical isolates.

**Table 3-6:** Comparison of MIC and MBC values of CHD as Na-salt with Na-citrate (CHD) to HCl-salt (CHD-HCl).

| Isolate | MIC [µg/mL] | | MBC [µg/mL] | |
|---|---|---|---|---|
| | CHD | CHD-HCl | CHD | CHD-HCl |
| *K. pneumoniae* 30 | 2 | 1.5 | 16 | 12 |
| *K. pneumoniae* 40 | 4 | 3 | 16 | 12 |
| *E. coli* 51 | 1 | 1.5 | 16 | 12 |
| *E. coli* 57 | 2 | 1.5 | 32 | 24 |
| *E. coli* AF48 | 4 | 3 | 16 | 12 |

**Table 3-7:** Comparison of MIC and MBC values of CDCHD as Na-salt with Na-citrate (CDCHD) to HCl-salt (CDCHD-HCl).

| Isolate | MIC [µg/mL] | | MBC [µg/mL] | |
|---|---|---|---|---|
| | CDCHD | CDCHD-HCl | CDCHD | CDCHD-HCl |
| *K. pneumoniae* 30 | 2 | 1.5 | 4 | 1.5 |
| *K. pneumoniae* 40 | 2 | 1.5 | 4 | 1.5 |
| *E. coli* 51 | 1 | 0.8 | 2 | 0.8 |
| *E. coli* 57 | 2 | 1.5 | 4 | 12 |
| *E. coli* AF48 | 4 | 1.5 | 4 | 1.5 |

For selected *K. pneumoniae* and *E. coli* isolates, MIC and MBC values were additionally determined in artificial urine at different pH values (Table 3-8). Both compounds showed pH-independent antibacterial activities, but pH-dependent bactericidal activities with MBC values varying by factor 2-16 depending on the adjusted pH. Compared to values determined in standard test medium (cation-adjusted Müller-Hinton broth, CAMHB), CHD and CDCHD showed 4-fold improved antibacterial activities with a concurrent increase of bactericidal activity in artificial urine (Table 3-9).

**Table 3-8:** Minimal inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of CHD and CDCHD against *E. coli* and *K. pneumoniae* clinical isolates determined in artificial urine at different pH values. Values denote median of three independent measurements per isolate.

| Isolate | pH | MIC [µg/mL] | | MBC [µg/mL] | |
|---|---|---|---|---|---|
| | | CHD | CDCHD | CHD | CDCHD |
| *E. coli* | 5.5 | 1 | 0.5 | 4 | 8 |
| | 6.5 | 1 | 1 | 4 | 2 |
| | 7.5 | 1 | 1 | 8 | 4 |
| | 8.5 | 1 | 1 | 8 | 2 |
| *K. pneumoniae* | 5.5 | 1 | 1 | 8 | 2 |
| | 6.5 | 1 | 1 | 8 | 4 |
| | 7.5 | 2 | 2 | 16 | 4 |
| | 8.5 | 2 | 2 | 16 | 32 |

**Table 3-9:** Minimal inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of CHD and CDCHD against *E. coli* and *K. pneumoniae* clinical isolates determined in artificial urine and standard CA-MHB test medium. Values denote median of three independent measurements per isolate.

| Test condition | Isolate (n=) | MIC [µg/mL] | | MBC [µg/mL] | |
|---|---|---|---|---|---|
| | | CHD | CDCHD | CHD | CDCHD |
| Artificial urine | *Escherichia coli* (7) | 1 | 1 | 8 | 2 |
| | *Klebsiella pneumoniae* (3) | 1 | 1 | 8 | 4 |
| CAMHB | *Escherichia coli* (7) | 4 | 4 | 32 | 8 |
| | *Klebsiella pneumoniae* (3) | 4 | 4 | 32 | 8 |

To estimate plasma protein binding (PPB), MIC values of CHD and CDCHD were assessed against *S. aureus*, *K. pneumoniae*, *A. baumannii* and *P. aeruginosa* in the presence of 50 % foetal calf serum (FCS). A shift of MIC was observed for both compounds, reflected in a 4- to 32-fold decrease of MIC values in the presence of 50 % FCS (Table-3-10).

**Table-3-10:** Serum effect on MIC values of CHD and CDCHD against Gram-positive and Gram-negative bacteria in the presence of 50 % foetal calf serum (FCS). n.d.: not determined. **values were determined at SANOFI as part of a collaboration.*

| Strain | MIC [µg/mL] | | | |
|---|---|---|---|---|
| | CHD | | CDCHD | |
| | | + 50% FCS | | + 50% FCS |
| *Staphylococcus aureus* ATCC-29213 | 8 | 64 | 8 | 64 |
| *Klebsiella pneumoniae* DSM-30104 | 4 | 16 | 4 | 16 |
| *Acinetobacter baumannii* ATCC-19606* | n.d. | n.d. | 8 | > 64 |
| *Escherichia coli* 35218* | n.d. | n.d. | 0.25 | 8 |
| *Pseudomonas aeruginosa* ATCC-27853* | n.d. | n.d. | 32 | > 64 |
| *Pseudomonas aeruginosa* PA14* | n.d. | n.d. | 32 | > 64 |

To examine the intracellular killing effect of CHD and CDCHD, murine macrophages (RAW264.7 cell line) were infected with *S. aureus* N315 at a multiplicity of infection (MOI)

of 10 and killing of intracellular bacteria was determined by CFU count after 18 h of treatment with 0.1- and 0.5-fold MIC of CHD and CDCHD. CHD was not able to reduce the number of intracellular bacteria at both tested sub-MIC concentrations (Figure 3-4) and for CDCHD no killing effect was observed at 0.1-fold MIC. CFU counts of macrophages treated with 0.5-fold MIC CDCHD indicated a killing effect, but microscopic investigations revealed a concurrent killing of RAW 264.7 cells. Due to high cytotoxic effects of CDCHD (section 3.1.1.3), no killing of intracellular *S. aureus* was achieved after treatment with CDCHD without affecting the host cells.

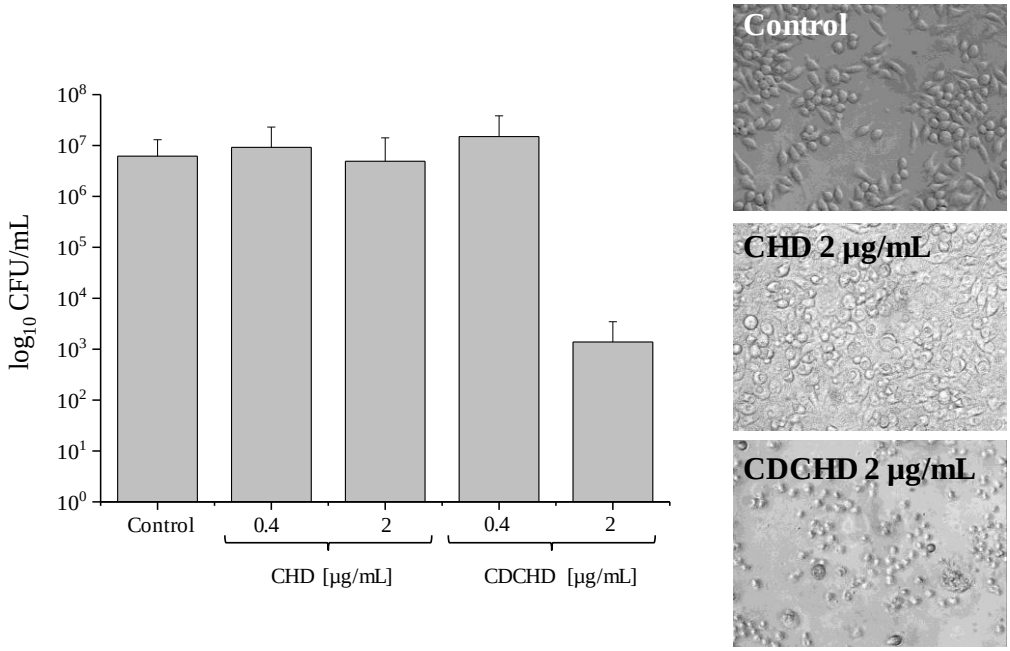



**Figure 3-4:** Killing of intracellular *Staphylococcus aureus* by CHD (MIC: 4µg/mL) and CDCHD (MIC: 4 µg/mL). Murine macrophages (RAW264.7 cell line) were infected with *S. aureus* N315 and treated with CHD and CDCHD for 18 h at 0.5- and 2-fold MIC. Intracellular bacterial load was determined by three independent CFU counts. Effects observed at 2 µg/mL CDCHD rely on the bactericidal and cytotoxic effect of CDCHD. Data represent mean ± standard deviation (SD). Images show cell morphology of control and treated RAW264.7 cells after 18 h.

### 3.1.1.2 STRUCTURE-ACTIVITY RELATIONSHIP

Several derivatives of CHD and CDCHD were generated through semisynthesis and genetic engineering (Figure 3-5, Table 3-11) and their antibacterial activities were tested against a panel of Gram-positive and Gram-negative bacteria including *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and *E. coli* TolC (efflux-deficient, Table 3-12). Positions $R_1$, $R_2$, $R_3$, $R_4$ and $R_5$ were chosen for derivatization (Figure 3-5).

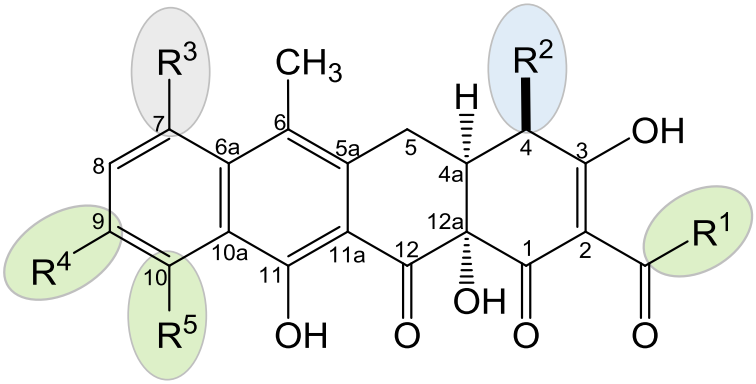



**Figure 3-5:** Positions used for CHD and CDCHD derivatives generated by semisynthesis (grey, blue ovals, compounds 12-25) or genetic engineering (green, blue ovals, compounds 3-11). Residues $R^1$-$R^5$ are listed in Table 3-11.

The efflux-deficient *E. coli* strain was virtually insusceptible to C-9 demethylated derivatives of CHD (**1**, **2**). With *S. aureus*, **1** and its C-4 epimer **2** displayed a 4- and 8-fold drop in MIC, respectively. Substitutions of $R^4$ to an *N*-dimethyl group (**3**-**5**) led to a drop in activity against Gram-negative bacteria of **5** by 16-fold, whereas **3** and **4** were inactive (MIC: > 64 µg/mL). Against *S. aureus*, **5** showed the same activity as the parent compound CDCHD, whereas **3** lost activity by 4-fold of the MIC value. Further derivatization of dimethylated derivatives by a glycosylation at position C-10 did not improve activity. Against *S. aureus*, **8** and **11** showed the same MIC values as determined for the parent dimethylated compound (4 µg/mL), whereas **9** and **10** were 16-fold less potent against Gram-positive bacteria. For all glycosylated and simultaneously dimethylated derivatives, activities against Gram-negative bacteria were completely abolished when tested against *E. coli* TolC. Furthermore, substitutions at C-4 by various functional groups (e.g. furoic acid, acetyl, biotin group **12**-**25**) led to a complete loss of activity against Gram-negative bacteria. Against *S. aureus*, **12**-**14**, **16**, **18** and **21**-**24** showed no improvement in comparison to CDCHD, but a 2- to 32-fold increased MIC value or a completely abolished activity

(MIC: > 64 µg/mL) was observed. Only **17** showed a slight improvement in activity against Gram-positive bacteria (2-fold increase) and **15**, **19** and **20** were as potent as the parent molecule CDCHD (4 µg/mL). Changes at C-7 by an addition of chlorine (**26**), bromine (**27**) or fluorine (**29**) led to a 2- to 8-fold decrease in Gram-negative activity and when tested against *S. aureus*, MIC values comparable to the parent compound CDCHD (4 µg/mL) were determined. Addition of a quinolone (**28**) or a nitrite (**30**) on C-7 were not tolerated and activity on all test Gram-negative bacteria was completely abolished (MIC: > 64 µg/mL). For several derivatives (**1**, **4**, **6**, **8**, **12**, **15**, **17**, **19**) the respective C-4 epimer (**2**, **5**, **7**, **9**, **13**, **16**, **18**, **20**) was available. Only for **8** and **17** the *S*-configured epimer was 16-fold less potent than the *R*-configured one and for all other derivatives, no difference in activities were observed when the compounds were compared to their respective epimer. However, it has to be noted that it cannot be excluded that spontaneous epimerization takes place under the test conditions.

**Table 3-11:** CHD and CDCHD derivatives generated by semisynthesis or genetic engineering. [a]Position used for derivatization ($R^1$ – $R^5$) are presented in Figure 3-5 (grey/green/blue ovals).

| Compound | Abbreviation | Position[a] | | | | |
|---|---|---|---|---|---|---|
| | | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ |
| CHD | CHD | $CH_3$ | $NH_2$ | H | $CH_3$ | OH |
| CDCHD | CDCHD | $NH_2$ | $NH_2$ | H | $CH_3$ | OH |
| desmethyl-CHD | **1** | $CH_3$ | $NH_2$ | H | H | OH |
| desmethyl-epi-CHD | **2** | $CH_3$ | $NH_2$ | H | H | OH |
| dimethyl-epi-CHD | **3** | $CH_3$ | $N(CH_3)_2$ | H | $CH_3$ | OH |
| dimethyl-CDCHD | **4** | $NH_2$ | $N(CH_3)_2$ | H | $CH_3$ | OH |
| dimethyl-epi-CDCHD | **5** | $NH_2$ | $N(CH_3)_2$ | H | $CH_3$ | OH |
| 586 | **6** | $CH_3$ | $N(CH_3)_2$ | H | $CH_3$ | Rhamnosyl |
| epi-586 | **7** | $CH_3$ | $N(CH_3)_2$ | H | $CH_3$ | Rhamnosyl |
| 572 | **8** | $CH_3$ | $N(CH_3)_2$ | H | H | Rhamnosyl |
| epi-572 | **9** | $CH_3$ | $N(CH_3)_2$ | H | H | Rhamnosyl |
| epi-587 | **10** | $NH_2$ | $N(CH_3)_2$ | H | $CH_3$ | Rhamnosyl |
| epi-573 | **11** | $NH_2$ | $N(CH_3)_2$ | H | H | Rhamnosyl |
| CDCHD-Phe | **12** | $NH_2$ | L-phenylalanine | H | $CH_3$ | OH |
| epiCDCHD-Phe | **13** | $NH_2$ | L-phenylalanine | H | $CH_3$ | OH |
| CDCHD-Asn | **14** | $NH_2$ | L-asparagine | H | $CH_3$ | OH |
| CDCHD-FuA | **15** | $NH_2$ | furoic acid | H | $CH_3$ | OH |
| epi-CDCHD-FuA | **16** | $NH_2$ | furoic acid | H | $CH_3$ | OH |
| CDCHD-AHA | **17** | $NH_2$ | aminohexanoic acid | H | $CH_3$ | OH |
| epi-CDCHD-AHA | **18** | $NH_2$ | aminohexanoic acid | H | $CH_3$ | OH |
| CDCHD-pFBA | **19** | $NH_2$ | *p*-fluorobenzoic acid | H | $CH_3$ | OH |
| epi-CDCHD-pFBA | **20** | $NH_2$ | *p*-fluorobenzoic acid | H | $CH_3$ | OH |
| CDCHD-NAc | **21** | $NH_2$ | acetyl | H | $CH_3$ | OH |
| CDCHD-$NSO_2$Me | **22** | $NH_2$ | methylsulfonyl | H | $CH_3$ | OH |
| CDCHD-CBz | **23** | $NH_2$ | benzyloxycarbonly | H | $CH_3$ | OH |
| CDCHD-biotin-A | **24** | $NH_2$ | biotin | H | $CH_3$ | OH |
| CDCHD-biotin-B | **25** | $NH_2$ | biotin | H | $CH_3$ | OH |
| CDCHD-Cl | **26** | $NH_2$ | $NH_2$ | Cl | $CH_3$ | OH |
| CDCHD-Br | **27** | $NH_2$ | $NH_2$ | Br | $CH_3$ | OH |
| CDCHD-Qu | **28** | $NH_2$ | $NH_2$ | quinolone | $CH_3$ | OH |
| CDCHD-F | **29** | $NH_2$ | $NH_2$ | fluor | $CH_3$ | OH |
| CDCHD-$NO_2$ | **30** | $NH_2$ | $NH_2$ | $NO_2$ | $CH_3$ | OH |

**Table 3-12:** MIC values of CHD and CDCHD derivatives against Gram-positive and Gram-negative bacteria. [a]Compounds (cpd) are listed in Table 3-11 and positions used for derivatization are presented in Figure 3-5; [b]TolC efflux deficient *E. coli; Sa: S. aureus*; *Kp: K. pneumoniae*; *Pa: P. aeruginosa*; n.d.: not determined.

| cpd [a] | MIC [µg/mL] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Sa* DSM-11822 | *Sa* Newman | *Sa* DSM-346 | *Kp* DSM-30104 | *Pa* PA14 | *Pa* DSM-1128 | *E. coli* TolC[b] |
| CHD | 4 | 4 | 8 | 2 | 16 | 64 | 0.5 |
| CDCHD | 4 | 4 | > 64 | 2 | 1 | 8 | 0.25 |
| **1** | 16 | n.d. | n.d. | n.d. | n.d. | n.d. | 64 |
| **2** | 32 | n.d. | n.d. | n.d. | n.d. | n.d. | 64 |
| **3** | 16 | n.d. | n.d. | > 64 | > 64 | n.d. | n.d. |
| **4** | n.d. | n.d. | n.d. | > 64 | n.d. | > 64 | n.d. |
| **5** | 4 | n.d. | n.d. | 32 | > 64 | n.d. | n.d. |
| **6** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | > 64 |
| **7** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. | > 64 |
| **8** | 4 | n.d. | n.d. | n.d. | n.d. | n.d. | > 64 |
| **9** | 64 | n.d. | n.d. | n.d. | n.d. | n.d. | > 64 |
| **10** | 64 | n.d. | n.d. | n.d. | n.d. | n.d. | > 64 |
| **11** | 4 | n.d. | n.d. | n.d. | n.d. | n.d. | > 64 |
| **12** | n.d. | 32 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **13** | n.d. | > 64 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **14** | n.d. | 64 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **15** | n.d. | 4 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **16** | n.d. | 8 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **17** | n.d. | 2 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **18** | n.d. | 32 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **19** | n.d. | 4 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **20** | n.d. | 4 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **21** | n.d. | n.d. | > 64 | > 64 | n.d. | > 64 | n.d. |
| **22** | n.d. | n.d. | > 64 | > 64 | n.d. | > 64 | n.d. |
| **23** | n.d. | n.d. | > 64 | > 64 | n.d. | > 64 | n.d. |
| **24** | n.d. | > 64 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **25** | > 256 | 128 | n.d. | > 256 | n.d. | n.d. | > 256 |
| **26** | n.d. | 4 | n.d. | 8 | n.d. | 16 | n.d. |
| **27** | n.d. | 8 | n.d. | 8 | n.d. | 32 | n.d. |
| **28** | n.d. | 32 | n.d. | > 64 | n.d. | > 64 | n.d. |
| **29** | 4 | n.d. | n.d. | 8 | 8 | n.d. | n.d. |
| **30** | n.d. | n.d. | > 64 | > 64 | n.d. | > 64 | n.d. |

### 3.1.1.3 CYTOTOXICITY

Cytotoxicity against human (HCT-116: colon carcinoma; HepG2: hepatocellular carcinoma; THP-1: acute monocytic leukemia; U-2 OS: bone osteosarcoma) and murine (RAW264.7: leukemia macrophages; L929: subcutaneous connective tissue, fibroblasts) eukaryotic cell lines was determined by tetrazolium salt-based assays (MTT) after 5 d treatment of cells with CHD and CDCHD in serial dilution. The half-inhibitory concentration ($IC_{50}$) was determined by sigmoidal curve fitting using Origin 6.1G software.

Against all tested human cell lines, CHD showed moderate toxicity with $IC_{50}$ values around 20 µg/mL, whereas CDCHD showed $IC_{50}$ values between 1 and 5 µg/mL (Table 3-13). Murine cell lines were less affected when tested against CHD ($IC_{50}$: 50-60 µg/mL) and CDCHD generally displayed more pronounced inhibitory effects ($IC_{50}$: 4-12 µg/mL).

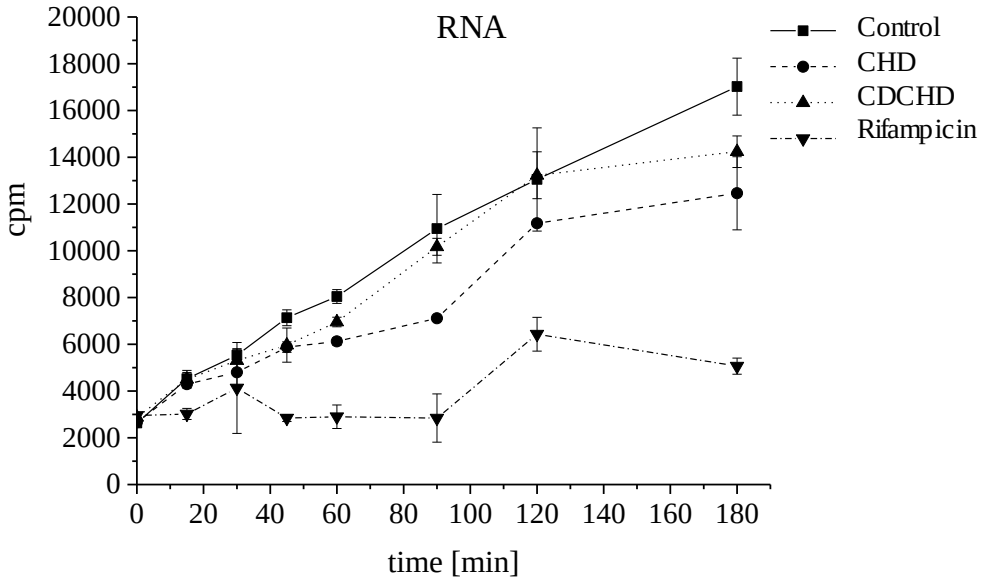
**Table 3-13:** $IC_{50}$ values of CHD and CDCHD against human and murine cell lines.

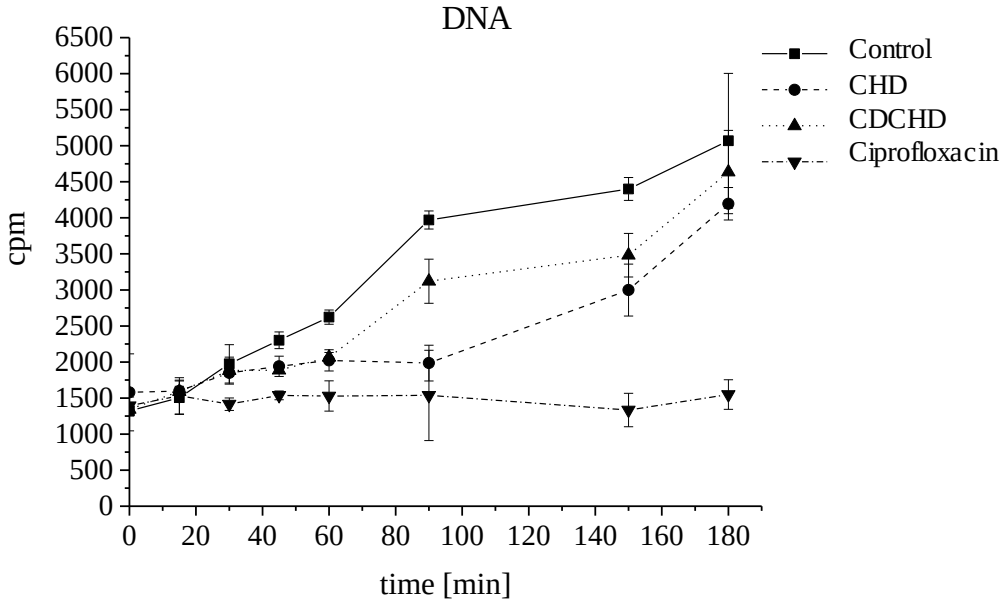
| Cell line | $IC_{50}$ [µg/mL] | |
|---|---|---|
| | CHD | CDCHD |
| Human | | |
| HCT-116 | 18.06 | 2.91 |
| HepG2 | 22.97 | 1.15 |
| THP-1 | 23.08 | 4.98 |
| U-2 OS | 16.09 | 4.98 |
| Murine | | |
| L929 | 58.54 | 11.63 |
| RAW264.7 | 47.74 | 3.74 |

### 3.1.1.4 MACROMOLECULE BIOSYNTHESIS

To examine the effect of CHD and CDCHD on essential cellular processes, macromolecule synthesis (MMS) assays were carried out and the incorporation of radioactive precursor molecules in *S. carnosus* DSM-20501 was assessed. The incorporation of $^{3}$H-uridine (RNA), $^{3}$H-thymidine (DNA), $^{3}$H-methionine (protein) and $^{3}$H-glucosamine (peptidoglycan) was determined over 180 min in cells treated with CHD and CDCHD at 0.5-fold MIC. Nisin, rifampicin, TET and ciprofloxacin served as positive controls for peptidoglycan, RNA, protein and DNA synthesis, respectively. For RNA, DNA and protein synthesis, CHD and CDCHD showed an unspecific slight inhibitory effect but final incorporation levels as determined by scintillation counts nearly reached the values of untreated control cells after 180 min of treatment (Figure 3-6). However, cell wall synthesis was delayed by

approximately 60 min compared to control cells in both cases (CHD and CDCHD treatment), but no complete inhibition was observed.



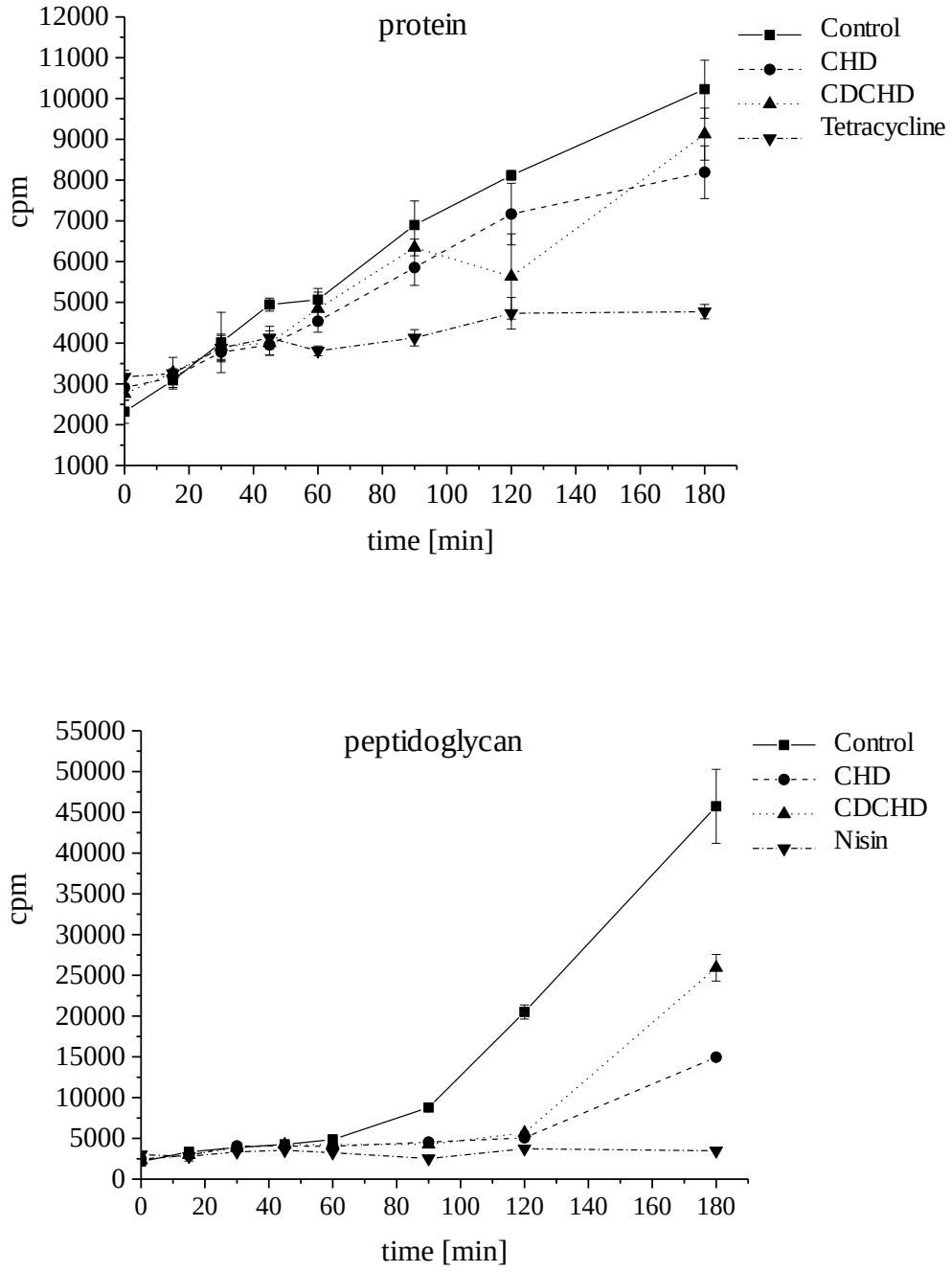



**Figure 3-6:** Effects of CHD and CDCHD on synthesis of macromolecules. *S. carnosus* DSM-20105 was treated with 0.5-fold MIC (MIC: 4 µg/mL) of CHD and CHD over 180 min and incorporation of and $^{3}$H-glucosamine (peptidoglycan), $^{3}$H-uridine (RNA), $^{3}$H-methionine (protein) and $^{3}$H-thymidine (DNA) was determined. Nisin (4-fold MIC), rifampicin (2-fold MIC), TET (4-fold MIC) and ciprofloxacin (2-fold MIC) served as positive controls, respectively. Radioactivity was measured in counts per minute (cpm) and data are represented as mean (n=3) ± standard deviation (SD).

### 3.1.1.5 *IN VITRO* TRANSLATION INHIBITION

Effects of CHD and CDCHD on translation compared to TET were examined using the RTS 100 *E. coli* HY Kit (5PRIME). For CHD and CDCHD a concentration range between 50 and 150 µM was chosen and TET was tested at concentrations between 3.06 and 50 µM. After 1 h exposure to compounds, luminescence was recorded and inhibition on *in vitro* translation was observed for all compounds, but the effect of CHD and CDCHD was less pronounced than observed for TET (Figure 3-7). Inhibition of translation by TET was already observed at 20 µM (approximate half-inhibitory concentration, $IC_{50}$), whereas CHD and CDCHD concentrations of ≥ 100 µM were needed to trigger similar inhibitory effects.

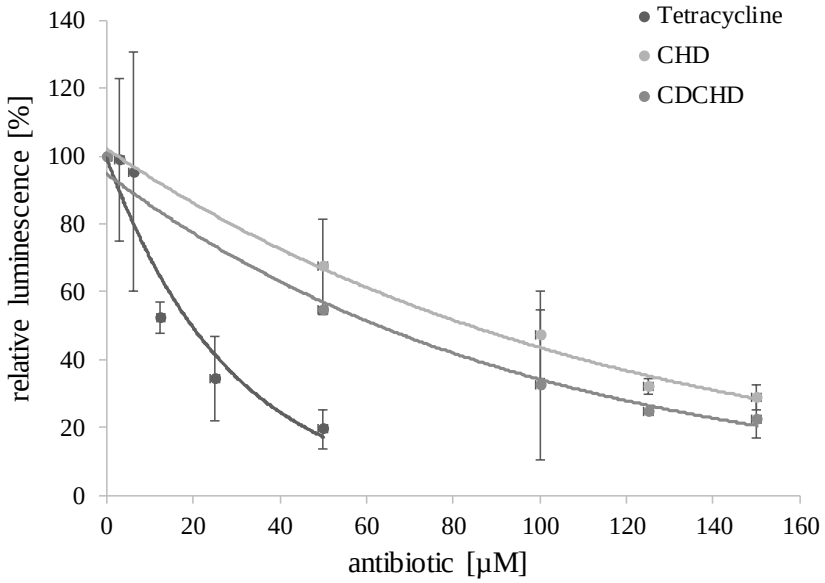



**Figure 3-7:** Effects of CHD, CDCHD and TET on *in vitro* translation. Luminescence was measured after 1 h of treatment with CHD and CDCHD at concentrations between 50 and 150 µM and TET between 3.06 and 50 µM. Data are represented as mean (n=3) ± standard deviation (SD).

### 3.1.1.6 MEMBRANE DEPOLARIZATION

Changes on the membrane potential (MP) after treatment with CHD, CDCHD and TET were examined using the BacLight™ Bacterial Membrane Potential Kit (Invitrogen). *S. aureus* Newman was exposed to compounds at 0.5-, 2- and 4-fold MIC and MP was measured after 0.5 and 2 h of treatment. To determine the MP, samples were stained with the cyanine dye 3,3‘-diethyloxacarbocyanin iodide ($DiOC_2(3)$) for 30 min and fluorescence was measured using a microplate reader [excitation.: 488nm; emission: 675 nm (green), 525 nm (red)]. In cells with intact MP, an intracellular accumulation of $DiOC_2(3)$ is observed and the emission

maximum turns towards red. A collapse of the MP leads to a decrease of the intracellular dye concentration and the emission maximum shifts towards green fluorescence. MP was calculated by the ratio of red to green fluorescence relative to untreated control cells. The proton ionophore carbonyl cyanide 3- chlorophenylhydrazone (CCCP) served as positive control.[185,186] Cells treated with 0.5-fold MIC of either CHD or CDCHD exerted only minor effects on the MP compared to untreated cells, displayed by a reduction of the MP by 10 % and 15 %, respectively (Figure 3-8). An increase of CHD and CDCHD concentrations up to 4-fold MIC led to a more pronounced effect showing a time- and concentration-dependent increase of membrane depolarization. After 2 h treatment at 2-fold MIC, CHD and CDCHD led to an MP decrease of 60 % and 70 %, respectively, approaching the effect caused by CCCP (90 % depolarization). For TET, neither a concentration- nor a time-dependent change of the MP was observed. MP of cells treated with TET showed an overall constant MP with a minor decline of 20 % at all tested conditions.

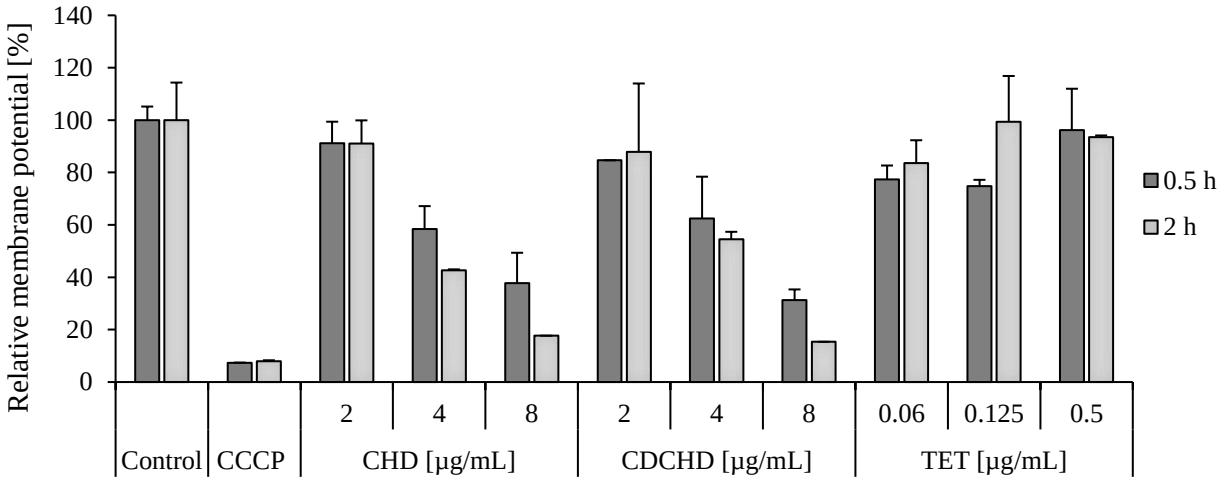



**Figure 3-8:** Membrane depolarization of *S. aureus* Newman caused by CHD (MIC: 4 µg/mL), CDCHD (MIC: 4 µg/mL) and TET (MIC: 0.125 µg/mL). Cells were treated with CHD, CDCHD and TET at 0.5-, 1- and 2- fold MIC for 0.5 and 2 h. CCCP (15 µM) served as positive control. Results show membrane potential relative to untreated cells and are represented as mean ± standard deviation (SD) of three independent samples. CCCP: carbonyl cyanide 3- chlorophenylhydrazone.

### 3.1.1.7 OUTER MEMBRANE PERMEABILIZATION

Permeabilization of the outer membrane was evaluated by the uptake of 1-*N*-phenylnaphtylamine (NPN). The lipophilic fluorescent probe NPN fluoresces strongly in

hydrophobic environments and weakly in aqueous environments. The lipopolysaccharide (LPS) layer of the outer membrane of intact cells serves as barrier to external lipophilic substances and a disruption of the membrane integrity enables NPN to pass into the hydrophobic lipid interior of outer and inner membrane, which in turn leads to an increase in fluorescence.[187,188] *E. coli* DSM-1116 was treated with CHD, CDCHD and TET at 0.5-, 1- and 2-fold MIC and kanamycin (KAN) and polymyxin B (PMB) served as negative and positive controls, respectively. After 2 h treatment, NPN was added and fluorescence was monitored within three minutes using a microplate reader [excitation: 355 nm; emission: 405 nm]. Effects on membrane permeability were represented as the relative NPN uptake factor, calculated as described in part 2.2.7. No increased uptake was observed when cells were treated with CHD, CDCHD, TET or KAN at all tested conditions, indicating no membrane permeabilizing effects (Figure 3-9). For PMB, membrane permeabilizing was observed, represented by an increased uptake of NPN compared to untreated control cells.

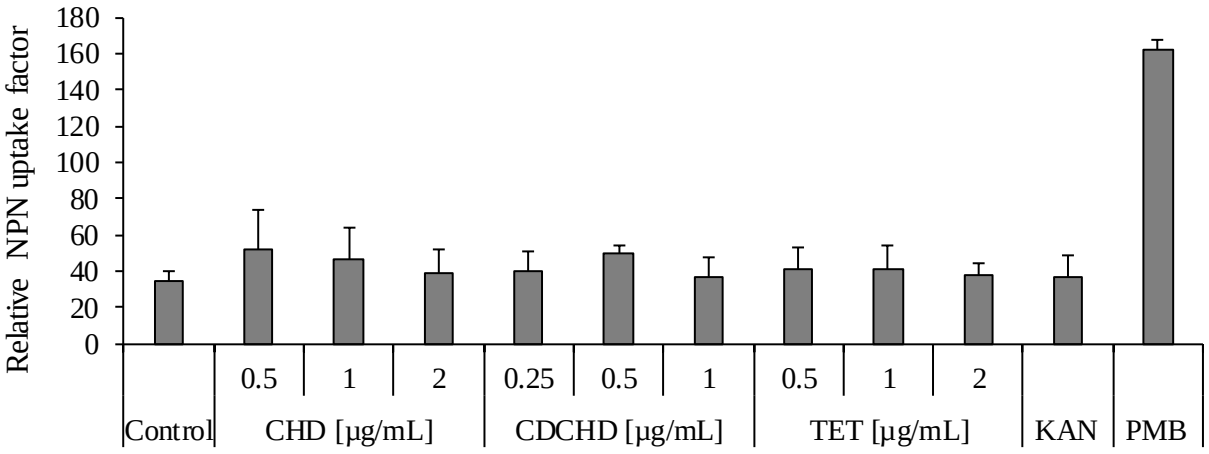



**Figure 3-9:** Membrane permeabilizing effects of CHD (MIC: 1 µg/mL), CDCHD (MIC: 0.5 µg/mL) and TET (MIC: 1 µg/mL). KAN and PMB (MIC for both: 4 µg/mL) served as negative and positive controls, respectively. *E. coli* DSM-1116 was treated with CHD, CDCHD and TET at 0.5-, 1- and 2- fold MIC, and KAN and PMB at 2-fold MIC for 2 h. Results show relative NPN uptake factors and data are represented as mean ± standard deviation (SD) of three independent samples. CHD: chelocardin; CDCHD: 2-carboxamindo-2-deacetyl-CHD; TET: tetracycline: KAN: kanamycin; PMB: polymyxin B.

Nevertheless, further studies on membrane permeabilization in an 18 h assay revealed effects on the membrane integrity of Gram-negative species by CHD comparable to those mediated by polymyxins. The effect of increased permeability towards hydrophobic agents was assessed by determining the MIC value of rifampicin against *A. baumannii* DSM-30008 in

the presence of CHD at sub-inhibitory concentrations. The addition of CHD led to an 8-fold increased MIC value compared to the activity of rifampicin in test medium without enhancer antibiotic; the same effect was observed when polymyxin B nonapeptide (PMBN) was present (Table 3-14).

**Table 3-14:** MIC values of rifampicin against *A. baumannii* DSM-30008 in the presence of CHD and PMBN (polymyxin B nonapeptide).

| Test medium | MIC of rifampicin [µg/mL] |
|---|---|
| CAMHB | 8 |
| + 3 µg/mL PMBN | 1 |
| + 1 µg/mL CHD | 1 |

### 3.1.2 RESISTANCE MECHANISM

The development of resistance against CHD was investigated by the generation of resistant mutants in two approaches with different wildtype strains. Despite several attempts, it was not possible to obtain CDCHD-resistant mutants.

#### 3.1.2.1 CHD-RESISTANT *ESCHERICHIA COLI* MUTANTS

Wildtype *E. coli* DSM-1116 was exposed to stepwise increasing CHD concentrations (2.5, 5, 7.7, 12.5, 15, 20, 30 µg/mL) obtaining seven independent resistant mutants (Mt30.1, Mt30.2, Mt30.4, Mt30.5, Mt30.6, Mt30.8, Mt30.10). At 30 µg/mL CHD, all mutants were able to grow but a concurrent fitness loss was observed, reflected in generation times of 2-3 weeks, compared to the wildtype which showed a normal growth behaviour. Cross-resistance was assessed directly after mutants had been generated and resistance against CHD was confirmed due to an observed 8- to ≥ 64-fold increase of determined MIC values (Table 3-15). All mutants were only 2- to 8-fold cross-/co-resistant to CDCHD, tetracycline, tigecycline, rifampicin and ciprofloxacin. With all other tested standard antibiotics, no changes in susceptibility of the mutants compared to the wildtype were observed.

Genomic DNA of wildtype and four mutants (Mt30.1, Mt30.2, Mt30.4, Mt30.8) was prepared by standard phenol-chloroform isolation and genome sequencing was performed. *In silico* comparison of genomic data from wildtype to mutants revealed the presence of serval single-nucleotide polymorphisms (SNPs) or frameshift mutations (FS) within the mutant genomes (Table 3-16). In total, 19 SNPs and 5 FS were present, but changes were

not consistent as no common mutant genotype was present. Of all SNPs, six were found in upstream regions without impact on amino acid sequences (of the respective genes; marked with °). Additionally, Mt30.2 and Mt.30.8 lost a naturally occurring 100kb plasmid, which was still present in Mt.30.1 and Mt30.8 as well as within the wildtype.

**Table 3-15:** Resistance pattern of *E. coli* CHD-resistant mutants (Mt30). Wt: *E. coli* DSM-1116 wildtype.

| Antibiotic compound | MIC [µg/mL] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Wt | Mt30.1 | Mt30.2 | Mt30.4 | Mt30.5 | Mt30.6 | Mt30.8 | Mt30.10 |
| CHD | 2 | 8 | 8 | 16 | 8 | > 64 | 16 | 8 |
| CDCHD | 0.5 | 2 | 2 | 2 | 1 | 2 | 4 | 2 |
| Tetracycline | 1 | 2 | 2 | 2 | 2 | 2 | 4 | 4 |
| Tigecycline | 1 | 2 | 1 | 2 | 4 | 1 | 2 | 2 |
| Rifampicin | 8 | 32 | 32 | 16 | 16 | 16 | 16 | 32 |
| Kanamycin | 4 | 8 | 4 | 4 | 2 | 2 | 1 | 4 |
| Erythromycin | 32 | > 64 | > 64 | 64 | 32 | 64 | 64 | > 64 |
| Polymyxin B | 0.5 | 0.5 | 1 | 0.5 | 1 | 0.25 | 0.5 | 0.5 |
| Chloramphenicol | 4 | 8 | 8 | 8 | 8 | 8 | 8 | 16 |
| Ciprofloxacin | 0.006 | 0.05 | 0.025 | 0.0125 | 0.0125 | 0.025 | 0.0125 | 0.05 |
| Vancomycin | > 128 | > 128 | > 128 | > 128 | 128 | 128 | > 128 | > 128 |
| Ampicillin | > 64 | > 64 | 64 | 32 | 64 | > 64 | > 64 | > 64 |

**Table 3-16:** List of mutations identified in CHD-resistant *E. coli* DSM-1116 mutants. °mutation found in upstream region of locus without impact on amino acid sequence; FS: frameshift; amino acids are denoted by three letter code, base pairs as single letter.

| Locus | Gene product and function | Mutation (base pair/amino acid #) | | | |
|---|---|---|---|---|---|
| | | Mt30.1 | Mt30.2 | Mt30.4 | Mt30.8 |
| ECW_m0440 | TauB: ATP-binding subunit of a taurine transporter[189] | | | Gln→His (3) | |
| ECW_m0536° | Putative operator region of AcrR[190,191]: repressor of AcrAB MDR efflux system[192–194] | A→C (32) | | | |
| ECW_m0536 | AcrR: repressor of AcrAB MDR efflux system[192–194] | | FS (190) | | |
| ECW_m0905 | Protein belonging to XerC family of integrase proteins | | | | Lys→Asn (5) |
| ECW_m1039 | OmpF: outer membrane porin F[195–197] | FS (123) | | | |
| ECW_m1113 | Protein with uncharacterized function | Lys→Asn (41) | | | |
| ECW_m1122 | RutA: pyrimidine monooxygenase; involved in the degradation of pyrimidine.[198–200] | | Asp→Asn (284) | | |
| ECW_m1152 | Protein with uncharacterized function | Gln→Leu (87) | | | |
| ECW_m1237 | PhoQ: sensor histidine kinase in two-component regulatory system with PhoP[201,202] | | | Ile→Asn (207) | |
| ECW_m1493° | Hypothetical protein (containing a lytic transglycosylase[203]) | | | | A→T (27) |
| ECW_m1551 | LysR-type transcriptional regulator | | | | FS (191) |
| ECW_m1820 | Putative ATP-dependent helicase | FS (1420) | | | |
| ECW_m2002 | ProQ: RNA chaperon[204] | | | Ser→Leu (219) | |
| ECW_m2153 | Shikimate transporter (shikimic acid, involved in the biosynthesis of aromatic amino acids[205]) | | Thr→Asn (270) | | |
| ECW_m2336 | PBP7: D-alanyl-D-alanine hydrolase (involved in cell wall synthesis[206,207]) | | Gly→Gly (195) | | |
| ECW_m2367° | kinase: catalyses phosphorylation of pseudouridine[208,209] | A→C (126) | | | |
| ECW_m3454 | PBP4: D-alanyl-D-alanine hydrolase (involved in cell wall synthesis[206,207]) | | | Val→Ile (287) | |
| ECW_m3617 | protein involved in filamentation induced by cyclic adenosine monophosphate[210,211] | | Arg→Gln (146) | | |
| ECW_m3661 | EnvZ: sensor histidine kinase in two-component regulatory system with OmpR[212,213] | | | | Leu→Ile (189) |
| ECW_m3728° | Hypothetical protein (uncharacterized) | | C→A (69) | | |
| ECW_m4224° | Protein of XRE (xenobiotic response element) family of transcriptional regulators (comprise a helix-turn-helix DNA-binding motif.[214,215]) | | | | G→A (51) |

To further investigate the mechanism underlying resistance exerted by CHD-resistant mutants (Table 3-15), activities of all sequenced mutants (Mt30.1, Mt30.2, Mt30.4, Mt30.8) were determined in the presence of the efflux pump inhibitor phenylalanine arginine β-naphthylamide dihydrochloride (PAβN) and in the presence of the membrane permeablizing agent PMBN. Susceptibility of all mutants against CHD, CDCHD, TET, ciprofloxacin and rifampicin increased in the presence of PAβN and MIC values approached activities determined for the wildtype (Table 3-17). The permeabilization of the outer membrane by PMBN led to similar result for all mutants, as an increase in susceptibility against all tested compounds was observed (Table 3-18). However, effects against rifampicin and ciprofloxacin were less pronounced than observed in the presence of PAβN and mutant Mt30.1 and Mt30.2, still showed co-resistance with ciprofloxacin.

**Table 3-17:** MIC values of selected antibiotics against *E. coli* DSM-1116 wildtype and CHD-resistant *E. coli* DSM-1116 mutants (Mt30.) in the presence of phenylalanine arginine β-naphthylamide dihydrochloride (PAβN).

| Compound | MIC [µg/mL] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wildtype | | Mt30.1 | | Mt30.2 | | Mt30.4 | | Mt30.8 | |
| | - | + PAβN | - | + PAβN | - | + PAβN | - | + PAβN | - | + PAβN |
| CHD | 2 | 2 | 8 | 4 | 8 | 2 | 16 | 1 | 16 | 0.5 |
| CDCHD | 0.5 | 0.25 | 2 | 0.5 | 2 | 0.25 | 2 | 0.125 | 4 | 0.25 |
| Tetracycline | 1 | 1 | 2 | 2 | 2 | 0.5 | 2 | 0.5 | 4 | 0.5 |
| Tigecycline | 1 | 2 | 2 | 2 | 1 | 1 | 2 | 4 | 2 | 1 |
| Ciprofloxacin | 0.006 | 0.006 | 0.05 | 0.006 | 0.025 | 0.006 | 0.0125 | 0.006 | 0.0125 | 0.003 |
| Rifampicin | 2 | ≤ 0.03 | 32 | ≤ 0.03 | 32 | ≤ 0.03 | 32 | ≤ 0.03 | 16 | ≤ 0.03 |

**Table 3-18:** MIC values of selected antibiotics against *E. coli* DSM-1116 wildtype and CHD-resistant *E. coli* DSM-1116 mutants (Mt30.) in the presence polymyxin B nonapeptide (PMBN).

| Compound | MIC [µg/mL] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wildtype | | Mt30.1 | | Mt30.2 | | Mt30.4 | | Mt30.8 | |
| | - | + PMBN | - | + PMBN | - | + PMBN | - | + PMBN | - | + PMBN |
| CHD | 2 | 2 | 8 | 4 | 8 | 2 | 16 | 2 | 16 | 0.5 |
| CDCHD | 0.5 | 0.25 | 2 | 0.5 | 2 | 0.5 | 2 | 0.25 | 4 | 0.5 |
| Tetracycline | 1 | 2 | 2 | 2 | 2 | 0.5 | 2 | 1 | 4 | 0.5 |
| Tigecycline | 1 | 2 | 2 | 2 | 1 | 2 | 2 | 4 | 2 | 2 |
| Ciprofloxacin | 0.006 | 0.006 | 0.05 | 0.025 | 0.025 | 0.025 | 0.0125 | 0.006 | 0.0125 | 0.006 |
| Rifampicin | 2 | 0.5 | 32 | 0.25 | 32 | 0.5 | 32 | 0.5 | 16 | 0.125 |

### 3.1.2.2 CHD-RESISTANT *KLEBSIELLA PNEUMONIAE* MUTANTS

In a second *in vitro* resistance development experiment, nine independent CHD-resistant *K. pneumoniae* mutants were generated by exposing wildtype strain *K. pneumoniae* DSM-30104 to stepwise increasing CHD concentrations (2, 4, 8 µg/mL). At 8 µg/mL all mutants were able to grow and no differences regarding fitness, compared to the wildtype were observed. Over 24 h, all mutants showed a normal, exponential growth behaviour with final cell densities comparable to the parent wildtype strain (Figure 3-10).

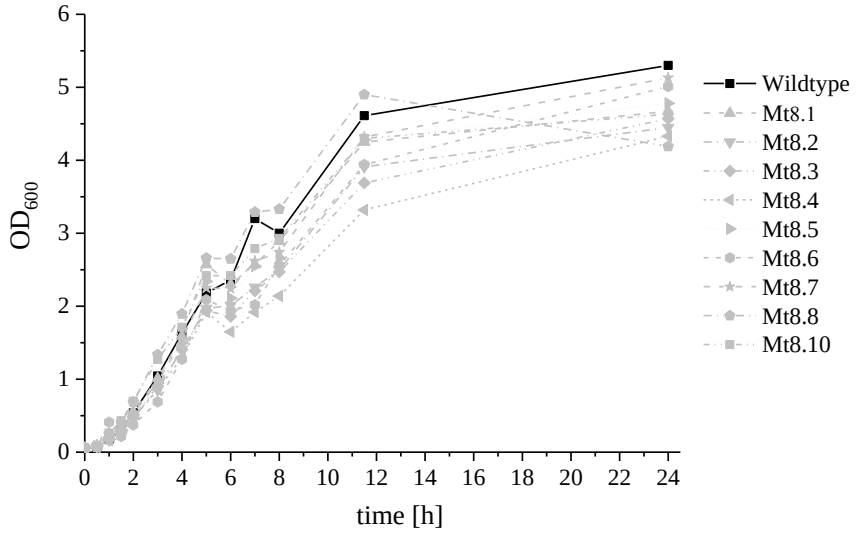



**Figure 3-10:** Growth curves of *K. pneumoniae* DSM-30104 wildtype and CHD-resistant mutants (Mt) over 24 h. Data represent $OD_{600}$ values at each time point.

Co-/Cross-resistance studies revealed resistance of all nine mutants against CHD as well as against chloramphenicol, ciprofloxacin and all other tested tetracyclines, but not against CDCHD (Table 3-19). Against erythromycin, co-resistance was observed as well, except for Mt8.3 and Mt8.7, which were more sensitive towards erythromycin compared to all other mutants. Against polymyxin B an increased sensitivity of all mutants was observed, represented by up to 16-fold increased activity compared to the wildtype. Against all other tested antibiotics, no co-resistance was observed.

To characterize the mechanism underlying resistance, genome sequencing of all mutants and the wildtype strain was performed. An *in silico* comparison of the wildtype genome to the mutants genomes revealed mutations to be present within the regulatory gene *ramR* (KPD_37740) in all nine mutants (Table 3-20). Mutations were identified as deletions (Mt.8.2, Mt8.3, Mt8.4, Mt.8.5, Mt8.6, Mt8.10), SNPs (Mt8.8) or insertions (Mt8.1, Mt8.7).

Additionally, a repeat expansion within a phosphoglyceromutase of Mt8.2 and Mt8.3 (KPD_35200) and a SNP within an ABC transporter permease of Mt8.7 (KPD_11910) were present.

**Table 3-19:** Resistance pattern of *K. pneumoniae* CHD-resistant mutants (Mt). Wt: *K. pneumoniae* DSM-30104 wildtype.

| Antibiotic compound | MIC [µg/mL] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wt | Mt8.1 | Mt8.2 | Mt8.3 | Mt8.4 | Mt8.5 | Mt8.6 | Mt8.7 | Mt8.8 | Mt8.10 |
| CHD | 2 | 16 | 8 | 16 | 16 | 32 | 16 | 16 | 16 | 8 |
| CDCHD | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Tetracycline | 4 | 64 | 32 | 64 | 64 | 64 | 64 | 64 | 16 | 32 |
| Minocycline | 4 | 64 | 64 | 64 | 64 | 64 | > 64 | 64 | > 64 | 64 |
| Oxytetracycline | 2 | 32 | 32 | 32 | 32 | 32 | 16 | 32 | 16 | 8 |
| Tigecycline | 0.125 | 1 | 2 | 2 | 2 | 1 | 2 | 2 | 1 | 4 |
| Rifampicin | 8 | 16 | 16 | 16 | 16 | 16 | 8 | 16 | 8 | 8 |
| Kanamycin | 2 | 2 | 2 | 1 | 1 | 1 | 0.5 | 2 | 1 | 0.5 |
| Erythromycin | 8 | 32 | 32 | 16 | 32 | 32 | 32 | 8 | 32 | 32 |
| Polymyxin B | 4 | 0.5 | 0.5 | 0.25 | 0.25 | 0.5 | 0.5 | 1 | 1 | 0.5 |
| Chloramphenicol | 1 | 32 | 32 | 32 | 16 | 8 | 8 | 16 | 16 | 8 |
| Ciprofloxacin | < 0.03 | 0.125 | 0.025 | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 | 0.125 |
| Vancomycin | > 64 | > 64 | 64 | 64 | 64 | 64 | 64 | > 64 | > 64 | > 64 |
| Ampicillin | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 |

**Table 3-20:** Mutations identified in CHD-resistant *K. pneumoniae* DSM-30104 mutants. Ins: insertion; Δ: deletion; bp: base pair; #: number of affected bp; number in brackets denote number of affected amino acid (denoted in three letter code): RE: repeat expansion.

| Locus | Gene product | mutation | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mt8.1 | Mt8.2 | Mt8.3 | Mt8.4 | Mt8.5 | Mt8.6 | Mt8.7 | Mt8.8 | Mt8.10 |
| KPD_37740 | RamR: TetR family transcriptional regulator | 11bp Ins | Δ4bp | Δ4bp | Δ4pb | Δ#550; 11bp Ins | Δ#550 | 11bp Ins | Ser→ Lys (44) | Δ#550 |
| KPD_35200 | Phosphoglycero-mutase | | 2x → 3x 7bp RE | 2x → 3x 7bp RE | | | | | | |
| KPD_11910 | branched-chain amino acid ABC transporter permease | | | | | | | Gly→ Ala(231) | | |

Further characterization focused on the function of *ramR*, as mutations within this gene were common within all mutants. A *K. pneumoniae* DSM-30104 *ramR* knockout mutant (KPΔ*ramR*) was generated with a λ-red based knock-out system and the gene knock-out was confirmed by sequencing. As expected, KPΔ*ramR* was less susceptible towards CHD with an 8-fold decrease in activity compared to wildtype *K. pneumoniae* and no significant changes in sensitivity were observed when CDCHD activity was determined (Table 3-21).

**Table 3-21:** Activity of *K. pneumoniae* DSM-30104 wildtype and RamR knockout mutant (Δ*ramR*) against CHD and CDCHD.

| *K. pneumoniae* DSM-30104 | MIC [µg/mL] | |
|---|---|---|
| | CHD | CDCHD |
| Wildtype | 4 | 4 |
| Δ*ramR* | 32 | 8 |

To explore the regulatory function of RamR with respect to the AcrAB-TolC efflux system, transcriptional levels of *ramA* (encoding RamA; positive regulator of AcrAB-TolC efflux system), *acrA* and *acrB* (encoding AcrA and AcrB, respectively; subunits of AcrAB-TolC efflux system) were analysed. Gene expression of all nine CHD-resistant *K. pneumoniae* mutants and KPΔ*ramR* was measured by qPCR and relative transcription levels compared to the wildtype were calculated by the $2^{-\Delta\Delta Ct}$ method[173] and normalized to 16S rRNA. Compared to the wildtype, upregulation of *ramA* up to 41-fold with a concurrent upregulation of *acrA* and *acrB* up to 12- and 67-fold, respectively (Table 3-22) was found within all mutants, indicating increased efflux mediated by AcrAB.

**Table 3-22:** Relative transcription levels of *ramA*, *acrA* and *acrB* genes of CHD-resistant *K. pneumoniae* mutants in comparison to *K. pneumoniae* DSM-30104 wildtype.

| *K. pneumoniae* DSM-30104 | Relative transcription level (fold change) | | |
|---|---|---|---|
| | *ramA* | *acrA* | *acrB* |
| Wildtype | 1 | 1 | 1 |
| Δr*amR* | 17.43 ± 2.42 | 1.38 ± 1.17 | 19.75 ± 0.73 |
| Mt8.1 | 18.47 ± 0.87 | 1.58 ± 0.74 | n.d. |
| Mt8.2 | 41.08 ± 1.05 | 11.78 ± 1.89 | 66.64 ± 0.58 |
| Mt8.3 | 14.01 ± 0.91 | 2.76 ± 0.73 | n.d. |
| Mt8.4 | 8.19 ± 0.55 | 8.40 ± 1.07 | 4.91 ± 0.80 |
| Mt8.5 | 23.46 ± 1.11 | 2.58 ± 0.71 | 1.41 ± 0.65 |
| Mt8.6 | 31.13 ± 1.30 | 4.33 ± 1.97 | 1.96 ± 1.13 |
| Mt8.7 | 17.78 ± 0.94 | 4.62 ± 2.11 | 1.28 ± 1.13 |
| Mt8.8 | 3.16 ± 0.97 | 9.37 ± 0.71 | 1.47 ± 0.94 |
| Mt8.10 | 10.40 ± 1.08 | 3.13 ± 1.87 | 1.28 ± 0.85 |

MIC values of all mutants were re-examined in the presence of PAβN and as expected, all mutants showed increased sensitivity towards CHD and no changes were observed in activity of CDCHD (Table 3-23). Additionally, MIC values against tetracycline, tigecycline, chloramphenicol and ciprofloxacin were determined and all mutants showed activity values comparable to the wildtype, indicating that former observed co-/cross-resistance (Table 3-19) relies on efflux. The inhibition of efflux pumps within the wildtype had no effect on activity, as same values were determined with and without PAβN. A comparison of MIC values of wildtype to mutants in the presence of PAβN showed a general recovery of activity upon efflux pump inhibition, however data indicated that a complete inhibition of efflux might have been not achieved, since mutants were overall less sensitive than the wildtype. However, higher concentrations of PAβN could induce unspecific toxic effects on the cells.[216]

**Table 3-23:** Activity of *K. pneumoniae* DSM-30104 wildtype and CHD-resistant mutants in the presence of phenylalanine arginine β-naphthylamide dihydrochloride (PAβN). TET: tetracycline; TIG: tigecycline; CM: chloramphenicol; CIP: ciprofloxacin.

| *K. pneumoniae* DSM-30104 | PAβN | MIC [µg/ml] | | | | | |
|---|---|---|---|---|---|---|---|
| | | CHD | CDCHD | TET | TIG | CM | CIP |
| Wildtype | - | 2 | 1 | 2 | 0.125 | 1 | ≤ 0.03 |
| | + | 2 | 1 | 2 | 0.25 | 2 | ≤ 0.03 |
| Δ*ramR* | - | 16 | 4 | 8 | 2 | 8 | 0.06 |
| | + | 8 | 2 | 4 | 0.25 | 1 | ≤ 0.03 |
| Mt8.1 | - | 8 | 2 | 16 | 1 | 32 | 0.125 |
| | + | 8 | 2 | 4 | 0.125 | 2 | ≤ 0.03 |
| Mt8.2 | - | 16 | 2 | 32 | 2 | 32 | 0.25 |
| | + | 8 | 2 | 4 | 0.5 | 2 | ≤ 0.03 |
| Mt8.3 | - | 16 | 2 | 32 | 2 | 32 | 0.125 |
| | + | 8 | 2 | 4 | 0.125 | 1 | ≤0.03 |
| Mt8.4 | - | 16 | 2 | 16 | 2 | 16 | 0.125 |
| | + | 2 | 2 | 4 | 0.25 | 1 | ≤ 0.03 |
| Mt8.5 | - | 16 | 2 | 16 | 1 | 8 | 0.125 |
| | + | 4 | 2 | 4 | 0.25 | 0.5 | ≤ 0.03 |
| Mt8.6 | - | 16 | 2 | 16 | 2 | 8 | 0.125 |
| | + | 4 | 2 | 2 | 0.5 | 1 | ≤ 0.03 |
| Mt8.7 | - | 16 | 2 | 32 | 2 | 16 | 0.125 |
| | + | 2 | 1 | 4 | 0.125 | 2 | ≤ 0.03 |
| Mt8.8 | - | 8 | 2 | 16 | 1 | 16 | 0.125 |
| | + | 4 | 1 | 2 | 0.125 | 1 | ≤ 0.03 |
| Mt8.10 | - | 16 | 2 | 32 | 4 | 8 | 0.125 |
| | + | 8 | 1 | 4 | 0.5 | 1 | ≤ 0.03 |

To confirm that CHD resistance mechanisms and co-resistance with ciprofloxacin and rifampicin are not related to mechanisms commonly observed for the reference antibiotics, activities against rifampicin-resistant ($RIF^R$) *S. aureus* Newman, ciprofloxacin-resistant *E. coli* (*E. coli* WT-3) and *marR*-deficient *E. coli* (*E. coli* WT-III; upregulated efflux) were determined. Against *S. aureus* $RIF^R$ [mutation within *rpoB*; encoding RNA polymerase β-subunit] no co-resistance with CHD or any other tested tetracycline was observed. Resistance mediated by mutation of *gyrA* [encoding DNA gyrase A-subunit] led also to no co-resistance for CHD or any other tested antibiotic when activity was tested against *E. coli* WT-3 (Table 3-24). Against *E. coli* WT-III, an 8-fold decrease in activity was observed for CHD and CDCHD, and tigecycline showed 4-fold less activity compared to wildtype *E. coli* (*E. coli* WT), supporting the assumption that resistance relies on efflux events.

**Table 3-24:** Minimal inhibitory concentrations (MIC) of selected antibiotics against *S. aureus* Newman wildtype, [a]rifampicin-resistant *S. aureus* Newman (mutations within *rpoB*), [b]*E. coli* wildtype (served as wildtype for generation of [c] and [d]), [c]ciprofloxacin-resistant *E. coli* (mutation of *gyrA* within amino acid # 644 ) and [d]*E. coli marR* deficient mutant (Δ72bp within *marR* gene).

| Compound | MIC [µg/mL] | | | | |
|---|---|---|---|---|---|
| | *S. aureus* Newman | *S. aureus* Newman ($RIF^R$)[a] | *E. coli* WT[b] | *E. coli* WT-3[c] | *E. coli* WT-III[d] |
| CHD | 4 | 8 | 4 | 4 | 16 |
| CDCHD | 2 | 2 | 2 | 2 | 2 |
| Tetracycline | ≤ 0.03 | ≤ 0.03 | 2 | 2 | 2 |
| Tigecycline | 8 | 8 | 8 | 16 | 32 |
| Ciprofloxacin | 0.1 | 0.1 | 0.03 | 2 | 0.05 |
| Rifampicin | ≤ 0.03 | > 64 | 8 | 8 | 8 |

To compare resistance mechanisms against CHD to mechanisms of CDCHD-resistant pathogens, it was planned to generate CDCHD-resistant mutants. Wildtype *K. pneumoniae* DSM-30104 *and S. aureus* N315 were exposed to stepwise increasing CDCHD concentrations (*K. pneumoniae*: 2, 4, 8 µg/mL; *S. aureus*: 4, 8, 12 µg/mL), generating eight independent mutants, respectively. *K. pneumoniae* mutants were able to grow at 8 µg/mL and *S. aureus* mutants at 12 µg/mL, but for all a tremendous fitness loss was observed, reflected in generation times up to 2 weeks, compared to the wildtype which showed a normal growth behaviour. Finally, repeating MIC assays with these mutants, resistance against CDCHD or CHD could not be confirmed for any *K. pneumoniae* or *S. aureus* mutant (Table 3-25, Table 3-26). Additionally, some mutants (*K. pneumoniae*: Mt8.7, Mt8.8, Mt8.9; *S. aureus*: Mt12.1, Mt12.9) were not able to grow after cryo-storage, underlining the strong influence of CDCHD on the mutants fitness and a further characterization of all mutants was omitted.

**Table 3-25:** Resistance pattern of *K. pneumoniae* CDCHD-resistant mutants (Mt). Wt: *K. pneumoniae* DSM-30104 wildtype. n.d.: not determined.

| *K. pneumoniae* DSM-30104 | MIC [µg/mL] | |
|---|---|---|
| | CHD | CDCHD |
| Wt | 8 | 4 |
| Mt8.1 | 16 | 8 |
| Mt8.2 | 16 | 8 |
| Mt8.3 | 16 | 4 |
| Mt8.4 | 16 | 4 |
| Mt8.7 | n.d. | n.d. |
| Mt8.8 | n.d. | n.d. |
| Mt8.9 | n.d. | n.d. |
| Mt8.10 | 16 | 8 |

**Table 3-26:** Resistance pattern of *S. aureus* CDCHD-resistant mutants (Mt). Wt: *S. aureus* N315 wildtype. n.d.: not determined.

| *S. aureus* N315 | MIC [µg/mL] | |
|---|---|---|
| | CHD | CDCHD |
| Wt | 8 | 4 |
| Mt12.1 | n.d. | n.d. |
| Mt12.2 | 8 | 4 |
| Mt12.3 | 8 | 4 |
| Mt12.5 | 8 | 8 |
| Mt12.6 | 8 | 4 |
| Mt12.7 | 8 | 8 |
| Mt12.8 | 8 | 8 |
| Mt12.9 | n.d. | n.d. |

## 3.2 DISCUSSION

### 3.2.1 ANTIBACTERIAL ACTIVITY

Chelocardin belongs to the family of atypical tetracyclines[127,135,217] and is known to be a broad-spectrum antibiotic with activity against Gram-positive and Gram-negative bacteria, including multidrug-resistant bacteria, but it lacks activity against *Pseudomonas aeruginosa*.[107,108,139] Compared to clinically used typical tetracyclines, CHD exhibits some structural differences and depicts a more planar and rather lipophilic molecule, which is thought to be the main reason for a different mode of action.[48,139] Typical tetracyclines are known to inhibit protein biosynthesis by blocking the acceptor site of aminoacyl-tRNA at the 30S subunit of the ribosome to prevent translation.[217–219] In contrast, atypical tetracyclines such as CHD are not believed to be involved in translation inhibition, but they rather interfere with the cytoplasmic bacterial membrane which leads to a disrupted function and a bacteriolytic effect.[127–129] One main difference of CHD to typical tetracyclines with regard to structural properties is a different aromatization pattern of ring C of CHD (Figure 3-11). This chemical feature leads to a more planar molecule, the reason why CHD might not be able to bind to the ribosome. Moreover, CHD bears an acetyl group at C-2, which is replaced by a carboxamido group at typical tetracyclines and known to be the main feature for tetracyclines antibacterial activities.[130,131] Based on this knowledge, biosynthetic engineering studies aimed at improving chelocardin and yielded a new chelocardin derivative, 2-carboxamindo-2-deacetyl-CHD (CDCHD, Figure 3-11), bearing a carboxamido group at C-2 and exhibiting improved antibacterial activities including inhibition of *P. aeruginosa*.[139]

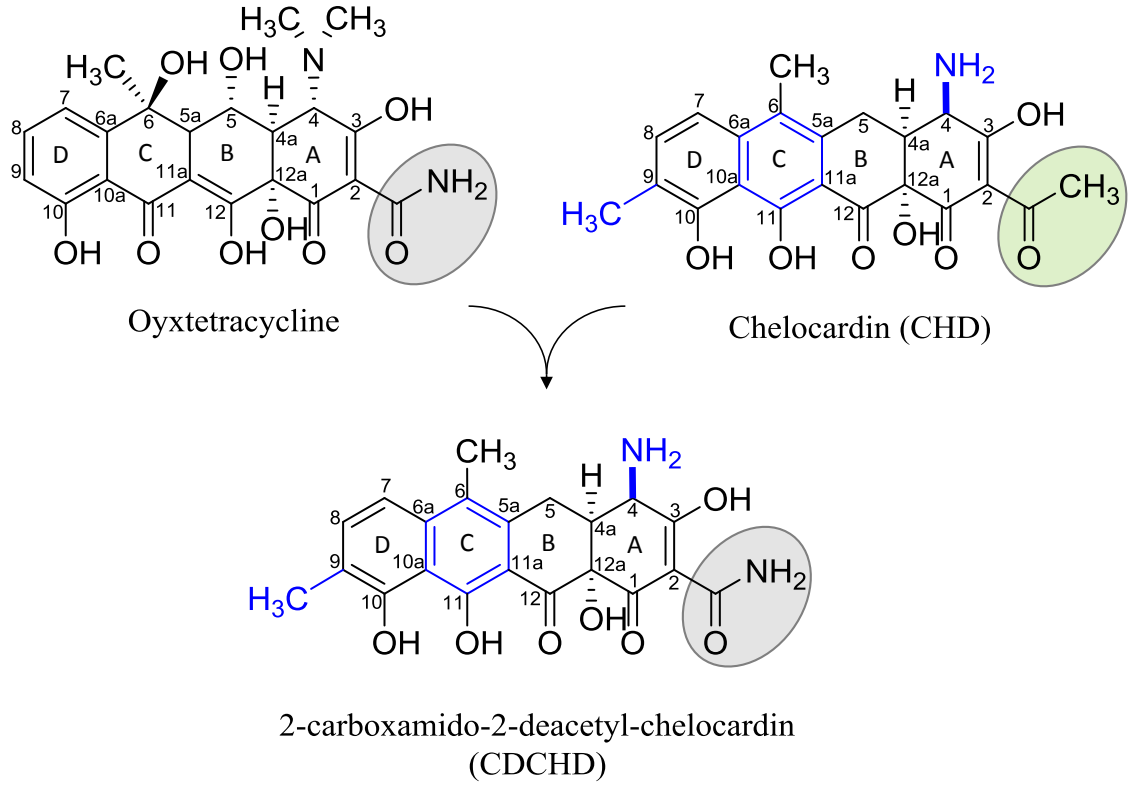



**Figure 3-11:** Combination of typical and atypical structural features within 2-carboxamido-2-deacetyl-CHD (CDCHD). Main structural characteristics of atypical tetracyclines (CHD, CDCHD) that differ from typical tetracyclines (oxytetracycline) are marked in blue.

Antibacterial activities of CHD and CDCHD were determined against Gram-positive and Gram-negative bacteria and broad-spectrum effectiveness, including inhibitory activity against TET-resistant pathogens, of both compounds were confirmed (Table 3-1, Table 3-2).[139] Throughout, CDCHD exhibited an improved activity pattern, especially against *P. aeruginosa* with overall 2- to 8-fold increased activities, compared to CHD. The difference of CHD to CDCHD in *P. aeruginosa* activity was further investigated by MIC determination against efflux-deficient *P. aeruginosa* PA14 mutants (Δ*mexAB*, Δ*mexCD*, Δ*mexEF*, Δ*mexXY*). It was shown that susceptibility relied on efflux mechanisms exhibited mainly by the MexAB-OprM efflux pump of *P. aeruginosa* (Table 3-3). A deletion of the efflux pump encoded by *mexAB* increased susceptibility of both compounds up to 16-fold, the same effect as observed for TET. Efflux pumps encoded by *mexCD*, *mexEF* and *mexXY* did not affect activities as they are thought not to be expressed under normal growth conditions. They are rather involved in acquired resistance based on mutations of regulatory genes what leads to an increased expression of the efflux systems.[46,220–223] In contrast, MexAB-OprM is known to cause intrinsic resistance towards antibiotics, including

tetracyclines.[224,225] Since CHD and TET are only effective against *P. aeruginosa* upon efflux pump inhibition and CDCHD was shown to be able to inhibit wildtype species of *P. aeruginosa*[139] (Table 3-1), the difference in activity must rely on structural characteristics of CDCHD, which enable the compound to at least partially overcome efflux. This is also in accordance to results from *in vitro* resistance development in *Klebsiella pneumoniae*, where it was not possible to obtain CDCHD resistant mutants and all CHD resistant clones displayed increased efflux via the AcrAB system (see chapter 3.2.3). Compared to typical tetracyclines, chelocardins contain a non-methylated amino group at C-4, as well as different aromatization patterns in the western part of the molecules (Figure 3-11). Since those features do not seem to be sufficient to cause inhibition of *P. aeruginosa* spp. (see CHD activity pattern), the combination of chemical features from typical and atypical tetracyclines, including a charged primary amine, merged within CDCHD, seems to be the reason to overcome intrinsic resistance mediated by *P. aeruginosa* and CDCHD's improved activity pattern.

Bringing together the facts that chelocardins are in general able to inhibit MDR species and tetracycline-resistant pathogens (Table 3-2) and the overall improved activity pattern of CDCHD compared to CHD, depicts CDCHD as a frontrunner molecule in the class of chelocardins. To further improve the potency of chelocardins and to generate a lead molecule, several derivatives were prepared through semisynthesis and genetic engineering, with a main focus on derivatization of CDCHD (Figure 3-5, Table 3-11). Structure activity relationships (SAR) of 30 derivatives bearing different moieties (Figure 3-5, Table 3-11) were investigated by determining activities against Gram-positive and Gram-negative bacteria including *S. aureus*, *K. pneumoniae*, *P. aeruginosa* and efflux-deficient *E. coli* TolC (Table 3-12). Modifications by structural features from typical tetracyclines, such as dimethylation of the amino group at C-4 or a demethylation at C-9, as well as substitutions or additions of several active groups were performed (Figure 3-12, Figure 3-13). So far, none of the generated derivatives showed improved antibacterial activity. A demethylation at C-9 of CHD led to a complete abolishment in antibacterial activity, confirming the importance of a methyl group at this position for the effectiveness of CHD[226]. A dimethylation or substitutions of various active groups at C-4 led to a loss or complete abolishment in activity for CHD and CDCHD against Gram-negative bacteria. However, *S. aureus* was still susceptible towards both compounds upon substitution of $R_4$ to a *N*-dimethyl group (at C-4), but no improvement in activity was achieved. In general, the *N*-dimethyl group (tertiary

amine) at C-4 leads to a declined activity pattern for both compounds and it seems to be crucial to bear a primary amine at C-4 to exert their broad-spectrum activity pattern. However, so far only the relative stereochemistry in CHD and CDCDH is known. It might be that difference in the overall topology of chelocardins in comparison to typical tetracyclines account for different binding and activity pattern. A combinatorial derivatization by a substitution of C-4 to a *N*-dimethyl group and a glycosylation at C-10 of either CHD or CDCHD led to a complete loss in activity and as expected, a concurrent demethylation at C-9 did not change the molecules properties. The addition of a sugar moiety to either CHD or CDCHD could positively influence the molecules properties, including an increased water-solubility or a decrease of their cytotoxicity[227,228]. However, to examine the properties of a glycosylated molecule in detail, it would be preferable to add the glycosylation to the parent molecules (CHD and CDCHD) bearing no further modification. Regarding derivatization at C-7, not all active groups were tolerated upon addition, but so far, this position seems to be promising to perform further derivatization. The addition of a chlorine or a fluorine still led to compounds inhibiting Gram-positive as well as Gram-negative bacteria comparable to the parent molecule CDCHD. Even though no improvement in activity was achieved, the molecule's broad-spectrum activity pattern was still present. Derivatization at C-7 of CDCHD seems to be a promising starting point to optimize the scaffold in terms of pharmacological properties including besides bioactivity also bioavailability or the molecules solubility.

From eight derivatives, their respective C-4 epimer was available for activity determination. For two derivatives their *S*-configured epimer was 16-fold less potent than the *R*-configured molecule, whereas for all other derivatives a comparison between both types of configuration did not show differences in terms of their antibacterial activity. In general, tetracyclines are known to undergo reversible epimerization at C-4 at acidic pH conditions which leads to a decrease of antibacterial potency.[229–232] Overall, chelocardins in (*4S*)-configuration did not seem to loose antibacterial activity, but it cannot be excluded that spontaneous epimerization to the (*4R*)-epimers takes place under assay conditions, which causes the observed antibacterial activities.

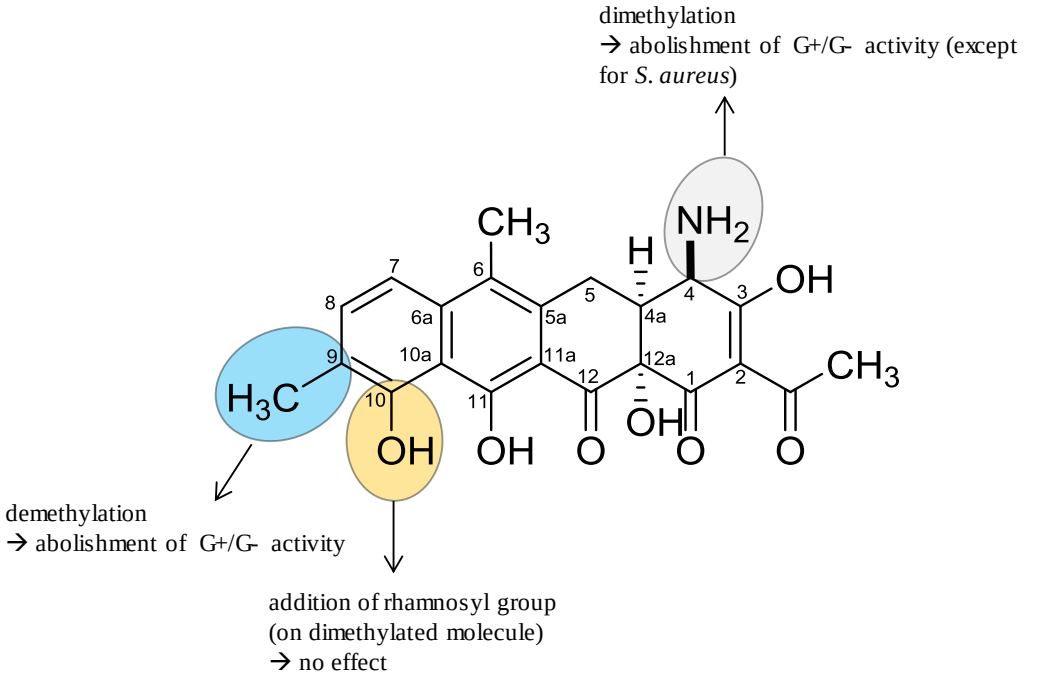



**Figure 3-12:** Structure activity relationship (SAR) of CHD derivatives in comparison to the parent molecule.

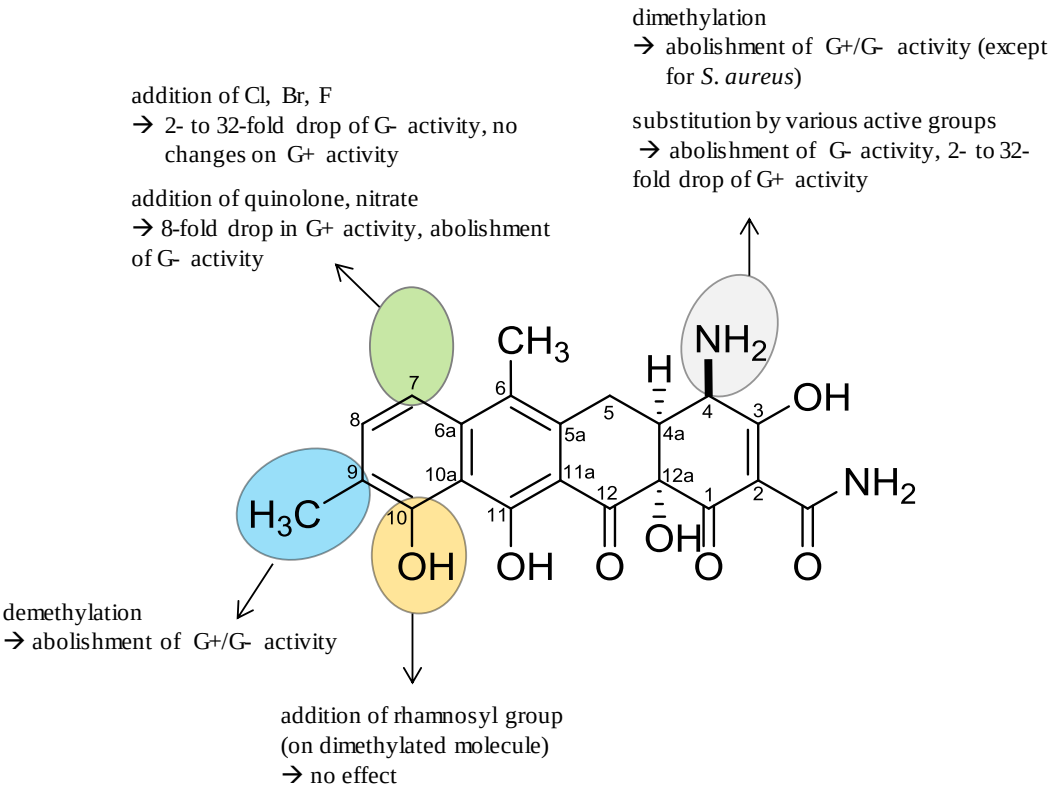



**Figure 3-13:** Structure activity relationship (SAR) of CDCHD derivatives in comparison to the parent molecule.

### 3.2.2 MECHANISM OF ACTION

One key element in the optimization of antibiotics is the elucidation of their mechanism of action and identification of the target molecules. Based on this information, molecules can be adapted to provide antibacterial agents with improved pharmaceutical properties to assure an optimum in effectiveness and to overcome the ability of bacteria to exert resistance towards antibiotics.[24,41] The target molecule of CHD is yet not identified and ever since the discovery of CHD[107], its mode of action is controversially discussed. Studies in the 1970s revealed CHD to exert the same mechanism of action as typical tetracyclines by inhibiting protein translation upon binding to ribosomal subunits[140], whereas more recent studies describe cell lysis as the primary effect, which is mediated by interference with the cell membrane, and no interaction with the ribosome.[127–129,135] Additionally, a current study predicted a dual mechanism of action involving both, inhibition of protein biosynthesis and targeting of the cell membrane in a concentration dependent manner.[141] To get further insight into the predicted mechanisms of chelocardins, effects on the outer membrane as well as effects on membrane integrity were analysed. Typical tetracyclines are known to enter Gram-negative cells through porins without affecting the permeability of the outer membrane.[48,233,234] This was also observed for *E. coli* treated with CHD and CDCHD at concentrations ranging from sub-inhibitory to 2-fold MIC over 2 h. None of the tested antibiotics increased permeability towards the lipophilic dye NPN (Figure 3-9), indicating that chelocardins are not able to change the composition and integrity of the OM under these conditions. However, in a 18 h assay, CHD revealed a similar effect than observed for polymyxins by sensitizing the outer membrane towards hydrophobic agents like rifampicin (Table 3-14), which are under normal conditions not able to penetrate the OM.[235,236] This may be triggered by membrane disturbing antibacterial effects at sub-lethal concentrations, which do not directly rely on membrane re-organization as observed with e.g. polymyxins. Thus, the observed sensitizing of *A. baumannii* could be caused indirectly and after CHD exerts its primary antibacterial mechanism.

Additionally, changes on the membrane potential (MP) caused by treatment with CHD and CDCHD in comparison the TET were examined. A concentration- and time-dependent depolarization of the MP was observed when *S. aureus* was treated with CHD or CDCHD (Figure 3-8). After 2 h treatment at 8 µg/mL both compounds affected MP (approx. 70 % depolarization) in a similar way as observed for the control CCCP, which caused almost a complete collapse (90 % decrease) of the MP by eliminating the proton gradient.[185,186] For

TET, neither a concentration- nor a time-dependent change of the MP was observed. Those results are in accordance with published data on the membrane potential upon treatment with CHD and TET in a short term study (6 min treatment).[141] Stepanek et al. described effects on the MP only at high CHD concentrations (12 µg/mL) and no effect at lower concentrations (3-6 µg/mL). The fact that Stepanek et al. had to use higher concentrations of CHD to observe membrane depolarization in a shorter time period (only 6 min treatment) supports the result (Figure 3-8) of a concentration and time-dependent depolarization effect caused by chelocardins. Differences of typical and atypical tetracyclines in terms of their membrane depolarization effects seem to rely on structural differences. Typical tetracyclines exist in an equilibrium of a lipophilic, non-ionized and a hydrophilic form.[128,132,237] To enter Gram-negative cells, typical tetracyclines form a positively charged cation complex, presumably with $Mg^{2+}$, what enables the uptake through porins (OmpF, OmpC) of the outer membrane.[233,234] The tetracycline-ion complex accumulates within the periplasm and presumably dissociates resulting in equilibrium of the zwitterion and a non-ionized form of the antibiotic. The neutral form is needed to further proceed through the cytoplasmic membrane. Once the molecule is present within the cytoplasm, a chelation is likely to happen again, which results in a hydrophilic ionized molecule which is able to bind to the ribosome.[48,233] This relies on a higher pH and a higher ion concentration within the cell compared to the periplasm.[234] In contrast, atypical tetracyclines solely exist in a lipophilic form which is probably caused by their more planar structure.[128] Due to the increased lipophilicity of CHD, the molecule is thought to stay longer in the membrane compared to typical tetraclycines[141], which might trigger membrane damaging effects, followed by a time-dependent altering of the MP of Gram-negative bacteria, which in turn leads to cell lysis.

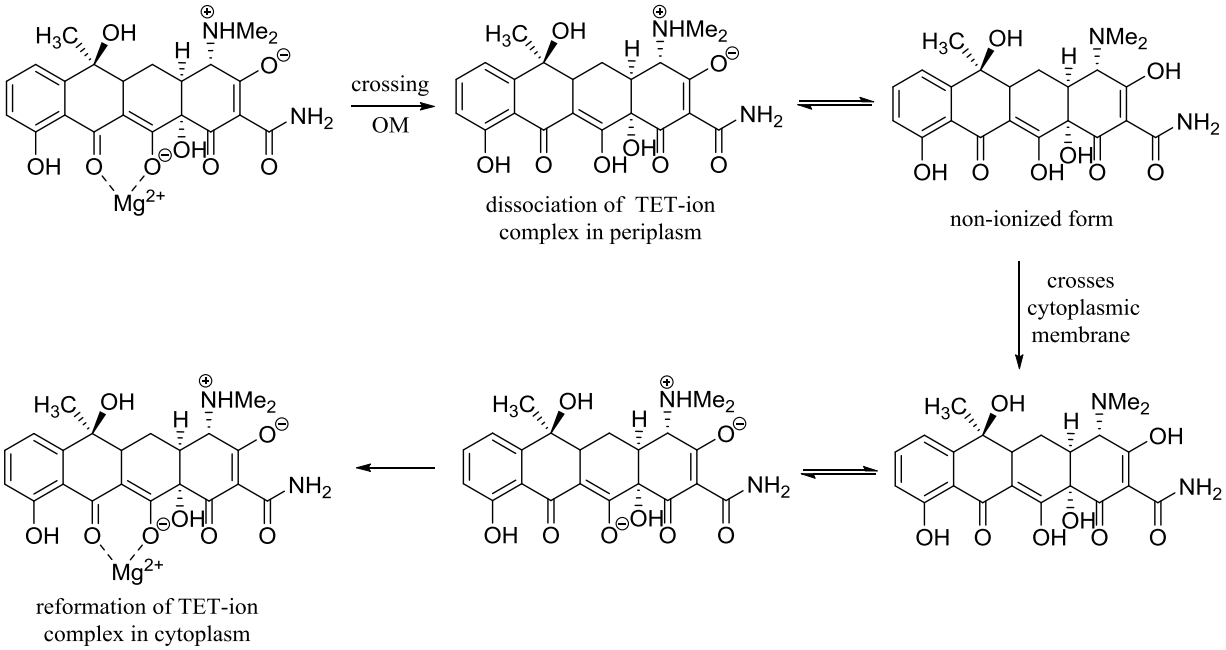



**Figure 3-14:** Formational changes during tetracycline (TET) uptake. A chelation of a TET-ion ($Mg^{2+}$) complex is needed to cross the outer membrane (OM). The complex dissociates within the periplasm and an equilibrium of the zwitterion and a non-ionized form is present. The latter form dissociates through the cytoplasmic membrane into the cytoplasm, where the molecule predominantly exists in a hydrophilic from. A reformation of the TET-ion complex is likely to happen and is required for ribosome binding.[238–240]

Besides membrane associated mechanisms, inhibitory effects of CHD on the synthesis of macromolecules were described.[128] In comparison to the nearly complete inhibition of RNA, DNA and protein synthesis described by Rasmussen et al.[128], contrary data were achieved within the present study (Figure 3-6). *S. carnosus* DSM-20105 was incubated with CHD and CDCHD at 0.5-fold MIC and macromolecule synthesis (MMS) was monitored over 180 min. Both compounds showed only a slight inhibition of RNA, DNA and protein synthesis, which can be neglected, as final incorporation levels of precursor molecules nearly reached the values achieved for untreated control cells. Additionally, effects on cell wall synthesis were analysed and apart from a delay in the synthesis by approximately 60 min compared to control cells, treatment with both compounds did not lead to complete inhibition. One limiting factor within this study was the concentration range of both chelocardins. Results depicted (Figure 3-6) were generated at sub-inhibitory concentrations (2 µg/mL), as the use of higher concentrations was not suitable within the assay set-up. Concentrations ≥ MIC led to fast killing and complete lysis of cells due to the strong bactericidal effect of chelocardins[127]. On the contrary, Rasmussen et al. performed their studies at higher concentrations (20-fold MIC), what might account for the discrepancies in observed effects. In contrast to the above described minor effects on protein synthesis on living cells, both

chelocardins displayed inhibition of translation in a cell-free system (Figure 3-7). However, > 5-fold higher concentrations of CHD and CDCHD compared to TET were needed to trigger similar inhibitory effects. Those findings are contradictory to the predicted mechanism of CHD by Stepanek et al.[141], as they stated that CHD caused inhibition of translation through ribosome binding at low CHD concentrations. Based on their data, results on MMS study would have been expected to be different, i.e. an early breakdown of methionine incorporation was expected. Indeed, cell wall synthesis appeared to be most affected when *S. carnosus* was treated at the 0.5-fold MIC. However, the discrepancies on the mechanism of CHD cannot be explained so far. Regarding results of the *in vitro* translation in a cell-free system, ribosome inhibition seems to contribute to CHD's antibacterial activity. However, based on the cell-based studies (MMS), translation inhibition was excluded as primary mechanism. Furthermore, effective concentrations in cells cannot be extrapolated form inhibitory concentrations in the *in vitro* experiment. The cell-free system overcomes the already mentioned bactericidal activity of CHD[127,129], which was shown to happen in a concentration dependent manner (Figure 3-2). Sub-inhibitory concentrations led to an either exponential growth or a bacteriostatic effect, whereas higher antibiotic concentrations (≥ MIC value) led to a 2- to 3-$\log_{10}$ reduction of counted CFU. For CDCHD, a similar bactericidal activity was observed and the effect was even more pronounced compared to CHD, killing > 3-$\log_{10}$ cells (Figure 3-3). Additionally, it was shown that chelocardins exert in general a stronger activity than observed for typical tetracyclines (Table 3-4), since they act more bacteriostatic.[127] Combining those results with the observed effects on translation, leads to the conclusion that the concentration range used in the cell-free translation assay would trigger a bactericidal effect in an assay with live cells by a rapid killing of the cells. It might be possible that membranes of bacterial cells are already affected irreversibly after CHD treatment and that simultaneous effects on the ribosome are negligible. This shows that the main mechanism of action of chelocardins rely on membrane associated effects and that it is not based on the inhibition of protein synthesis. Although the mode-of-action of chelocardin is still not completely understood, a potential *in vivo* use was already described in the 1970s. A small Phase II clinical study in the 1970s reported the cure of twelve patients suffering from urinary tract infections after treatment with CHD[138]. To compare *in vivo* efficacy of CHD to CDCHD, both compounds were initially investigated in a thigh infection mouse model (data not shown; Dr. Katharina Rox, HZI, Braunschweig). Both compounds were tolerated up to 50 mg/kg/day and reduction of bacterial burden (*K. pneumoniae*) was determined in muscles, blood and kidneys. Based on

CFU count, antibacterial effects in blood samples were more pronounced compared to effects in muscles. However, both compounds revealed best potency in kidneys and similar or even better effects compared to the control levofloxacin were observed. The overall promising results from *in vivo* infection experiments along with results from extensive pharmacokinetic (PK) studies in mice, that were performed by Dr. K. Rox as part of a DZIF-funded project, hinted towards an accumulation of the compounds in kidneys and urine and the potential use of chelocardins to treat urinary tract infections (UTI). Thus, both chelocardins were assessed in an *E. coli* UTI mouse model at Statens Serum Institute in Copenhagen, Denmark.[241] As expected, CHD as well as CDCHD were able to induce a significant reduction of bacterial burden in urine and bladder, but antibacterial effects in kidneys were somewhat less pronounced and only CDCHD treatment resulted in significant reduction of bacterial load. Due to limitations with respect to solubility of CHD and CDCHD and their bioavailability further scaffold improvement is required to achieve even better *in vivo* efficacy. However, the results from *in vivo* mouse models, in particular from the *E. coli* UTI model, qualify CDCHD as a valid starting point for lead generation (hit-to-lead program). UTIs are classified into complicated an uncomplicated infections, whereas the latter type is additionally subdivided into lower UTI (cystitis) and upper UTI (pyelonephritis).[242,243]. Uncomplicated UTIs are typically described in otherwise healthy patients whereas complicated UTIs occur in association with urinary tract abnormalities including structural or neurological changes.[244] UTIs are mainly caused by Enterobacteriaceae, with an annual estimated occurrence of about 150 million cases.[245] Uropathogenic *E. coli* (UPEC) are the most common pathogens causing UTI, but e.g. *K. pneumoniae*, *P. mirabilis*, *Enterobacter* spp. and *P. aeruginosa* are also associated with UTIs (Figure 3-15). Besides Gram-negative bacteria, Gram-positive bacteria such as Enterococci or Staphylococci, and fungi are also capable of causing UTI.[242,246] The increasing number of MDR pathogens, in particular among Gram-negative species, severely hampers the effectiveness of drugs such as fluoroquinolones or β-lactam antibiotics, which are typically used to treat UTIs.[247–249] Elderly patients appear to be most affected by drug-resistant uropathogens. Furthermore, the spread from hospitals to the community including special-care homes is alarming.[250,251] The combination of increasing prevalence of MDR bacteria among uropathogenic strains, an extensive spread of the pathogens into the community, and a concurrent emergence of ineffective drugs depicts the importance of finding alternative or rather new effective antibiotics.

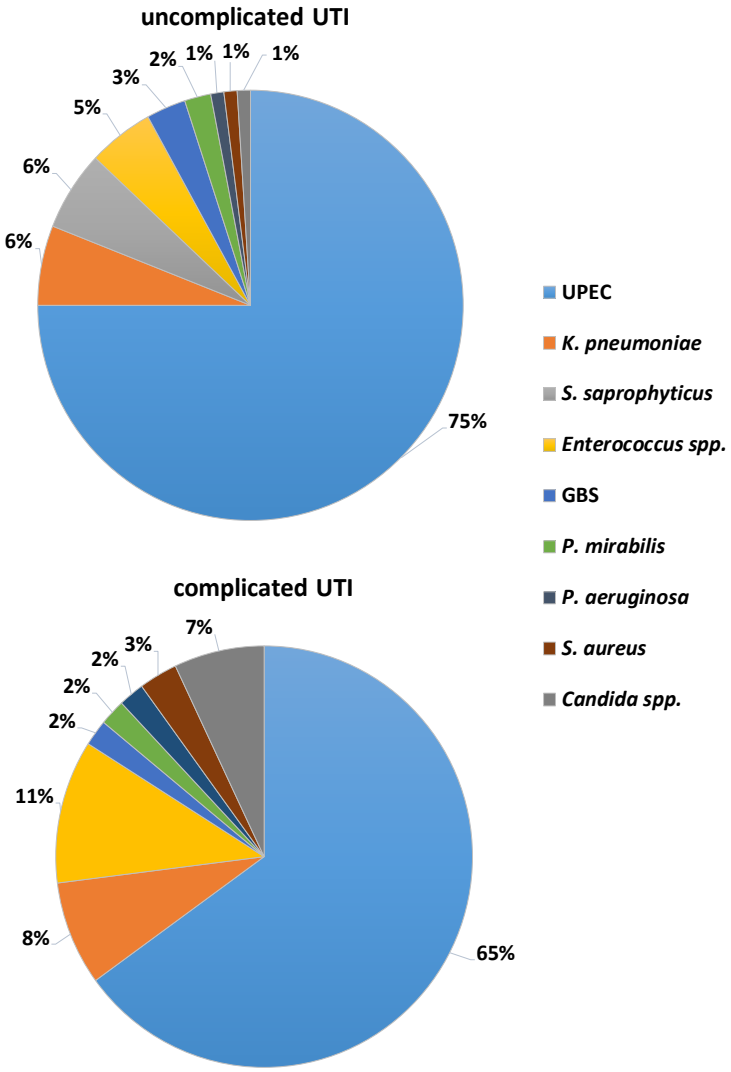



**Figure 3-15:** Epidemiology of uncomplicated urinary tract infections (UTI) and complicated UTI. UPEC: uropathogenic *E. coli*; GBS: group B *Streptococcus*. Adapted from Flores-Mireles et al.[242]

To further investigate the potential use of CHD and CDCHD against UTIs, the *in vitro* activity of both compounds was determined against uropathogenic laboratory strains and clinically relevant isolates, including MDR isolates. Both chelocardins inhibited laboratory strains (Table 3-1) and as expected CDCHD showed improved activity compared to CHD (2- to 8-fold increased MIC values). In addition, CHD and CDCHD revealed inhibitory effects against clinical isolates and intriguingly, they were able to inhibit colistin-resistant pathogens and pathogens expressing TEM-β lactamases or extended-spectrum β-lactamases (Table 3-5). Moreover, approximately 4-fold improved potency of both compounds was observed when tested against selected isolates in artificial urine, compared to standard test medium (Table 3-8, Table 3-9) and determined MIC values were pH-independent (pH 5.5 to pH 8.5). However, MBC values varied by a factor 2-16 depending on the adjusted pH. This effect could not be attributed to changed stability of CHD and CDCHD at different pH

values and it was more pronounced with the *K. pneumonia*e isolate (compared to *E. coli*), whereas potency at acidic pH was by tendency better. Remarkably, an opposite line-up of CHD and CDCHD in terms of their antibacterial potency was observed. CDCHD showed better activities against laboratory strains, whereas CHD was the more potent molecule against clinical isolates and additionally, CDCHD lost its ability to efficiently inhibit *P. aeruginosa* when tested against clinical isolates. Those data are contradictory to initial reported MIC values[139] on clinical isolates. It was assumed that a changed formulation of CHD and CDCHD caused the reverted potency. Initially, chelocardins were prepared as HCl adducts in methanol and reported data[139] rely on using CHD and CDCHD in this solution. Due to stability issues (e.g. spontaneous epimerization), which were discovered later on, both chelocardins were provided in a modified formulation as sodium salts with citrate as stabilizer. However, it was shown, that the change in formulation did not cause reverted potency of CHD and CDCHD (Table 3-6, Table 3-7). Throughout, MIC values determined with CHD and CDCHD in either MeOH (HCl adducts) or water (Na salts with Na-citrate) were comparable and thus, it could be confirmed that CDCHD is by tendency less active compared to CHD on uropathogenic clinical isolates. In addition, both compounds were in general less potent against clinical isolates compared to laboratory strains, which probably relies on intrinsic resistance mechanism (e.g. higher efflux rates) that are *per se* found frequently in clinical isolated pathogens. Moreover, the slight difference in potency can probably also be neglected as CDCHD appears to be the preferred scaffold due to its significantly more pronounced bactericidal effects, which was throughout shown to be stronger compared to CHD, regardless if determined against laboratory strains (Figure 3-3) or clinical isolates (Table 3-5, Table 3-8, Table 3-9).

These results further substantiate the assumption that chelocardins, in particular CDCHD-derived compounds, are useful early lead structures for the preclinical development towards an UTI drug. Moreover, experimental *in vivo* PK/PD studies in mice and reports on the phase II clinical trial with CHD did not reveal any severe *in vivo* toxicity or adverse effects.[138] Those results underline a good tolerance of chelocardins *in vivo* and leading to the conclusion, that cytotoxic effect observed *in vitro* can be neglected. However, *in vitro* cytotoxicity against human (HCT-116: colon carcinoma; HepG2: hepatocellular carcinoma; THP-1: acute monocytic leukemia; U-2 OS: bone osteosarcoma) and murine (RAW264.7: leukemia macrophage; L929: subcutaneous connective tissue, fibroblasts) cell lines (Table 3-13) hampered some of the cell-based studies. The analysis of killing efficacy on intracellular bacteria within macrophages by CHD and CDCHD failed as the assay set-up

was particularly limited; half-inhibitory concentrations of CHD and CDCHD on eukaryotic cells ($IC_{50}$ on RAW264.7: 4.5 and 0.7 µg/mL) were in the same range than MIC values on the intracellular pathogen (*S. aureus* MIC: 4 µg/mL) (Figure 3-4). Concentrations $\geq$ MIC/$IC_{50}$ would have led to killing of bacteria with a concurrent disruption and lysis of macrophages. For CDCHD, the general higher toxicity was even more limiting as concentrations $\leq IC_{50}$ (0.7 µg/mL) would have been too low to cover the concentration ranges needed to determine any antibacterial effect (MIC: 4 µg/mL). *In vitro* cytotoxicity probably relies on membrane interaction of CHD and CDCHD similar to that observed for bacterial species. The lack of pronounced *in vivo* toxicity might be explained by the fact that cells, which are organized in e.g. a tissue are usually more tolerant to external stressors.[252]

### 3.2.3 RESISTANCE MECHANISM

The development of resistance against CHD and CDCHD was investigated in Gram-positive and Gram-negative bacteria. Despite several attempts, it was not possible to obtain CDCHD-resistant mutants. The exposure of stepwise increasing concentrations of CDCHD to wildtype *K. pneumoniae* DSM-30104 and *S. aureus* N315 led to a severe loss in fitness of the treated bacteria and MIC determination revealed all mutants not to be resistant (Table 3-25, Table 3-26). However, by exposing wildtype *E. coli* DSM-1116 and *K. pneumoniae* DSM-30401 to stepwise increasing CHD concentrations, independent CHD-resistant mutants were obtained. The exposure to wildtype *E. coli* yielded seven independent CHD-resistant mutants, but the generation was accompanied by a severe reduction in fitness of the mutants compared to the wildtype. This was reflected in generation times of the mutants of 2-3 weeks. A second *in vitro* resistance development performed with wildtype *K. pneumoniae* DSM-30104 yielded nine independent mutants, which were all able to grow at 8 µg/mL Compared to CHD-resistant *E. coli* mutants, no influence on fitness compared to the respective wildtype was observed for CHD-resistant *K. pneumoniae* DSM-30104 mutants (Figure 3-10).

*In silico* comparison of four CHD-resistant *E. coli* mutants (Mt30.1, Mt30.2, Mt30.4, Mt30.8) to wildtype *E. coli* DSM-1116 revealed the presence of an inconsistent mutation pattern within each mutant (Table 3-15) and no common genotype was observed. Moreover, the loss of the natural occurring 100kb plasmid (pRK1) in mutants Mt30.2 and Mt30.8 does not seem to rely on mechanisms of resistance towards CHD. The characterization of pRK1 by Archer et al. revealed no resistance genes present on the plasmid and additionally, they

suggested pRK1 to be unstable.[253,254] Even though no direct evidence pointing towards plasmid stability were stated, this might explain the loss of pRK1in Mt30.1 and Mt30.2. In summary, it was not possible to draw any conclusion on the major CHD resistance mechanism in *E. coli* but data hinted towards a combination of several physiological alterations that probably contributes to resistance of all four genome sequenced mutants. Bacteria are able to adapt towards antibiotic use via different resistance mechanisms. Those include the production of detoxifying enzymes which can lead to antibiotic degradation, increased efflux to export compounds or the alteration of the composition of the outer membrane to act as barrier for the influx of antibiotics.[22,23] The latter two mechanisms are the most common pathways bacteria undergo to protect themselves. Increased efflux is carried out by overexpression of efflux systems through alteration of transcriptional or post-transcriptional mechanisms which are involved in efflux pump regulation.[255] Structural changes of the outer membrane are known to be mediated by altered porin profiles like the expression of different types of porins or by mutations leading to decreased expression of porins.[23] Importantly, different resistance mechanisms are often carried out simultaneously like alteration of the outer membrane with a concurrent increased efflux.[56] With regard to this, several mutations identified within CHD-resistant *E. coli* mutants can be correlated to general resistance mechanisms. In summary, major putative resistance factors identified within CHD-resistant *E. coli* mutants were increased efflux via AcrAB modification of porins and interestingly, some peptidoglycan modifying enzymes were found to be mutated (PhoQ, PBP4, PBP7). The latter supports the finding from MMS assays where an influence on cell wall biosynthesis was observed after treatment of *S. carnosus* with sub-lethal concentrations of CHD and CDCHD. Furthermore, co-/cross-resistance studies revealed the involvement of efflux systems and the outer membrane as barrier for antibiotic uptake. The increase of the permeability of the outer membrane by PMBN as well as the presence of the efflux pump inhibitor PAβN increased sensitivity of all *E. coli* mutants towards all tested antibiotics (Table 3-18). However, mutants Mt30.1 and Mt30.2 were still co-resistant to ciprofloxacin (CIP) in the presence of PMBN and without PAβN (permeabilized cells; efflux mechanisms unaffected). This can be correlated to the mutations identified within *acrR* and its putative operator region in these mutants (Table 3-15). *acrR* encodes the repressor (AcrR) of the AcrAB multidrug efflux system of *E. coli* spp. and mutations of *acrR* lead to a malfunction of AcrR as repressor, what in turn leads to a permanent expression of the efflux pump AcrB (encoded by *acrB*) and the efflux of CIP.[192–194] PAβN represents an inhibitor of major efflux pumps that are involved in multidrug-resistance[256], including the AcrAB-TolC

system of *E. coli* spp.. Thus, the increased efflux of CIP, which is based on the identified mutations of Mt30.1 and Mt30.2, is inhibited by PAβN, but still ongoing when only PMBN is present, as no efflux inhibition occurs. Regarding resistance mediated by the alteration of the outer membrane, Mt30.1 and Mt30.8 revealed mutations of genes involved in porin formation, *ompF*[195–197] and *envZ*[212,213], respectively. *ompF* encodes the outer membrane protein F (OmpF) which is a major component of the outer membrane and involved in transport systems of *E. coli* cells.[195–197]A loss of the OmpF or mutations within *ompF* are known to be involved in antibiotic resistance[54,257] by structural changes of porins. Those changes hinder hydrophilic antibiotics to traverse the outer membrane. In this context, the mutation of *envZ* of Mt30.8 can also be linked to changes of porin levels within the membrane of bacterial cells. *envZ* encodes a senor histidine kinase (EnvZ) within the two-component regulatory system with OmpR and enables the phosphorylation of OmpR what leads to the expression of the porins encoded by *ompF* and *ompC*.[22,212,213] A loss in functional *envZ* might prevent the phosphorylation of OmpR, what hinders the enzyme to mediate further steps and leads in turn to a hampered porin expression. Mutations that might lead to modifications of the cell envelope were present within mutant Mt30.2 and Mt30.4. Mutant Mt30.4 yielded mutations within *phoQ*, a gene encoding the sensor histidine kinase PhoQ, which is part of a two component regulatory system together with the response regulator PhoP and is involved in the regulation of transcription.[201,202] By interaction with metal ions (such as $Mg^{2+}$), PhoQ autophosphorylates and leads to a transphosphorylation of PhoP. This enables PhoP to bind the DNA and triggers downstream events like the regulation of the PmrA-PmrB system which is involved in the modification of lipopolysaccharides (LPS).[258,259] Mutations of *phoQ* were previously shown to be involved in antibiotic resistance which is mediated by altered LPS of Gram-negative bacteria.[258–260] An altered lipid composition of the OM acts as a barrier to hydrophobic compounds which hinders the antibiotic to enter the cell through the lipid layer.[56] Additionally, mutations in genes that are linked to the synthesis of the cell wall were present in mutant Mt30.2 and Mt30.4. The affected genes *dacB* (Mt30.4) and *pbpG* (Mt30.2) encode enzymes PBP4 and PBP7, respectively. Both enzymes depict periplasmic peptidoglycan (PG) hydrolases which are involved in hydrolysing peptide bridges of PG during cell growth.[207,261] They contribute to the metabolism of the PG layer by hydrolysing polymers enabling the incorporation of subunits into the murein sacculus. Even though it seems obvious that mutations of these genes might contribute to resistance by an altered cell wall, previous studies described that a loss of either PBP4 or PBP7 did not contribute to cellular changes.[262,263] This indicates that

the resistance mediated by mutant Mt30.2 and Mt30.4 rather relies on changes caused by the additional mutations that are present in both mutants (efflux; porin altering).

Taking together the above summarized findings, resistance of CHD-resistant *E. coli* mutants relies on general mechanisms executed by the bacteria to protect against antibacterial compounds, but no CHD-specific resistance mechanism was identified.

In order to gain further insights into CHD resistance mechanisms, a second *in vitro* resistance development with *K. pneumoniae* DSM-30104 was performed. The genomic comparison of CHD-resistant *K. pneumoniae* mutants to the wildtype strain (Table 3-20) revealed a repeat expansion of 7 bp in *gpmA* of Mt8.2 and Mt8.3, a gene encoding a phosphoglyceromutase (2,3-bisphosphoglycerate-dependent) which is involved in energy metabolism by catalysing the interconversion of 2-phosphoglycerate to 3-phosphoglycerate.[264] Within Mt8.7 an amino acid transition from alanine to glutamine was found in a gene which encodes a permease of the branched-chain amino acid ABC transporter family.[265] For all nine CHD-resistant *K. pneumoniae* DSM-301014 mutants, mutations in *ramR* were present. *ramR* encodes the TetR family transcriptional regulator RamR which is found within the *ram* locus (*ramR-romA-ramA*). So far, the *ram* locus was described to be present in *Klebsiella* spp., *Citrobacter* spp., *Enterobacter* spp., and *Salmonella* spp., whereas the latter lacks the *romA* gene. The *ramR* gene was first described in *Salmonella enterica* and it was identified as repressor of *ramA*.[266] Its protein product RamA is a positive regulator of the AcrAB-TolC efflux system.[255] This efflux system belongs to the resistance-nodulation-division (RND) family and pumps of this family are involved in intrinsic as well as acquired resistance of Gram-negative bacteria.[267–269] RND efflux pumps (AcrB in the present case) are located in the inner membrane and are associated with a periplasmic membrane fusion protein (MFP; AcrA in the present case) and with an outer membrane channel protein (OMF: outer membrane factor family of proteins; TolC in the present case; Figure 3-16).[270,271] Among the RND family of efflux pumps, AcrB is a major, constitutively expressed pump and is involved in resistance towards compounds of multiple classes of antibiotics, including tetracyclines (incl. glycylcyclines such as tigecycline) or quinolones.[268,272,273] The AcrAB-TolC efflux system is one of the best characterized efflux systems and is found in Enterobacteriaceae including *K. pneumoniae* and *E. coli*.[255,256] Among other Gram-negative bacterial species, homologous of the AcrAB-TolC efflux system are described, like the Mex systems (major systems: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM) present in *Pseudomonas* spp..[255,274] RND family efflux pumps are regulated by global transcription factors as well as via local repression. In *E. coli*, *Klebsiella* spp. and *Salmonella* spp. the local repression of AcrAB-

TolC is mediated by the regulatory gene *acrR*[194,255] (encoding AcrR) as already mentioned in context with CHD-resistant *E. coli*. AcrR belongs to the TetR family of transcriptional regulators which control genes involved in metabolism or the production of antibiotics as well as in antibiotic resistance.[275]

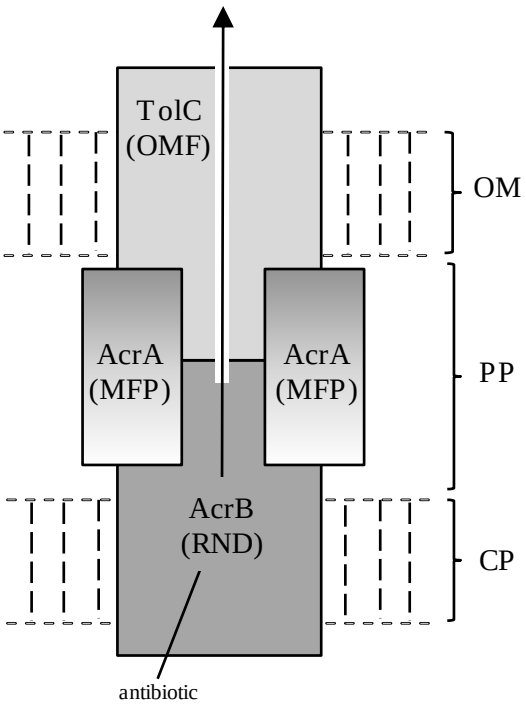



**Figure 3-16:** Schematic representation of the AcrAB-TolC efflux system. OMF: out membrane factor; MFP: membrane fusion protein; RND: resistance-nodulation-division; OM: outer membrane; PP: periplasm; IM; inner membrane; CP: cytoplasmic membrane. Adapted from Poole[271]

Global transcriptional events in *E. coli* are regulated by the multiple antibiotic resistance (Mar) operon.[255,276] The Mar operon consists of three genes: *marR*, *marA* and *marB*. The repressor protein of the Mar operon (MarR) is encoded by *marR* and constitutively expressed under normal conditions. Genomic changes mediated for instance by mutations of *marR* lead to an increase in the expression of the Mar operon. This leads to further downstream events such as the expression of *marA.* The global transcriptional activator MarA (encoded by *marA*) belongs to the AraC/XylS family of proteins, whose expressions lead to activation of genes involved in efflux, like *acrAB* and *tolC*.[277,278] MIC determination of a *marR*-efflux-deficient *E. coli* mutant compared to the respective *E. coli* wildtype illustrated the above described resistance mechanism mediated by mutations of *marR*. A decrease in activity for chelocardins as well as tigecycline due to the upregulation of the AcrAB pump were observed (Table 3-24). Within *Salmonella* spp., *Enterobacter* spp. and *Klebsiella* spp. a

homologue of MarA, RamA is present.[266] As already mentioned above, the expression of RamA is regulated by the repressor protein RamR and induces the activation of the AcrAB-TolC efflux system (Figure 3-17). Studies in *S. enterica* and *K. pneumoniae* identified the binding site of the RamR protein in the upstream region of the *romA-ramA* locus (only *ramA* locus in *Salmonella*), where RamR binds to a palindromic conserved region that overlaps with the transcriptional starting site in the promoter region of *romA-ramA* (Figure 3-17, black box).[279–281] Moreover, mutations in *ramR* were shown to be involved in resistance of clinical isolates of *Salmonella* spp. against ciprofloxacin and *Klebsiella* spp. against tigecycline.[282,283] The identified mutations disabled RamR to bind to the *ramA* promoter region, which in turn led to the expression of AcrAB-TolC efflux system.[255]

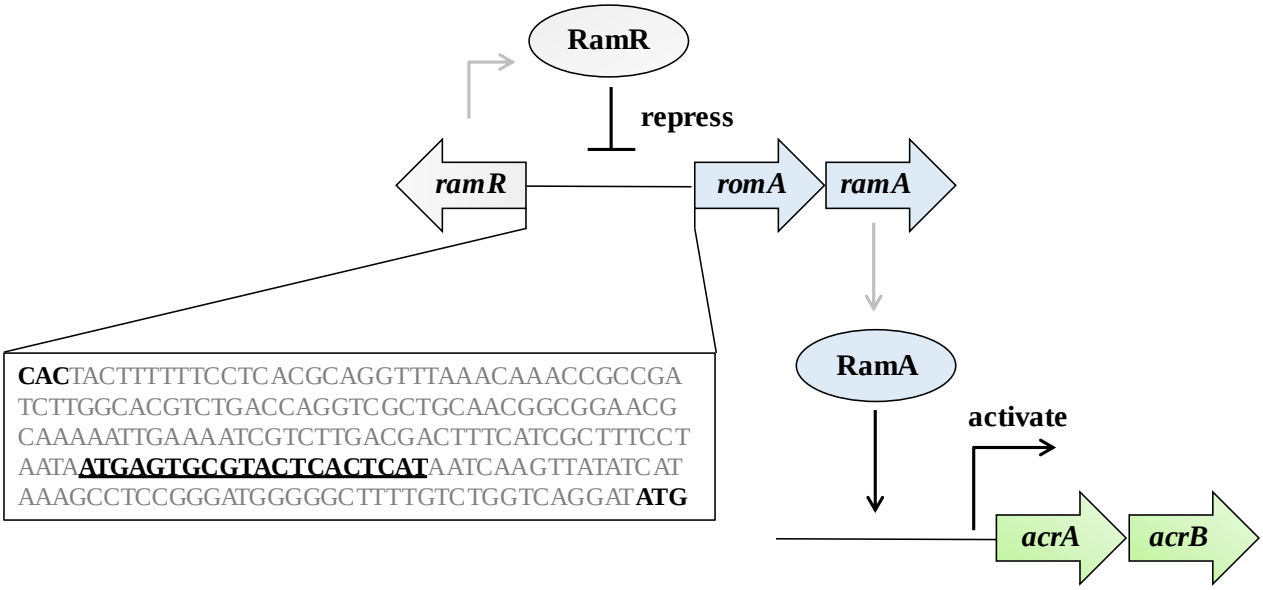



**Figure 3-17:** Schematic representation of the genetic regulation by RamR. *ramR* encodes the repressor protein RamR whose expression leads to repression of *romA-ramA*. A repression of RamR leads to the expression of RamA, which induces the expression of *acrAB,* encoding the AcrAB efflux system. Inserted black box: upstream region of *romA-ramA* locus. Bold triplets indicate start codons of either *ramR* and *romA* genes and bold underlined bases indicate the palindromic binding site of RamR. Adapted from Majumdar et al.[280] and Yamaski et al..[284]

Determination of the expression levels of genes regulated by RamR/RamA (AcrAB efflux system) of CHD-resistant *K. pneumoniae* mutants revealed increased expression, compared to wildtype *K. pneumoniae* DSM-30104 genes (Table 3-22). Gene expression measured by qPCR revealed upregulation of *ramA* up to 41-fold with a concurrent upregulation of *acrA* and *acrB* up to 12- and 67-fold. A complete deletion of *ramR* in *K. pneumonia* led to significantly reduced susceptibility towards CHD by factor eight (Table 3-21), which proves

the involvement of RamR and the AcrAB-TolC efflux pump in the resistance mechanism of CHD. Additionally, co-/cross-resistance studies in the presence of PAβN proved furthermore the involvement of efflux systems in resistance towards CHD (Table 3-23) and explained resistance of all mutants towards all other tested antibiotics including ciprofloxacin, chloramphenicol, tetracyclines and tigecycline (Table 3-19). Tigecycline depicts the first member of the glycylcyclines[123,285], a family of semisynthetic derivatives of minocycline. It exhibits broad-spectrum antibacterial activity including inhibition of tetracycline-resistant pathogens[124], comparable to chelocardins. In the context of resistance, several cases of resistance towards tigecycline in clinical isolates of *K. pneumoniae* were described and assigned to inactivation-mutations of *ramR*[279,283,286], the same resistance mechanism as observed for CHD. Surprisingly, all mutants were more susceptible to polymyxin B treatment compared to the wildtype strain. Underlying mechanisms are not entirely clear, but the CHD-resistant phenotype of *Klebsiella* might involve some alterations of the bacterial membrane, which facilitate the interaction of polymyxins.[287,288]

Importantly, CHD-resistant mutants as well as the *ramR* knockout mutant were not cross-resistant to CDCHD. This result shows that resistance through mutations of *ramR* exclusively affects CHD but not CDCHD, which indicates – along with the fact that no CDCHD-resistant mutant could be generated *in vitro* – resistance-breaking properties for CDCHD. Taken together, the results from studies on chelocardin-related resistance mechanisms in Gram-negative species, qualifies CDCHD as a new early lead structure for further development of urgently needed broad-spectrum antibiotics. However, further scaffold improvements including studies to enhance better physicochemical and pharmacological properties need to be performed.

## 3.3 SUMMARY AND OUTLOOK

Chelocardin depicts a natural product produced by *Amycolatopsis sulphurea* with broad-spectrum antibacterial activity including antibacterial activity against MDR pathogens. Previous bioengineering studies yielded the amidated analogue CDCHD, which exhibits improved antibacterial activities compared to the parent molecule CHD. Both compounds belong to the family of atypical tetracyclines and are able to overcome resistance mechanisms of tetracycline-resistant pathogens. The mode of action of CHD is controversially discussed, but previous studies hint towards a mechanism different to that of typical tetracyclines. In the course of this study, CHD and CDCHD along with several derivatives from semi-synthesis and bioengineering were profiled in terms of their *in vitro* antibacterial properties with the goal to identify a preferred molecule as starting point for preclinical development. Structure-activity relationships (SAR) of 30 derivatives bearing structural features from typical tetracyclines or substitutions/additions of several active groups were investigated. However, none of the so far generated derivatives showed an improved profile in terms of physicochemical properties (e.g. solubility) or antibacterial activities. Thus, further studies with the aim to elucidate CHD's mechanism of action and associated resistance mechanisms were solely performed with the parent molecules CHD and CDCHD. The biological characterization revealed both compounds to be toxic on murine and human cell lines at low micromolar concentrations. However, *in vitro* cytotoxicity was rated to be negligible since chelocardins show a good tolerance *in vivo*. Both compounds revealed broad-spectrum activities against Gram-positive and Gram-negative bacteria laboratory strains, including inhibition of clinical isolates and multidrug-resistant pathogens. Throughout, CDCHD exhibited improved activities in comparison to CHD. Both molecules exert strong bactericidal (and bacteriolytic) effects as shown with some laboratory strains and clinical isolates of uropathogenic strains. This compound behaviour, in turn, hampered some of the cellular assays and CHD and CDCHD could mostly be assessed only at sub-inhibitory concentrations to avoid false-positive results due to rapid cell lysis. Thus, in standard MMS assays, CHD and CDCHD did not show a differential response in *S. carnosus* and none of the typical antibiotic mechanisms (inhibition of RNA, DNA, protein or cell wall synthesis) could be proven or ruled out. However, both compounds led to a concentration-dependent membrane depolarization, confirming the proposed membrane-associated mechanism. Even though no unambiguous insight into the mechanism of action of CHD could be gained, the assumption of a mode of action away

from protein synthesis inhibition and towards a cell membrane-based mechanism was consolidated. However, to completely understand the mechanism underlying chelocardin activity, more detailed studies in particular on cell wall activity need to be performed.

Additionally, it was shown that CDCHD exerts a similar mechanism that is even more pronounced compared to CHD and importantly, CDCHD exhibits resistance-breaking properties. Compared to CHD, CDCHD is able to inhibit *P. aeruginosa* wildtype species and the generation of CDCHD-resistant mutants *in vitro* could not be achieved. Moreover, the resistance mechanism towards CHD, which was identified as increased efflux mediated by mutations of *ramR*, does not affect the activity of CDCHD. The amidated compound was still able to inhibit the growth of all CHD-resistant mutants bearing mutations or a loss of *ramR*.

Even though the mechanism of action is still not completely understood CDCHD (and CHD) was progressed in the course of a DZIF-funded project to further development as an antibiotic with broad-spectrum antibacterial activity. Both chelocardins showed *in vitro* and *in vivo* potency against UTI pathogens. Interestingly, a discrepancy of the efficacies of CHD compared to CDCHD was observed when activity was determined against uropathogenic clinical isolates compared to laboratory strains. CHD was more potent against clinical strains whereas CDCHD showed improved activities against laboratory strains. However, due to an overall significantly more pronounced bactericidal activity of CDCHD compared to CHD, CDCHD appears to be the preferred scaffold. Nevertheless, further synthesis to improve pharmacological properties including bioavailability or increased solubility need to be performed. Additionally, to further develop the scaffold of chelocardins, in particular CDCHD and to obtain a lead molecule, it is crucial to identify the target molecule and to achieve a full characterization of the molecule.

In summary, chelocardins depict a promising class of molecules with potent broad-spectrum antibacterial activity and they exhibit a mode of action different to typical tetracycline antibiotics Bioengineering yielded an improved derivative, CDCHD, which was shown to be more efficient with regards to *in vivo* efficacy and resistance-breaking properties. Thus, the CDCHD scaffold can serve as starting point to develop a new antibiotic drug able to overcome multidrug-resistance.

# 4 TELOMYCINS

## CONTRIBUTIONS

Jennifer Herrmann (HIPS-MINS) determined some of the presented MIC values and generated resistant mutants. Antoine Abou Fayad (HIPS-MINS) isolated natural products, synthesized derivatives and tool compounds, and performed NMR experiments. Armin Bauer (Sanofi) provided the natural product and two semi-synthetic derivatives. Katarina Cirnski (HIPS-MINS) generated *S. aureus* gene disrupted mutants. Nestor Zaburannyi (HIPS-MINS) performed *in silico* genome analyses. In collaboration with Prof. Dr. Markus Bischoff (UKS Homburg, Medical Microbiology and Hygiene) $MIC_{50}$/$MIC_{90}$ values of clinical isolates were determined. Hans Prochnow (HZI Braunschweig, group CBIO) performed direct lipid binding assays. Protein identification by MALDI-TOF/MS and LC-MS/MS was performed in collaboration with Prof. Dr. Lothar Jänsch (HZI Braunschweig, group CPRO). Jan Pippel (HZI Braunschweig, group SFPR) performed heterologous expression of proteins used for SPR studies. Anna Müller (University of Bonn, Institute for Pharmaceutical Microbiology) performed intracellular localization studies with *S. aureus*. All other presented experiments were performed by the author.

## 4.1 RESULTS

### 4.1.1 MECHANISM OF ACTION

#### 4.1.1.1 DETERMINATION OF MINIMAL INHIBITORY CONCENTRATIONS

Initially, the antibacterial activity of TM and two derivatives, TM-Dodec and TM-*N*-Oct (Figure 4-1), was evaluated against a panel of Gram-positive bacteria in EBS medium. Growth inhibition was assessed by visual inspection and MIC (minimal inhibitory concentration) values were determined as the lowest concentration of antibiotic at which no visible growth was observed. Against all tested bacteria, TM was 16- to 128-fold less active compared to the two semi-synthetic derivatives, which were active in the low µg/mL range (0.5-4 µg/mL, Table 4-1). A re-screen of the activity of all telomycins (TMs) against *Staphyloccocus aureus* str. Newman in medium containing various concentrations of $CaCl_2$ (0-50 mM) revealed a $Ca^{2+}$-dependent increase of TM's antibacterial activity. At $Ca^{2+}$ concentrations around the physiological level of free calcium in blood (1.25 mM), an 8-fold improvement of activity was observed and at concentrations over 10 mM no further improvement was observed (Table 4-2). TM displayed an MIC of 2 µg/mL at highest $Ca^{2+}$ concentrations ($\geq$ 10 mM) and thus, was comparably active to the two semi-synthetic derivatives with MIC values of 2 and 0.5 µg/mL for TM-Dodec and TM-*N*-Oct, respectively. Interestingly, the addition of calcium ions had no effect on the activity of both derivatives.

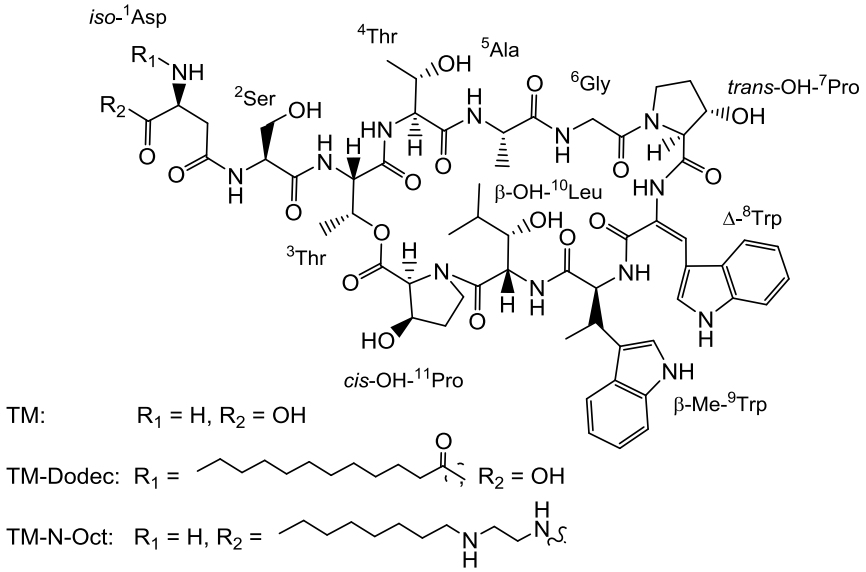



**Figure 4-1:** Chemical structures of telomycin (TM) and two semi-synthetic derivatives (TM-Dodec and TM-*N*-Oct).

**Table 4-1:** MIC values of TM, TM-Dodec and TM-*N*-Oct against Gram-positive bacteria.

| Strain | MIC [µg/mL] | | |
|---|---|---|---|
| | TM | TM-Dodec | TM-*N*-Oct |
| *Enterococcus faecium* | | | |
| DSM-20477 | 16 | 2 | 1 |
| DSM-17050 | 8 | 2 | 0.5 |
| *Staphylococcus aureus* | | | |
| Newman | 32 | 2 | 1 |
| N315[a] | 32 | 2 | 1 |
| Mu50[b,c] | > 64 | 4 | 1 |
| *Streptococcus pneumoniae* DSM-11865[d] | 64 | 1 | 0.5 |

[a]methicillin-resistant *S. aureus* (MRSA); [b]multidrug-resistant (MDR) *S. aureus*; [c]vancomycin-intermediate *S. aureus* (VISA); [d]penicillin-resistant

**Table 4-2:** MIC values of TM, TM-Dodec and TM-*N*-Oct against *S. aureus* Newman in the presence of various concentrations of $CaCl_2$.

| $Ca^{2+}$ [mM] | MIC [µg/mL] | | |
|---|---|---|---|
| | TM | TM-Dodec | TM-*N*-Oct |
| 0 | 32 | 2 | 0.5 |
| 0.1 | 16 | 2 | 0.5 |
| 0.5 | 8 | 2 | 0.5 |
| 1 | 4 | 2 | 0.5 |
| 5 | 4 | 2 | 0.5 |
| 10 | 2 | 2 | 0.5 |
| 25 | 2 | 2 | 0.5 |
| 50 | 2 | 2 | 0.5 |

A follow-up screen of all TMs in the presence of 1.25 mM $CaCl_2$ was performed against a panel of Gram-positive and Gram-negative bacteria and mycobacteria. All TMs were inactive against all tested Gram-negative bacteria, including a membrane permeabilized *Escherichia coli* TolC-efflux deficient strain (addition of polymyxin B nonapeptide as permeabilizer), indicating an intrinsic resistance pattern (Table 4-3). In contrast, all TMs display broad spectrum antibacterial activity against Gram-positive bacteria, except for *Mycobacterium smegmatis* and *Nocardia asteroides*, which were not susceptible. In contrast, slow-growing mycobacteria (*M. tuberculosis*, *M. bovis*) are susceptible. On average, TM was 2- to 8-fold less potent compared to the semi-synthetic derivatives and by tendency, TM-*N*-Oct was slightly more active than TM-Dodec, in particular on Staphylococci. Interestingly, TM was virtually inactive on two resistant *S. aureus* strains, *S. aureus* DSM-11822 (MDR) and *S. aureus* Mu50 (VISA/MRSA). A comparison of the activity of all TMs

to daptomycin (DAP) revealed TMs to be as potent as daptomycin and activity spectra are overlapping.

**Table 4-3:** MIC values of TM, TM-Dodec, TM-*N*-Oct and DAP against Gram-positive and Gram-negative bacteria in the presence of 1.25 mM $CaCl_2$.

| Classification | Strain | MIC [µg/ml] | | | |
|---|---|---|---|---|---|
| | | TM | TM-Dodec | TM-*N*-Oct | DAP |
| Gram-negative | *Acinetobacter baumannii* DSM-30008 | > 64 | > 64 | > 64 | > 64 |
| | *Burkholderia cenocepacia* DSM-16553 | > 64 | > 64 | > 64 | > 64 |
| | *Citrobacter freundii* DSM-30039 | > 64 | > 64 | > 64 | > 64 |
| | *Enterobacter aerogenes* DSM-30053 | > 64 | > 64 | > 64 | > 64 |
| | *Escherichia coli* | | | | |
| | DSM-1116 | > 64 | > 64 | > 64 | > 64 |
| | ATCC-25922 | > 64 | > 64 | > 64 | > 64 |
| | TolC (+ PMBN) | > 64 | > 64 | > 64 | > 64 |
| | *Klebsiella pneumoniae* DSM-30104 | > 64 | > 64 | > 64 | > 64 |
| | *Pseudomonas aeruginosa* | | | | |
| | PA14 | > 64 | > 64 | > 64 | > 64 |
| | DSM-24599 | > 64 | > 64 | > 64 | > 64 |
| | *Proteus vulgaris* DSM-2140 | > 64 | > 64 | > 64 | > 64 |
| | *Serratia marcescens* DSM-30121 | > 64 | > 64 | > 64 | > 64 |
| Gram-positive | *Bacillus subtilis* DSM-10 | 2 | 1 | 0.5 | 0.5 |
| | *Bacillus megaterium* DSM-32 | 0.25 | 1 | 0.25 | 1 |
| | *Corynebacterium glutamicum* DSM-20300 | 0.5 | 1 | 0.5 | 0.5 |
| | *Enterococcus faecalis* DSM-20478 | 16 | 8 | 8 | 4 |
| | *Enterococcus faecium* DSM-20477 | 32 | 8 | 4 | 16 |
| | *Streptococcus pneumoniae* DSM-11865 | 4 | 1 | 1 | 0.25 |
| | *Micrococcus luteus* | | | | |
| | DSM-1790 | 0.25 | 1 | 0.25 | 0.25 |
| | DSM-20030 | 0.25 | 1 | 0.25 | ≤ 0.03 |
| | *Mycobacterium smegmatis* $mc^2155$ | > 64 | > 64 | 64 | > 64 |
| | *Mycobacterium bovis BCG* DSM-43990 | 4-8 | 32 | 4-8 | |
| | *Mycobacterium tuberculosis* H37RvMA | 3.8 | 7.2 | 2.7 | |
| | *Nocardia asteroides* DSM-43757 | 64 | 64 | 64 | > 64 |
| | *Staphylococcus epidermidis* DSM-28765 | 1 | 2 | 0.5 | 1 |
| | *Staphylococcus carnosus* DSM-20501 | 1 | 1 | 0.5 | 0.25 |
| | *Staphylococcus aureus* | | | | |
| | DSM-346 | 1 | 1 | 1 | 0.5-1 |
| | DSM-11822[a] | > 64 | 8 | 4 | 1 |
| | ATCC-29213 | 32 | 4 | 4 | 1 |
| | Cowan1 | 2-4 | 1 | 1 | 1 |
| | Newman | 2 | 2 | 1 | 1 |
| | N315[b] | 2 | 2 | 1 | 1 |
| | Mu50[a,c] | 64 | 8 | 4 | 4 |

DAP: daptomycin. PMBN: polymyxin B nonapeptide. [a]multidrug-resistant (MDR) *S. aureus*; [b]methicillin-resistant *S. aureus* (MRSA); [c]vancomycin-intermediate *S. aureus* (VISA)

Screening of TMs against clinical isolates (n=98) of *Staphylococcus aureus* confirmed the potency of both derivatives (Table 4-4). $MIC_{50}$ and $MIC_{90}$ values demonstrated superior activity of TM-Dodec and TM-*N*-Oct compared to TM. For TM, a $MIC_{50}$ value of 8µg/mL and $MIC_{90}$ value of 64 µg/mL was determined, whereas TM-Dodec and TM-*N*-Oct showed 8-fold and 32-fold lower $MIC_{50}$ and $MIC_{90}$ values, respectively ($MIC_{50}$: 1 µg/mL; $MIC_{90}$: 2µg/mL).

**Table 4-4:** $MIC_{50}$ and $MIC_{90}$ values of TM, TM-Dodec and TM-*N*-Oct against *S. aureus* isolates.

| Compound | n = | Number of *S. aureus* isolates inhibited by the indicated concentration (MIC in µg/mL) | | | | | | | | | | | | | | $MIC_{50}$ | $MIC_{90}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ≤ 0.03 | 0.03 | 0.06 | 0.13 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | > 64 | | |
| TM | 98[a] | | | | | | | 2 | 12 | 13 | 23 | 16 | 18 | 12 | 2 | 8 | 64 |
| TM-Dodec | 98[a] | | | | | | 17 | 44 | 32 | 5 | | | | | | 1 | 2 |
| TM-*N*-Oct | 98[a] | | | | 1 | 8 | 22 | 39 | 26 | 2 | | | | | | 1 | 2 |

[a]90 clinical isolates, 8 laboratory strains.

### 4.1.1.2 KILLING OF INTRACELLULAR BACTERIA

To examine the intracellular killing effect of TMs, murine macrophages (RAW264.7 cell line) were infected with *Staphyloccocus aureus* N315 at a multiplicity of infection (MOI) of 10 and killing of intracellular bacteria was determined by CFU count after 18 h of treatment with 1- and 4-fold MIC of TMs (Figure 4-2). At 1-fold MIC of TM, only a slight killing of intracellular *Staphylococcus* was achieved (approx. 0.5-$log_{10}$ reduction) but an increase to 4-MIC led to 2-$log_{10}$ reduction of bacterial load. TM-*N*-Oct and TM-Dodec were already able to reduce bacterial concentration up to 2-$log_{10}$ at 1-fold MIC and increasing concentrations to 4-fold MIC only slightly improved the killing capacity of both derivatives.

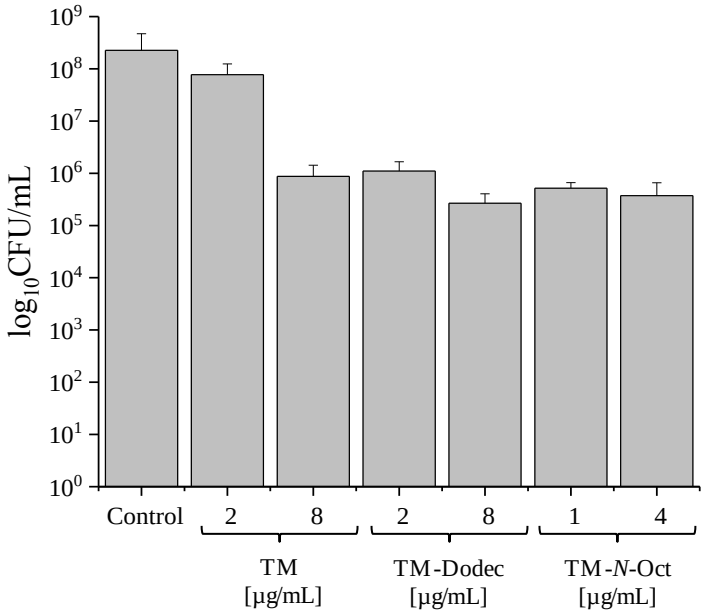



**Figure 4-2:** Killing of intracellular *Staphyloccocus aureus* by TM (MIC: 2 µg/mL), TM-Dodec (MIC: 2 µg/mL) and TM-*N*-Oct (MIC: 1 µg/mL). Murine macrophages (RAW264.7) were infected with *S. aureus* N315 and treated with TMs for 18 h at 1- and 4-fold MIC. Intracellular bacterial concentrations were determined by three independent CFU counts. Data represent mean ± standard deviation (SD).

#### 4.1.1.3 CALCIUM-DEPENDENT MEMBRANE DEPOLARIZATION

Due to TM‘s similarity to the lipopeptide antibiotic daptomycin in terms of calcium-dependency and antibacterial spectrum, the influence on the membrane potential (MP) of *S. carnosus* was assessed after treatment with TM in the presence of increasing concentrations of $Ca^{2+}$ by using the BacLight™ Bacterial Membrane Potential Kit (Invitrogen). *S. carnosus* DSM-20501 was exposed to 5-fold MIC of TM in the presence of 0-10 mM $CaCl_2$ and MP was measured after 2.5 h of treatment. In order to determine the relative MP, samples were stained with the cyanine dye 3,3‘-diethyloxacarbocyanin iodide ($DiOC_2(3)$) for 30 min and fluorescence was measured using a microplate reader [excitation.: 488 nm; emission: 675 nm (green), 525 nm (red)]. In cells with intact MP, an intracellular accumulation of $DiOC_2(3)$ is observed and the emission maximum turns towards red. A collapse of the MP leads to a decrease of the intracellular dye concentration and the emission maximum shifts towards green fluorescence. Relative MP was calculated as the ratio of red to green fluorescence and the value of untreated control cells was set to 100 %. The proton ionophore carbonyl cyanide 3- chlorophenylhydrazone (CCCP) served as positive control[185,186]. As expected, the effect of membrane depolarization increased proportional to the concentration of $CaCl_2$ along with improved antibacterial activity of TM in the presence of $Ca^{2+}$ at concentrations higher than

0.5 mM (MIC in one-digit µg/mL range). A drop of MP to less than 20 % was observed when calcium was present at concentrations ≥ 0.25 mM (Figure 4-3), indicating a membrane associated mechanism of action of TM in the presence of calcium. The results are also in accordance with previously determined MIC values.

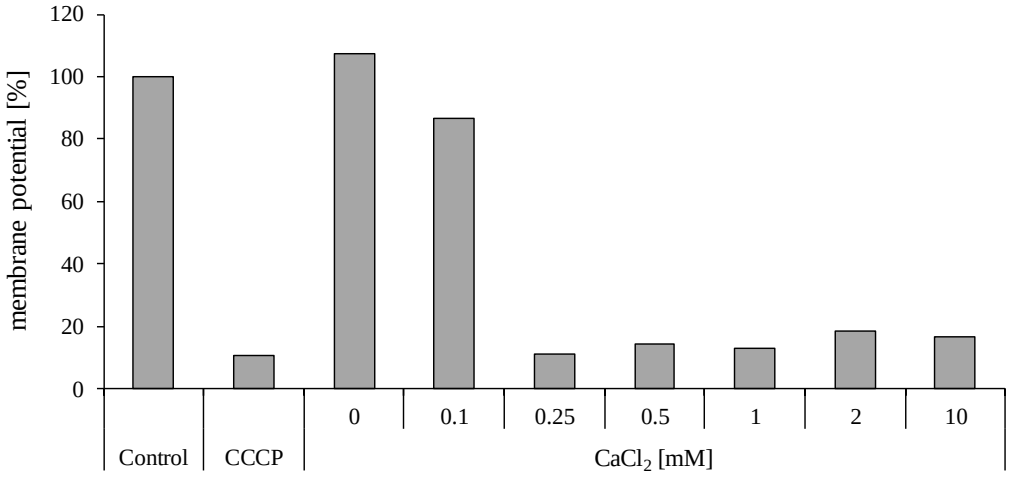
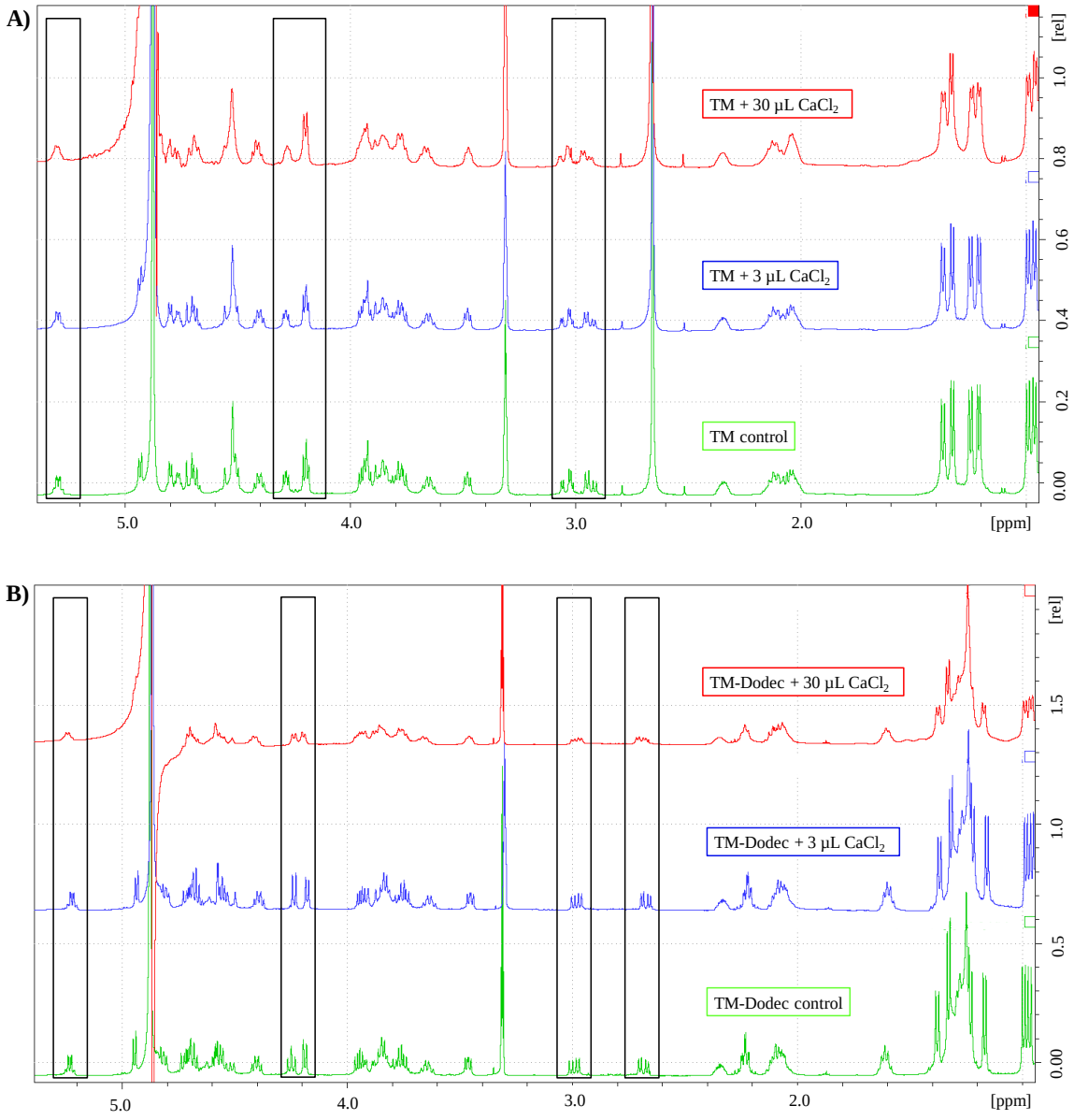



**Figure 4-3:** Membrane depolarization of *S. carnosus* DSM-20501 caused by TM (MIC: 1 µg/mL) in a $Ca^{2+}$-dependent manner (c = 0-10 mM). Cells were treated with TM at 5-fold MIC in the presence of 0-10 mM $CaCl_2$ for 2.5 h. CCCP (15 µM) served as positive control. Results show membrane potential (MP) relative to untreated cells. Positive control CCCP: carbonyl cyanide 3- chlorophenylhydrazone.

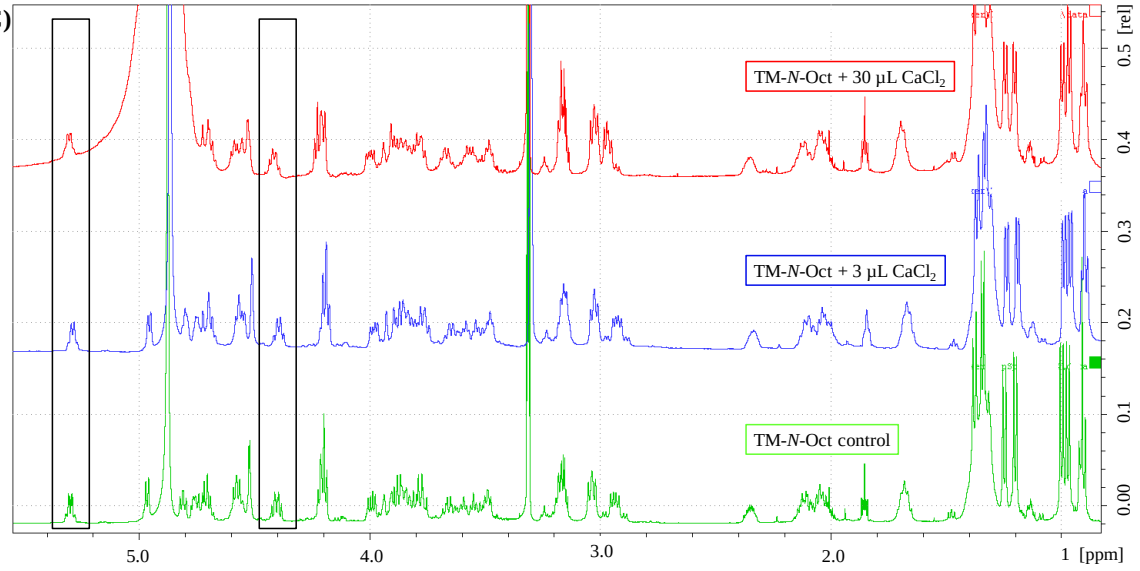
#### 4.1.1.4 CALCIUM-DEPENDENT CONFORMATIONAL CHANGES

Potential conformational changes upon calcium addition were analysed by proton ($^1$H) NMR. The interaction of TMs with $Ca^{2+}$ was studied in the presence of increasing amounts of a 0.1 M $CaCl_2$ solution. Broadening of some proton signals (Figure 4-4 A-C, black boxes), which was observed in a $Ca^{2+}$-concentration-dependent manner, indicating binding of $Ca^{2+}$ to TM, TM-Dodec and TM-*N*-Oct. The more $Ca^{2+}$ ions were present, the broader the recorded signal was. This effect was slightly more pronounced in the case of TM compared to the two semi-synthetic derivatives. In addition, preliminary 2D ROESY NMR data (not shown) indicated potential conformational changes of TM upon the addition of calcium which seemed to affect mostly amino acids $^3$Thr, $^4$Thr, $^2$Ser and *iso*-$^1$Asp (Figure 4-4 D). This was not observed for TM-Dodec and TM-*N*-Oct. Additionally, the interaction of TM with other metal ions was tested and monovalent ions did not show any effect even at high concentrations, whereas $Cu^{2+}$ showed the most pronounced effect, followed by $Ba^{2+}$ and $Ca^{2+}$ (Figure 4-5, Table 4-5). In terms of biological activity, calcium ions had a major effect when added at physiological concentrations (1.25 mM; Table 4-6). Addition of magnesium ions

to TM resulted in only minor effects on NMR spectra, which is in accordance to the results from MIC testing (Table 1.6), where the antibacterial effect of TM did not improve at physiological concentration of $Mg^{2+}$ (0.8 mM). $Ba^{2+}$ and $Cu^{2+}$ could not be used for activity determination due to their high toxicity.



C)




D)

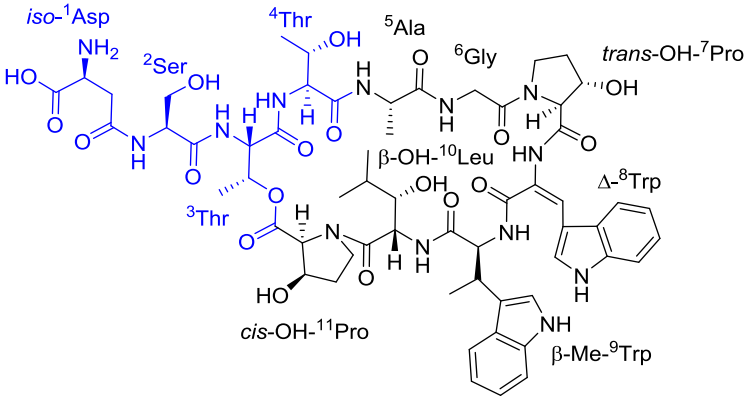



**Figure 4-4:** $^{1}$H-NMR studies on the interaction of TM (A), TM-Dodec (B) and TM-*N*-Oct (C) with increasing amounts of $CaCl_2$ (0.1 M). NMR shifts pointing towards binding of TMs to $Ca^{2+}$ (broadening of some signals; black boxes). The addition of $Ca^{2+}$ to TM mainly affected signals of amino acids $^{3}$Thr, $^{4}$Thr, $^{2}$Ser and *iso*-$^{1}$Asp (D).

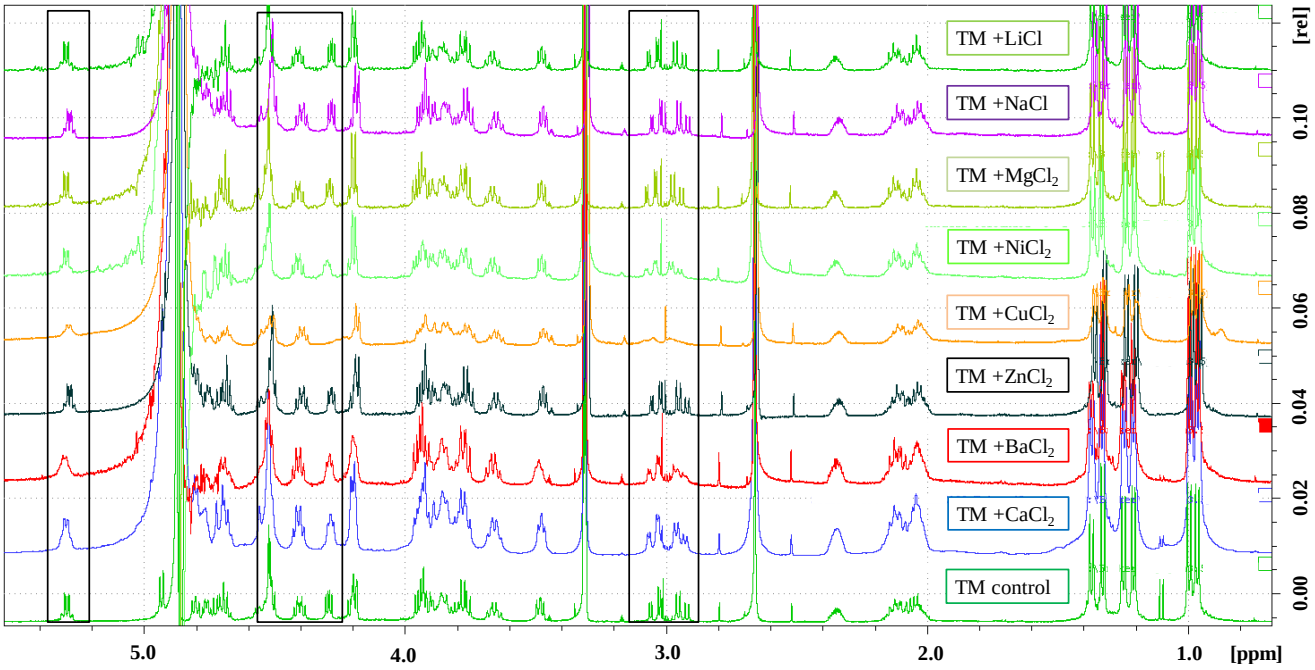



**Figure 4-5:** $^{1}$H-NMR structural studies on TM and its interaction with various monovalent and divalent metal ions. NMR shifts pointing towards binding of TM to the respective ions (broadening of some signals upon addition of $CuCl_2$, $BaCl_2$, and $NiCl_2$; black boxes).

**Table 4-5:** Interaction (signal broadening in $^{1}$H NMR spectra) of monovalent and divalent ions with TM. -: no binding; +: binding (the more +, the stronger the effect).

| Ions tested | Interaction (NMR) |
|---|---|
| $Li^{+}$ | - |
| $Na^{+}$ | - |
| $Mg^{2+}$ | + |
| $Ni^{2+}$ | + |
| $Cu^{2+}$ | +++++ |
| $Zn^{2+}$ | - |
| $Ba^{2+}$ | ++++ |
| $Ca^{2+}$ | +++ |

**Table 4-6:** Influence of $Ca^{2+}$ and $Mg^{2+}$ on biological activity of TM in MHB medium (contains 0.4 mM $Mg^{2+}$ and 0.25 mM $Ca^{2+}$). Bold values: adjusted to physiological concentration.

| c($Mg^{2+}$/$Ca^{2+}$) [mM] | MIC of TM on MSSA* [µg/mL] |
|---|---|
| 0.4/0.25 | 32 |
| 0.4/**1.25** | 2 |
| **0.8**/0.25 | 32 |

* MSSA: methicillin sensitive *S. aureus* Newman

### 4.1.2 RESISTANCE MECHANISM AND BINDING TO CARDIOLIPIN

#### 4.1.2.1 GENERATION OF TELOMYCIN-RESISTANT MUTANTS

Wildtype *S. aureus* Newman was exposed to stepwise increasing concentrations of either TM (8, 30, 50, 100 µg/mL) or TM-Dodec (10, 20, 50, 100 µg/mL) in the presence of either 10 or 0.25 mM $CaCl_2$, respectively. This yielded seven independent TM-resistant mutants (Mt-T.1, Mt-T.3, Mt-T.4, Mt-T.6, Mt-T.7, Mt-T.8, Mt-T.9) and nine independent TM-Dodec-resistant mutants (Mt-D.1, Mt-D.3, Mt-D.4, Mt-D.5, Mt-D.6, Mt-D.7, Mt-D.8, Mt-D.9, Mt-D.10). All mutants finally grew at a concentration of 100 µg/mL TM and TM-Dodec, respectively, without any obvious fitness cost. The frequency of developing *S. aureus* Newman resistant mutants was determined by exposing the wildtype strain to 4-foldMIC of the respective compound at a final cell concentration of $10^7$ (TM) or $10^8$ (TM-Dodec) CFU/plate. *S.* aureus Newman resistant mutants developed at frequencies of $4 \times 10^{-6}$ for TM and $8.5 \times 10^{-9}$ for TM-Dodec.

The resistance phenotype of all TM-resistant mutants was confirmed (MIC of TM > 64 µg/mL). However, these mutant strains were still susceptible towards TM-Dodec, TM-*N*-Oct and DAP with a maximum two-fold MIC shift (Table 4-7). Resistance of TM-Dodec-resistant mutants towards TM-Dodec was also confirmed by MIC determination and interestingly, all nine mutants showed additionally cross-resistance with TM and TM-*N*-Oct but not with DAP (Table 4-8). TM- and TM-Dodec-resistant mutants displayed no co-resistance with reference antibiotics, indicating that TMs may have a new antibacterial target and probably exhibit a novel mechanism of action that differs from that of established antibiotic classes.

**Table 4-7:** Resistance pattern of *S. aureus* Newman TM-resistant mutants. Wt*: S. aureus* Newman.

| Antibiotic compound | MIC [µg/mL] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Wt | Mt-T.1 | Mt-T.3 | Mt-T.4 | Mt-T.6 | Mt-T.7 | Mt-T.8 | Mt-T.9 |
| TM | 4 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 |
| TM-Dodec | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| TM-*N*-Oct | 2 | 2 | 4 | 4 | 2 | 2 | 2 | 2 |
| Daptomycin | 1 | 1 | 0.5 | 1 | 1 | 1 | 1 | 1 |
| Vancomycin | 1 | 2 | 2 | 1 | 0.5 | 1 | 2 | 2 |
| Ciprofloxacin | 0.125 | 0.125 | 0.125 | 0.06 | 0.125 | 0.125 | 0.125 | 0.125 |
| Erythromycin | 0.5 | 0.25 | 0.125 | 0.125 | 0.25 | 0.25 | 0.25 | 0.25 |
| Kanamycin | 2 | 4 | 2 | 2 | 2 | 4 | 4 | 4 |
| Ampicillin | 1 | 1 | 0.5 | 1 | 1 | 1 | 1 | 2 |
| Tetracycline | 1 | 0.06 | 0.25 | 1 | 0.5 | 1 | 0.5 | 0.5 |
| Rifampicin | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤0.03 | ≤ 0.03 | ≤ 0.03 |
| Polymyxin B | > 64 | 64 | > 64 | 64 | 64 | 64 | 64 | 64 |
| Chloramphenicol | 2 | 2 | 4 | 4 | 2 | 4 | 4 | 2 |

**Table 4-8:** Resistance pattern of *S. aureus* Newman TM-Dodec-resistant mutants. Wt*: S. aureus* Newman.

| Antibiotic compound | MIC [µg/mL] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Wt | Mt-D.1 | Mt-D.3 | Mt-D.4 | Mt-D.5 | Mt-D.6 | Mt-D.7 | Mt-D.8 | Mt-D.9 | Mt-D.10 |
| TM | 4 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 | > 64 |
| TM-Dodec | 2 | 32 | 32 | 32 | > 64 | 32 | 8 | > 64 | 32 | 64 |
| TM-*N*-Oct | 2 | 16 | 16 | 16 | 16 | 16 | > 64 | 16 | 16 | 32 |
| Daptomycin | 1 | 0.5 | 2 | 0.5 | 0.5 | 1 | 2 | 1 | 0.5 | 0.5 |
| Vancomycin | 1 | 2 | 1 | 2 | 4 | 4 | 2 | 1 | 4 | 4 |
| Ciprofloxacin | 0.125 | 0.25 | 0.25 | 0.25 | 0.06 | 0.125 | 0.03 | 0.25 | 0.25 | 0.25 |
| Erythromycin | 0.5 | 0.5 | 0.25 | 0.5 | 0.25 | 0.5 | 0.25 | 0.5 | 0.5 | 0.25 |
| Kanamycin | 2 | 2 | 4 | 4 | 4 | 4 | 2 | 4 | 2 | 4 |
| Ampicillin | 1 | 0.5 | 0.5 | 0.5 | 0.5 | 1 | 1 | 1 | 1 | 1 |
| Tetracycline | 1 | 1 | 1 | 1 | 1 | ≤ 0.03 | 1 | 1 | 2 | 1 |
| Rifampicin | ≤ 0.03 | ≤0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 | ≤ 0.03 |
| Polymyxin B | > 64 | 64 | > 64 | > 64 | 64 | > 64 | 64 | 64 | > 64 | > 64 |
| Chloramphenicol | 2 | 2 | 4 | 4 | 1 | 0.5 | 4 | 4 | 4 | 4 |

#### 4.1.2.2 GENOME SEQUENCING AND ANALYSIS OF MUTATIONS

To further investigate the mechanisms underlying resistance towards TMs, genomic DNA of all TM- and TM-Dodec-resistant mutants, as well as from wildtype *S. aureus* Newman was prepared by standard phenol-chloroform extraction and samples were sequenced (Illumina). Sequencing reads based on Illumina technology were mapped to the published reference genome (accession number NC_009641). An alignment of the wildtype genome to the mutant genomes revealed several single-nucleotide polymorphisms (SNPs) and deletions to be present within all mutants. All TM-resistant mutants contained mutations within *cls2* gene (NWMN_1992), identified as base pair deletions (Mt-T.1, Mt-T.6) or SNPs (Mt-T.3, Mt-T.4, Mt-T.7, Mt-T.8, Mt-T.9) leading to the formation of an in frame stop codon in MT-T.4, MT-T.7, MT-T.8 and MT-T.9. In addition, SNPs within a gene encoding for a diacylglycerol kinase (NWMN_1836) were present in Mt.T-1, Mt-T.4, Mt-T.7, Mt-T.8 an Mt-T.9 (Table 4-9). Additionally, inconsistent mutations were identified in the genomes of Mt-T.3 (NWNM_1272), Mt-T.7 (NWNM_1591) and Mt-T.9 (NWNM_0285/1344/2133), whereas Mt-T.9 was the most affected with three additional mutated genes. Interestingly, all TM-Dodec-resistant mutants contained SNPs (Mt-D.1, Mt-D.3, Mt-D.4, Mt-D.8, Mt-D.10) or base pair deletions (Mt-D.5, Mt-D.6, Mt-D.7, Mt-D.9) within the same *cls2* gene (NWMN_1992) as identified for TM-resistant mutants. SNPs found within *cls2* of MT-D.1 and MT-D.3 led to a stop codon, whereas all other SNPs were identified as base pair deletions (MT.D-5, MT-D.6, MT-D.7, MT-D.9) or missense point mutations (MT-D.4, MT-D.8, MT-D.10). Additionally, in all TM-Dodec-resistant mutants deletions (Mt-D.3, Mt-D.10) or SNPs (Mt-D.1, Mt-D.4, Mt-D.5, Mt-D.6, Mt-D.7, Mt-D.8, Mt-D.9) were present within the gene *pmtR* encoding the GntR family regulator PmtR (NWMN_1870, Table 4-11). For seven TM-Dodec-resistant mutants (MT.D-1, MT-D.4, MT-D.4, MT-D.6, MT-D.7, MT-D.8, MT-D.9) which bear a mutation within the gene encoding PmtR, the same amino acid was affected and the mutation led to a premature stop codon. Besides these commonly found genetic changes, serval inconsistent mutations in other genes were present within eight TM-Dodec-resistant mutants, identified as SNPs (Mt-D.1, Mt-D.3, Mt-D.4, Mt-D.5, Mt-D.6, Mt-D.8, Mt-D.9), deletions (Mt-D.4) or insertions (Mt-D.7, Mt-D.8).

As all TM-resistant mutants (TM-/TM-Dodec-resistant) had mutations of *cls2* in common, its gene product seems to play an important role to mediate resistance against TMs. Due to this overlap, further steps to characterize resistance to TMs focused on the function of the *cls2* gene. However, data indicated that further genetic changes are needed to gain higher

resistance towards acylated TM derivatives (mutations of PmtR found in all TM-Dodec resistant mutants).

**Table 4-9:** Mutations identified in TM-resistant *S. aureus* Newman mutants. Amino acids are denoted by three letter code.

| Locus | Gene product and function | Mutation (amino acid position is given in brackets, WT→mutant) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Mt-T.1 | Mt-T.3 | Mt-T.4 | Mt-T.6 | Mt-T.7 | Mt-T.8 | Mt-T.9 |
| NWMN_0285 | Hypothetical protein | | | | | | | Ile→Met (4) |
| NWMN_1272 | Lysyltransferase flippase domain MprF: involved in structural changes of the cell envelope | | Ser→Leu (337) | | | | | |
| NWMN_1344 | Ebh: cell-wall associated fibronectin binding protein | | | | | | | Δ4kb (902-4679) |
| NWMN_1591 | Transposase for insertion sequence like element *IS1272*[289,290] | | | | | Glu→Lys (218) | | |
| NWMN_1836 | Diacylglycerol kinase (DgkB) | Lys→Glu (15) | | Gly→Cys (276) | | Gly→Asp (275) | Pro→Leu (279) | Gly→Val (275) |
| NWMN_1992 | Cls2: cardiolipin synthase II | Δ15bp (744-759) | Asp→Gly (288) | Leu→Stop (97) | Δ15bp (744-759) | Glu→Stop (168) | Ser→Stop (413) | Ser→Stop (491) |
| NWMN_2133 | 50S ribosomal protein L15 | | | | | | | Asp→Val (137) |

Δ: deletion; bp: base pair; kb: kilo base.

**Table 4-10:** Mutations identified in TM-Dodec-resistant *S. aureus* Newman mutants. Amino acids are denoted by three letter code.

| Locus | Gene product and function | Mutation (amino acid position is given in brackets, WT→mutant) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Mt-D.1 | Mt-D.3 | Mt-D.4 | Mt-D .5 | Mt-D.6 | Mt-D.7 | Mt-D.8 | Mt-D.9 | Mt-D.10 |
| NWMN_0472 | Hypoxanthine phosphoribosyltransferase: involved in the conversion of purine bases into nucleotides[291] | Gln→Stop (72) | | | | | 1bp Ins (119) | 1bp Ins (136) | | |
| NWMN_0674 | SaeS: two-component sensor histidine kinase | | | Δ1bp (218) | | | | Ile→Thr (183) | | |
| NWMN_0676 | Uncharacterized membrane protein | Glu→Lys (147) | | | | | | | | |
| NWMN_1103 | Isoleucyl-tRNA ligase: catalysing function | | | | | Pro→Ser (57) | | | | |
| NWMN_1120 | guanylate kinase: catalyses the generation of guanosine diphosphate | | | Thr→Ile (141) | | | | | | |
| NWMN_1285 | tryptophan synthase subunit α: involved in catalysing tryptophan | Gln→Stop (238) | | | | | | | | |
| NWMN_1870 | PmtR: ABC transporter repressor protein | Leu→Stop (30) | Δ4bp (96) | Leu→Stop (30) | Leu→Stop (30) | Leu→Stop (30) | Leu→Stop (30) | Leu→Stop (30) | Leu→Stop (30) | Δ12bp (120-123) |
| NWMN_1956 | Subunit TsaD of adenosine(37)-N6)-threonylcarbamoyltransferase complex | | His→Arg (114) | | | | | | | |
| NWMN_1992 | Cls2: cardiolipin synthase II | Leu→Stop (390) | Glu→Stop (329) | Asn→Lys (233) | Δ1bp (184) | Δ83bp (90-117) | Δ1bp (61) | Gly→Arg (139); Asp→Ala (420) | Δ1bp (278) | Gly→Asp (170) |
| NWMN_2020 | Threonylcarbamoyl-AMP synthase | | | | Pro→Leu (241) | | | | | |
| NWMN_2145 | 50S ribosomal protein L16 | | | | | | | | Arg→Cys (6) | |

Ins: insertion; Δ: deletion; bp: base pair

### 4.1.2.3 *S. AUREUS CLS1* AND *CLS2* DISRUPTION MUTANTS

The *cls2* gene of *S. aureus* encodes for the main cardiolipin synthase and an alternative enzyme is encoded by the *cls1* gene. However, the latter one was not mutated in any of the TM- and TM-Dodec-resistant strains. To examine the effects of a loss in function of *cls1* and *cls2* towards susceptibility of *S. aureus* Newman against TMs and on cellular levels of cardiolipin, *S. aureus* disruptions strains of both genes were generated through gene disruption by intron insertions by using TargeTron®-Gene-Knockout-System Kit (Sigma Aldrich). MIC values confirmed that only an inactivation of *cls2* led to a decrease in activity against all TMs, with a significantly more pronounced effect on TM activity (MIC > 64 µg/mL; Table 4-11). Semi-synthetic derivatives were only 4- to 8-fold less active on the *cls2* mutant strain and the activity of DAP was not affected. A disruption of *cls1* had no effect on the sensitivity of *S. aureus* Newman towards TM, TM-Dodec and TM-*N*-Oct

and against DAP, compared to the wildtype. In addition, the disruption of *cls1* and *cls2* had no influence on the fitness of mutants, reflected in a similar growth behaviour as observed for *S. aureus* Newman wildtype (Figure 4-6).

**Table 4-11:** MIC values of TM, TM-Dodec, TM-*N*-Oct and DAP on *S. aureus* Newman wildtype compared to mutants with disruption within *cls1* and *cls2*.

| *S. aureus* Newman | MIC [µg/ml] | | | |
|---|---|---|---|---|
| | TM | TM-Dodec | TM-*N*-Oct | DAP |
| Wildtype | 1 | 0.5 | 0.5 | 0.5 |
| disrupted *cls2* | > 64 | 4 | 2 | 0.25 |
| disrupted *cls1* | 2 | 1 | 0.5 | 0.5 |

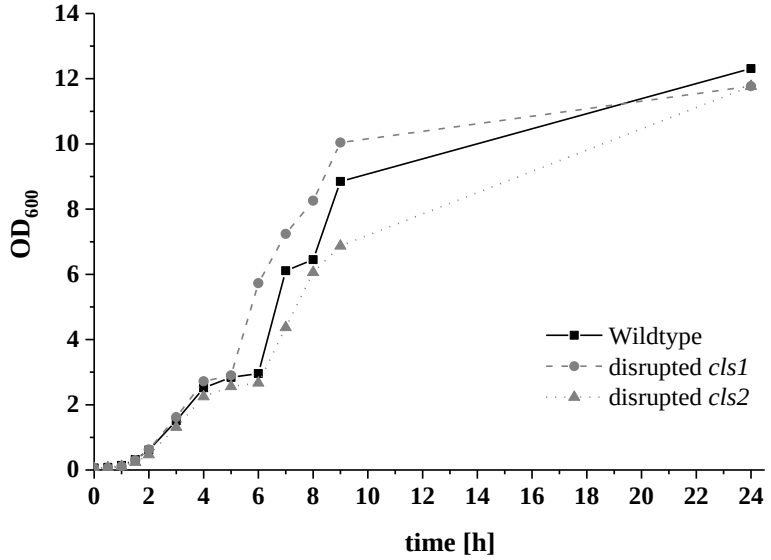



**Figure 4-6:** Growth curves of *S. aureus* Newman wildtype and *cls1* and *cls2* disruption mutant over 24 h. Data represent $OD_{600}$ values of each time point.

The presence of phospholipids within *S. aureus* Newman wildtype and *cls* disrupted mutants was analysed by thin-layer chromatography (TLC). Lipids were extracted with a mixture of chloroform-MeOH-$H_2O$ (2:2:1; *v/v/v*), applied to silica TLC plates and developed with chloroform-hexane-MeOH-acetic acid (50:30:10:5; *v/v/v/v*). Plates were sprayed with $CuSO_4$ and heated to detect lipids. For *S. aureus* Newman wildtype and *cls1* disrupted mutant, all common lipid species (CL: cardiolipin; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; LPG: lysyl-phosphatidylglycerol) were detected, whereas the

inactivation of *cls2* led to a loss of CL (Figure 4-7) with a concurrent increase of PG compared to wildtype and *cls1* mutant.

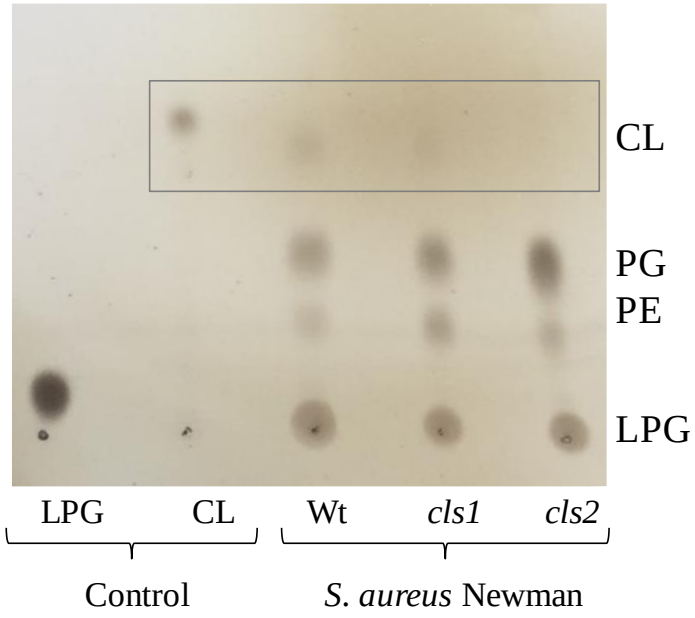



**Figure 4-7:** Composition of phospholipids of *S. aureus* Newman wildtype and *cls1* and *cls2* disrupted mutants. Wt: wildtype; *cls1*: *cls1* disrupted mutant; *cls2*: *cls2* disrupted mutant; CL: cardiolipin; PG: phosphatidylglycerol; PE: phosphatidylethanolamine; LPG: lysyl-phosphatidylglycerol.

As the alternative cardiolipin synthase 1 (Cls1) is able to compensate the loss of functional Cls2 under conditions of osmotic or pH stress[292,293], it was investigated whether the *cls2* disrupted mutant regains susceptibility towards TM under stress conditions. *S. aureus* Newman wildtype and *cls2* disrupted mutant were incubated under osmotic stress (4 M NaCl), low pH (pH 2, pH 4) and normal (PBS buffer) growth conditions for 60 min, followed by lipid extraction and MIC determination. All tested stress conditions had no major influence on the susceptibility of *S. aureus* Newman wildtype towards TM, TM-Dodec and TM-*N*-Oct (Table 4-12). However, low pH stress (pH 2) led to a slight improvement of activity of TM, as MIC values dropped from > 64 to 32 µg/mL. Against DAP, wildtype and mutant showed 16- and 8-fold increased susceptibility, respectively, under pH 2 stress conditions. The other stress-inducing conditions (4 M NaCl; pH 4) did not or only marginally influence the susceptibility towards DAP. To analyse the presence of CL in *S. aureus* Newman wildtype and *cls2* disrupted mutant under stress, lipids were extracted and analysed by LC-MS. Only trace amounts of CL were detected (partly below limit of detection) and peak intensities were too low to proceed with further analysis.

**Table 4-12:** Activity of *S. aureus* Newman wildtype and *cls2* disrupted mutant under conditions of osmotic or low pH stress.

| Stress condition | MIC [µg/mL] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *S. aureus* Newman wildtype | | | | *S. aureus* Newman disrupted *cls2* | | | |
| | TM | TM-Dodec | TM-*N*-Oct | DAP | TM | TM-Dodec | TM-*N*-Oct | DAP |
| Control (PBS) | 4 | 4 | 2 | 1 | > 64 | 8 | 4 | 1 |
| 4 M NaCl | 8 | 4 | 1 | 0.5 | > 64 | 8 | 4 | 1 |
| pH 2 | 4 | 4 | 1 | 0.0625 | 32 | 8 | 4 | 0.125 |
| pH 4 | 8 | 8 | 2 | 1 | > 64 | 8 | 4 | 0.5 |

#### 4.1.2.4 FITNESS OF MUTANTS AND BIOFILM FORMATION

TM-resistant mutants bearing disruptions mutations in *cls2* showed a similar growth behaviour as wildtype *S. aureus* Newman (Figure 4-8 A) but some grew slightly slower, particularly in early exponential phase (Figure 4-8 A inserted diagram). However, this slightly reduced growth profile cannot be linked to any common genotype of the mutants. Mutants generated under exposure of TM-Dodec (mutations in *cls2* and *pmtR*) showed a retarded growth compared to the wildtype (Figure 4-8 B) indicating a significant fitness loss due to the additional mutations in the gene encoding the GntR-type transcriptional regulator PmtR. However, final cell densities of mutants were comparable to the bacterial cell number in wildtype culture (24 h cultivation).

#### 4.1.2.5 IMPACT OF MUTATED GNTR-TYPE REGULATOR ON *S. AUREUS* BIOFILM

The gene that was additionally affected by mutations within TM-Dodec-resistant mutants was characterized to encode the GntR-type regulator PmtR.[294] Since its gene product is involved in the regulation of an export system for toxins and the presence of toxins is furthermore related to influence biofilm formation[294,295] (structuring and detachment), the impact on biofilm formation of TM-Dodec-resistant mutations was investigated. Compared to wildtype *S. aureus* Newman, an impaired biofilm formation for TM-Dodec-resistant mutants was observed (Figure 4-9). The biofilm formation was measured after 24 and 48 h by staining with crystal violet and determination of the optical density at 450 nm. Compared to *S. aureus* Newman wildtype, all mutants revealed a decreased biofilm formation after 24 h

and 48 h. In contrast to the wildtype, no further increase of biofilm formation after the initial 24 h incubation period was observed. For some mutants (Mt-D.4, Mt-D.6) biofilm mass even decreased after prolonged incubation.

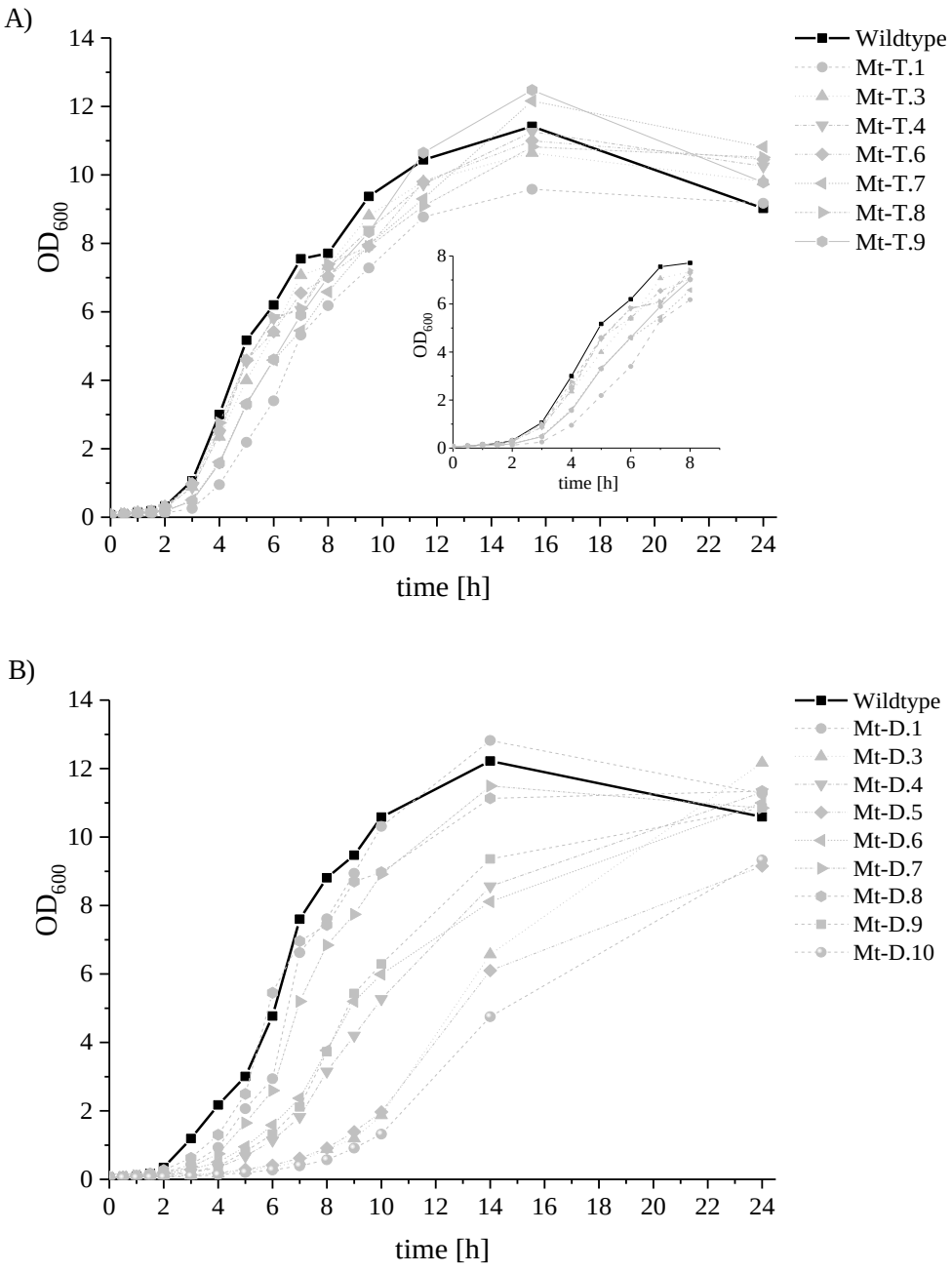



**Figure 4-8:** A) Growth curves of *S. aureus* Newman wildtype and TM-resistant mutants (Mt-T) over 24 h. Inserted diagram: growth over 8 h (early exponential phase). B) Growth curves of *S. aureus* Newman wildtype and TM-Dodec-resistant mutants (Mt-D) over 24 h. Data represent $OD_{600}$ values of each time point.

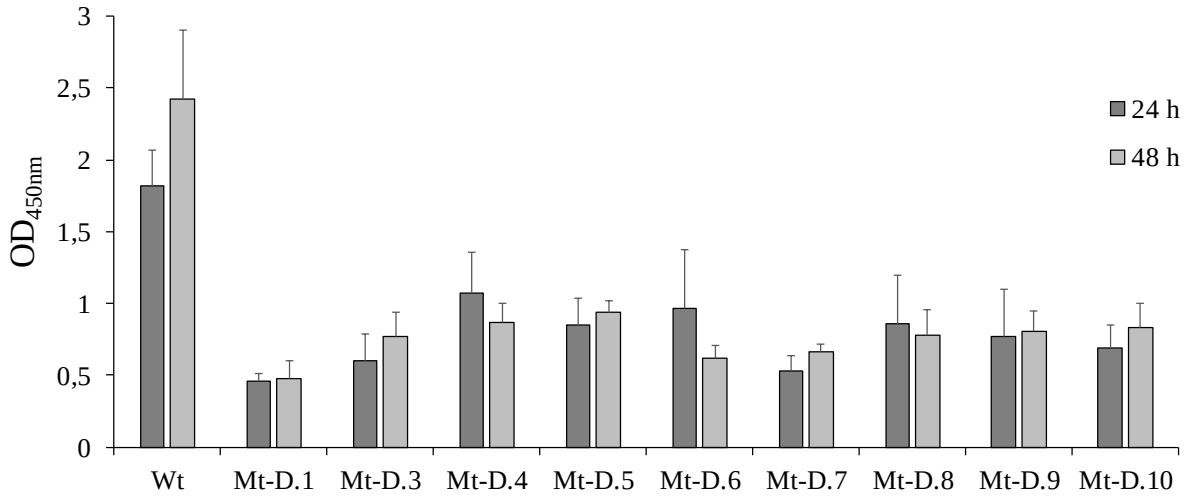


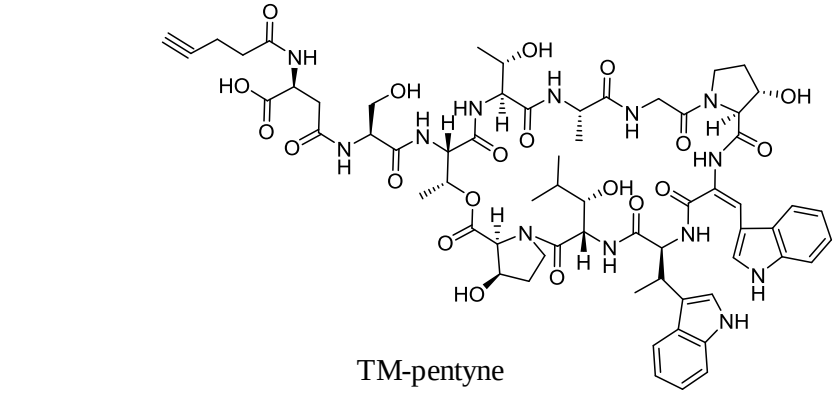

**Figure 4-9:** Biofilm formation of TM-Dodec-resistant *S. aureus* mutants (Mt-D.) in comparison to wildtype (Wt) *S. aureus* Newman monitored over 24 and 48 h. Data are represented as mean ± standard deviation (SD).

### 4.1.2.6 BINDING OF TELOMYCINS TO CARDIOLIPIN

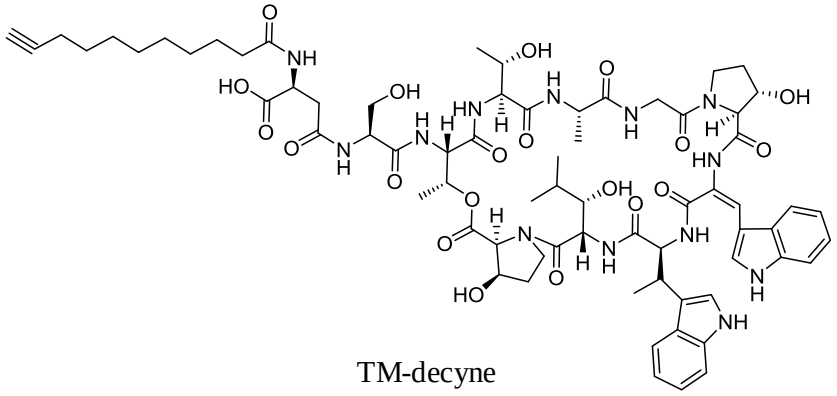
**Direct Binding Assay**

Two semi-synthetic alkyne-coupled TM derivatives, bearing a pentynoic acid (TM-pentyne) or an undecynoic acid group (TM-decyne, mimicking TM-Dodec; Figure 4-10) were synthetized to characterize the binding of TM to membrane lipids and lipoteichoic acid (LTA). Both derivatives were determined to exhibit antibacterial activities against Gram-positive bacteria in low µg/mL ranges comparable to the parent molecule TM (Table 4-13). Several lipid species (PE: phosphatidylethanolamine, PC: phosphatidylcholine, PG: phosphatidylglycerol, CL: cardiolipin, LPG: lysyl-phosphatidylglycerol) and LTA coated to surface treated 96-well plates (Nunc Polysorp; lipids: 128 pmol/well: LTA: 128ng/well) and incubated with alkyne-coupled TM derivatives at a final concentration of 0.7 µM/well. A click reaction was performed with biotin-azide and subsequent incubation with streptavidin-coupled horseradish peroxidase (HRP), revealed lipid binding partners of the two tool compounds in a colorimetric reaction using 1x TMB ELISA substrate solution (eBiosciences) as the substrate for HRP. Binding was exclusively observed for CL; all other lipid species as well as LTA showed no interaction with TM (Figure 4-11). As TM exerts a calcium-dependent mechanism, the binding of TM-pentyne to different amounts of CL (4-128 pmol/well) was assessed in the same assay in the presence of 10 mM $CaCl_2$. As expected, the binding affinity of TM-pentyne to CL can be further increased at the higher $Ca^{2+}$

concentration (Figure 4-12). Additionally, binding was clearly dependent on the amount of coupled CL with a maximum binding capacity in the presence of 64 pmol CL per well. Interestingly, no binding to CL was observed under calcium-depleted conditions, which were simulated by the addition of 10 mM of the strong chelating agent ethylenediaminetetraacetic acid (EDTA).



TM-pentyne



TM-decyne

**Figure 4-10:** Chemical structure of semi-synthetic alkyne-coupled TM derivatives.

**Table 4-13:** MIC values of TM and alkyne coupled TM-derivatives on Gram-positive bacteria.

| Indicator strain | MIC [µg/ml] | | |
|---|---|---|---|
| | TM | TM-pentyne | TM-decyne |
| *S. aureus* Newman | 2 | 2 | 4 |
| *B. subtilis* DSM-10 | 2 | 2 | 1 |
| *B. megaterium* DSM-32 | 0.25 | 0.5 | 0.5 |

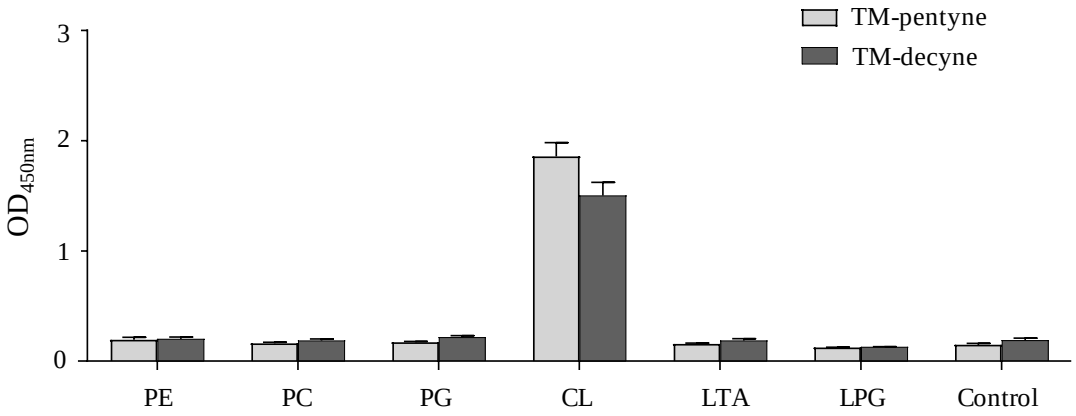



**Figure 4-11:** Relative binding affinity of TM to membrane lipids and LTA. Lipids and LTA were coated to surface treated 96-well plates (lipids: 128 pmol/well: LTA: 128ng/well) and incubated with alkyne-modified TM derivatives. After clicking to biotin-azide and incubation with streptavidin-coupled HRP, lipid binding partners of TM were analysed in a colorimetric reaction by measuring OD at 450nm. Data represent mean ± SEM. PE: phosphatidylethanolamine; PC: phosphatidylcholine; PG: phosphatidylglycerol; CL: cardiolipin; LTA: lipoteichoic acid; LPG: lysyl-phosphatidylglycerol.

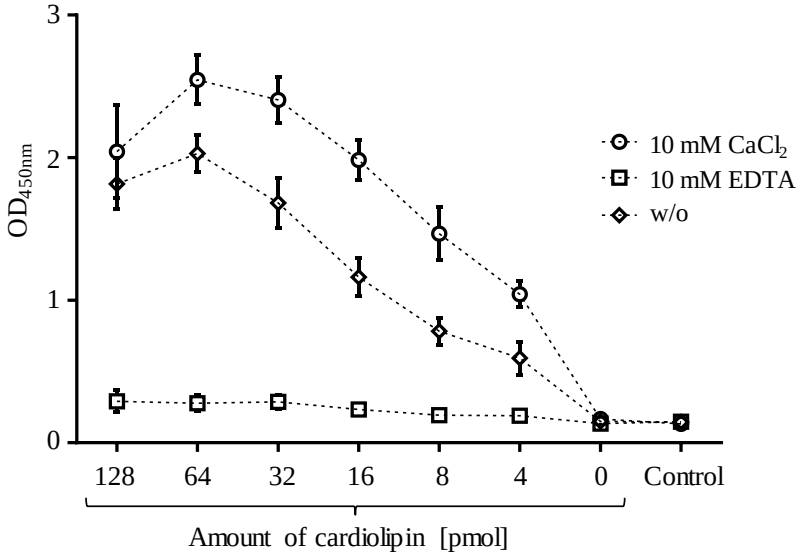



**Figure 4-12:** Binding affinity of TM-pentyne to CL in the presence of $CaCl_2$ and varying amounts of chip-coupled CL. ETDA served as negative control. Data represent mean ± SEM.

### Intracellular localisation

To localize TMs within or attached to Gram-positive cells, fluorescein- (-F) and rhodamine (-R) coupled TM (TM-F/R), TM-Dodec (TM-Dodec-R) and TM-*N*-Oct (TM-*N*-Oct-F/R) were prepared via the established semi-synthesis route. The fluorophore-tagged derivatives were less active than the respective parent compounds and displayed MIC values in the low to mid µg/mL range, whereas TM-Dodec-R and TM-F were virtually inactive (Table 4-14, Table 4-15). *Bacillus megaterium* was chosen as BSL1 model organism and was initially incubated with 50 µM of the respective fluorophore-tagged TM derivative and 50 µM fluorescein or rhodamine (control) for 30 min. Imaging of live cells revealed an accumulation of all TMs at septa and poles of treated cells (Figure 4-13 A,B). Both negative controls (fluorescein, rhodamine) showed an unspecific distribution within the cells. Incubation of *S. aureus* SG511 with 10 µg/mL of either TM-R or TM-*N*-Oct-R led to the same results, as an accumulation within septa and poles of both TMs was observed (Figure 4-13 C). To confirm the presence of CL within these regions, *B. megaterium* was treated with fluorescein-labelled TMs and co-incubated with acridine orange 10-nonyl bromide (NAO), a red fluorescent indicator for CL in membranes and bilayers[296]. Accumulation of telomycin was observed again at septa and poles of the cells and the co-incubation with NAO further confirms that TMs preferentially localize in CL-rich compartments (complete overlap of TM and NAO fluorescence).

**Table 4-14:** MIC values of TM, TM-*N*-Oct and their respective fluorescein-labelled derivative (-F) against Gram-positive strains.

| Strain | MIC [µg/mL] | | | |
|---|---|---|---|---|
| | TM | TM-F | TM-*N*-Oct | TM-*N*-Oct-F |
| *B. megaterium* DSM-32 | 0.25 | 32 | 0.25 | 2 |
| *S. aureus* Newman | 2 | > 64 | 1 | 4 |

**Table 4-15:** MIC values of TM, TM-Dodec, TM-*N*-Oct and their respective rhodamine-labelled derivative (-R) against Gram-positive strains.

| Strain | MIC [µg/mL] | | | | | |
|---|---|---|---|---|---|---|
| | TM | TM-R | TM-Dodec | TM-Dodec-R | TM-*N*-Oct | TM-*N*-Oct-R |
| *B. subtilis* DSM- 10 | 2 | 4 | 1 | 64 | 0.5 | 2 |
| *B. megaterium* DSM-32 | 0.25 | 2 | 1 | 64 | 0.25 | 0.5 |
| *S. aureus* Newman | 2 | 8 | 1 | 64 | 2 | 4 |

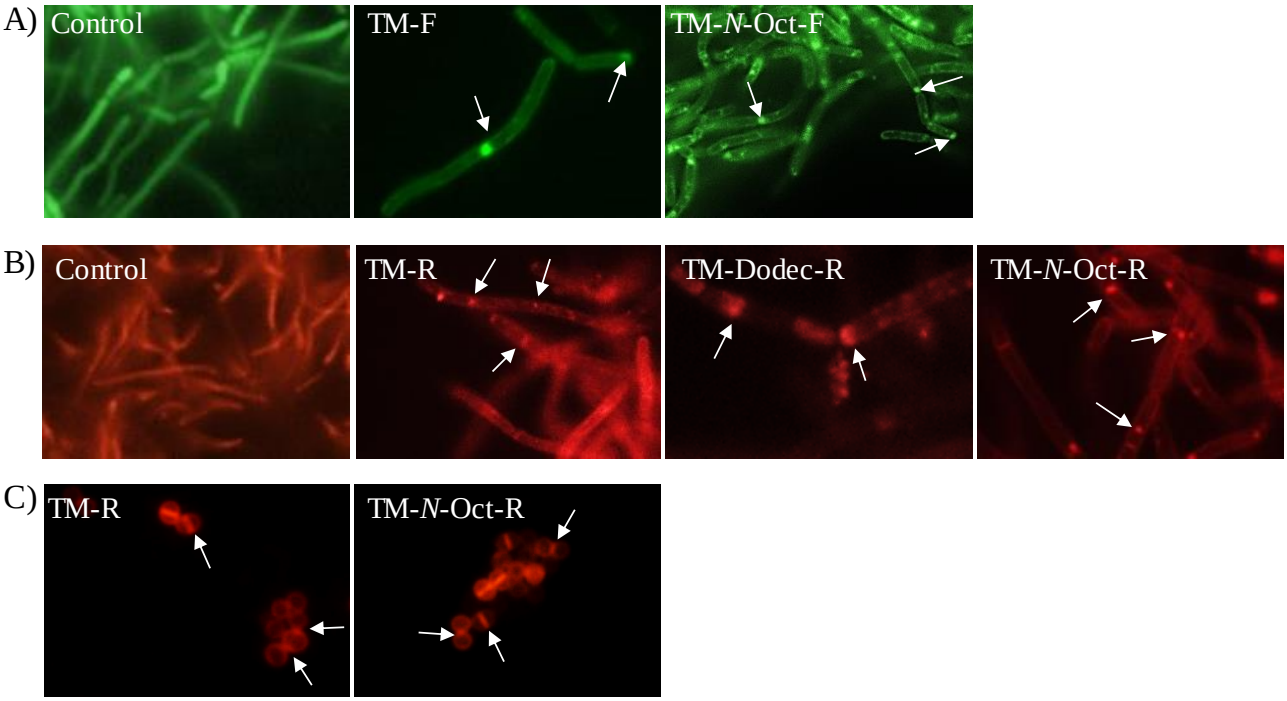



**Figure 4-13:** Localization of TM, TM-Dodec and TM-*N*-Oct within in Gram-positive cells (indicated by arrows). A/B) *B. megaterium* DSM-32 was incubated for 30 min with 50 µM fluorescein- (-F) or rhodamine (-R) labelled TM (TM-F/R), TM-Dodec (TM-Dodec-F/R) TM-*N*-Oct (TM-*N*-Oct-F/R) and 50 µM fluorescein/rhodamine as control. C) *S. aureus* SG511 was incubated with 10 µg/mL rhodamine-labelled TM (TM-R) and TM-*N*-Oct (TM-*N*-Oct-R).

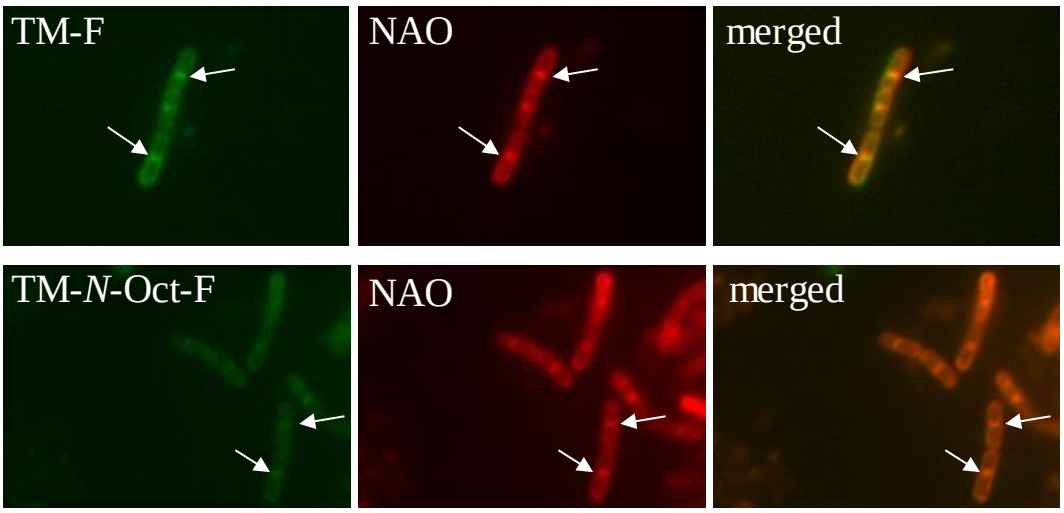



**Figure 4-14:** Localization of fluorophore-tagged TMs in CL-rich (NAO stained) regions of Gram-positive cells (indicated by arrows). *B. megaterium* DSM-32 was incubated with 50 µM fluorescein-labelled TM (TM-F) or TM-*N*-Oct (TM-*N*-Oct-F) for 30 min and co-incubated with 0.5 µM acridine orange 10-nonyl bromide (NAO).

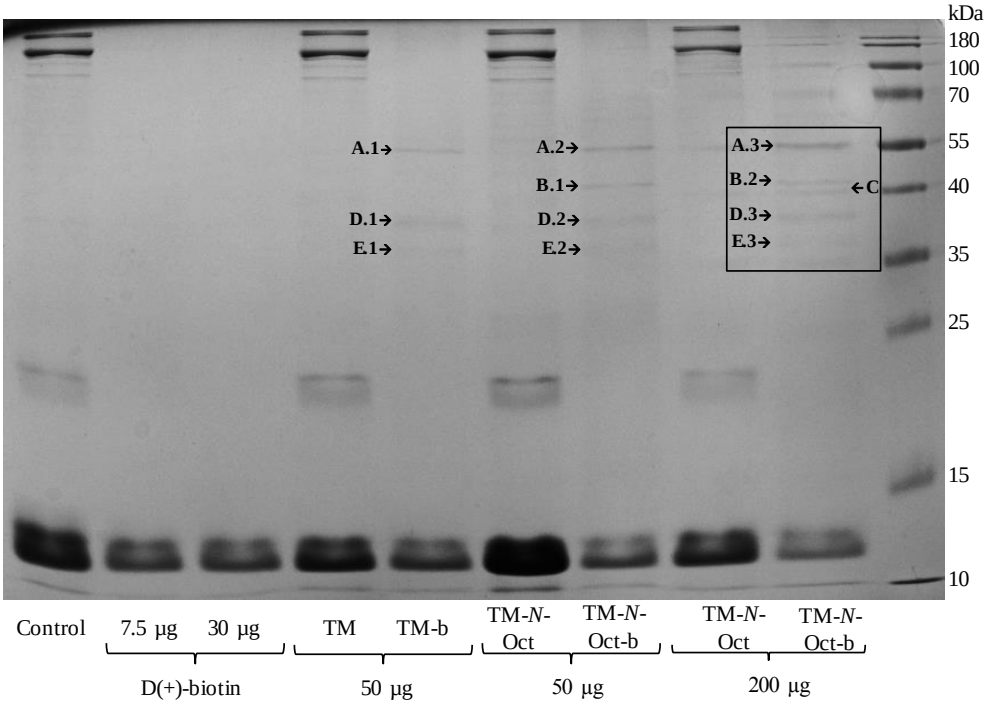
### 4.1.3 PROTEIN TARGET IDENTIFICATION

Although CL was found to be a binding partner of TMs, it seemed likely that additional targets for TMs exist. Thereof, a protein pull down experiment was performed to identify potential binding partners of TMs. Proteins were isolated from *Bacillus subtilis* DSM-10, incubated for 2 h with biotin-tagged TM (TM-b) or TM-*N*-Oct (TM-*N*-Oct-b), and streptavidin-captured proteins were separated by SDS-PAGE. Proteins that were only present within samples incubated with TM-b or TM-*N*-Oct-b or appeared to be enriched in the presence of biotin-tagged TMs were regarded as potential binding partners (Figure 4-15, protein bands A-E). Generally, all tested conditions (50 µg TM-b, 50 µg TM-*N*-Oct-b, 200 µg TM-*N*-Oct-b) led to the same protein band pattern on the SDS gel. Only bands B and C (Figure 4-15) were not present in the TM-b incubated samples. As proteins "fished" from *B. subtilis* lysate in the presence of 200 µg TM-*N*-Oct-b showed the highest intensity, only those proteins were selected for further analysis by mass spectrometry (Figure 4-15 A black box). Trypsin digested proteins were analysed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) and identified by searching against a protein database (UniProtKB/Swiss-Prot) on a MASCOT server. Proteins were identified as αβ-subunit of ATP synthase, a putative lipoprotein YcdA, subunit II (QoxA) of the quinol oxidase, and flagellin (Figure 4-15 B). As binding of TMs to flagellin is unlikely to be an antibiotic mechanism of action (as depicted in chapter 4.2.3), the binding to flagellin was excluded, therefor only YcdA, QoxA and ATP synthase were chosen for further characterization.

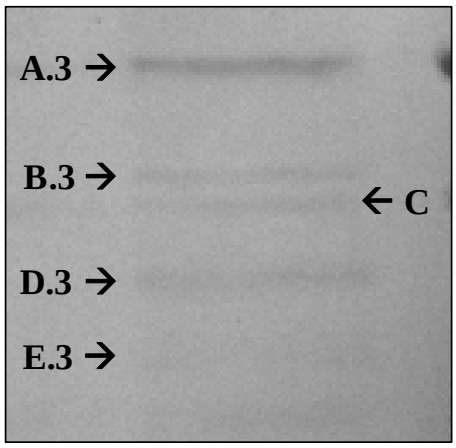
TMs were analysed in terms of direct binding to YcdA and QoxA (heterologously expressed in *E. coli* and purified) by surface plasmon resonance (SPR) spectroscopy on a Biacore instrument. Proteins were immobilized to a sensor chip by standard amine coupling and binding to increasing concentrations of either TM, TM-Dodec, TM-*N*-Oct or DAP in the presence or absence of 5 mM $CaCl_2$ was monitored. In accordance to results from protein pull-down, binding to YcdA was exclusively observed for TM-*N*-Oct (Table 4-16, Figure 4-16) with an equilibrium dissociation constant ($K_D$) of 1.1 and 0.7 µM in the absence and presence of calcium, respectively. Dissociation rates ($k_d$) were 4.3 x $10^{-3}s^{-1}$ and association rates were in the range of approximately 4000 $M^{-1}s^{-1}$. A direct interaction with QoxA was only detected for TM with a $K_D$ of 50 µM in the absence of calcium and an improved binding affinity in the presence of calcium ions ($K_D$: 8 µM, Figure 4-17). The determination of the

association and dissociation rates of TM to QoxA was not possible, thus, $K_D$ values were determined by steady-state affinity analyses.

A)




B)




| Sample | Identified protein | Mass [kDa] |
|---|---|---|
| A.3 | ATP synthase subunit αβ | 55 |
| B.2 | YcdA: putative lipoprotein | 39 |
| C | YcdA: putative lipoprotein | 39 |
| D.3 | flagellin | 33 |
| E.3 | QoxA: quinol oxidase subunit II | 36 |

**Figure 4-15:** Identification of protein binding partners for TMs. A) A cell lysate of *B. subtilis* DSM-10 was incubated for 2 h with biotin-tagged TM (TM-b, 50 µM) or TM-*N*-Oct (TM-*N*-Oct-b, 50 and 200 µM) and proteins were separated by SDS-PAGE. Untreated cell lysate (Control) as well as untagged compounds (TM, TM-*N*-Oct) and D(+)-biotin (at concentrations respective to free biotin per molecule telomycin) served as controls. Unspecific protein bands were identified as streptavidin (~12 kDa), biotin-carrier proteins (~20 kDa) or pyruvate carboxylase (~ 130 kDa). Black arrows indicate potential binding partners (protein bands that were only present or appeared enriched within biotin-tagged TMs samples). B) Proteins identified by MALDI-TOF/MS and LC-MS/MS. Black box: analysed protein bands; Table: identified proteins and their respective masses.

**Table 4-16:** Binding affinity of TM, TM-Dodec, TM-*N*-Oct and DAP to YcdA and QoxA in the absence (- $CaCl_2$) or presence (+ $CaCl_2$) of 5 mM $CaCl_2$. Equilibrium dissociation constants ($K_D$) were either determined by the ratio of association rate ($k_a$) to dissociation rate constant ($k_d$) or by steady-state affinity analysis. n.d.: not determinable.

| Protein | Compound | Concentration range [µg/mL] | - $CaCl_2$ | | | + $CaCl_2$ | | |
|---|---|---|---|---|---|---|---|---|
| | | | $K_D$ [µM] | $k_d$ [$s^{-1}$] | $k_a$ [$M^{-1}$ $s^{-1}$] | $K_D$ [µM] | $k_d$ [$s^{-1}$] | $k_a$ [$M^{-1}$ $s^{-1}$] |
| YcdA | TM | 0.8 - 50 | - | - | - | - | - | - |
| | TM-Dodec | 0.04 - 30 | - | - | - | - | - | - |
| | TM-*N*-Oct | 0.04 - 30 | 1.13 | $4.3 \times 10^{-3}$ | 3752 | 0.73 | $4.3 \times 10^{-3}$ | 5941 |
| | DAP | 0.8 - 50 | - | - | - | - | - | - |
| QoxA | TM | 0.8 - 50 | 50 | n.d. | n.d. | 8 | n.d. | n.d. |
| | TM-Dodec | 0.04 - 30 | - | - | - | - | - | - |
| | TM-*N*-Oct | 0.04 - 30 | - | - | - | - | - | - |
| | DAP | 0.8 - 50 | - | - | - | - | - | - |

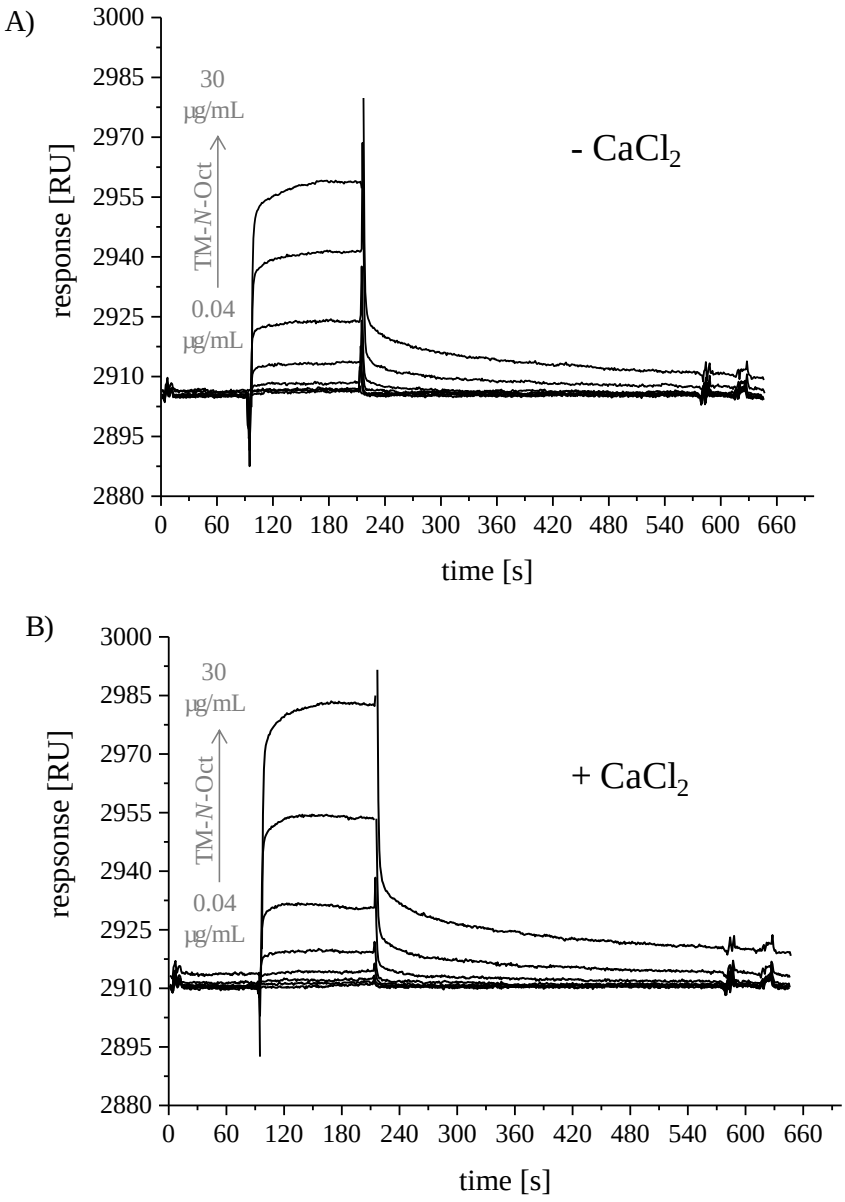



**Figure 4-16:** SPR sensorgrams of binding of increasing concentrations TM-*N*-Oct (0.04-30 µg/mL) to YcdA in the absence (A) and presence (B) of 5 mM $CaCl_2$.

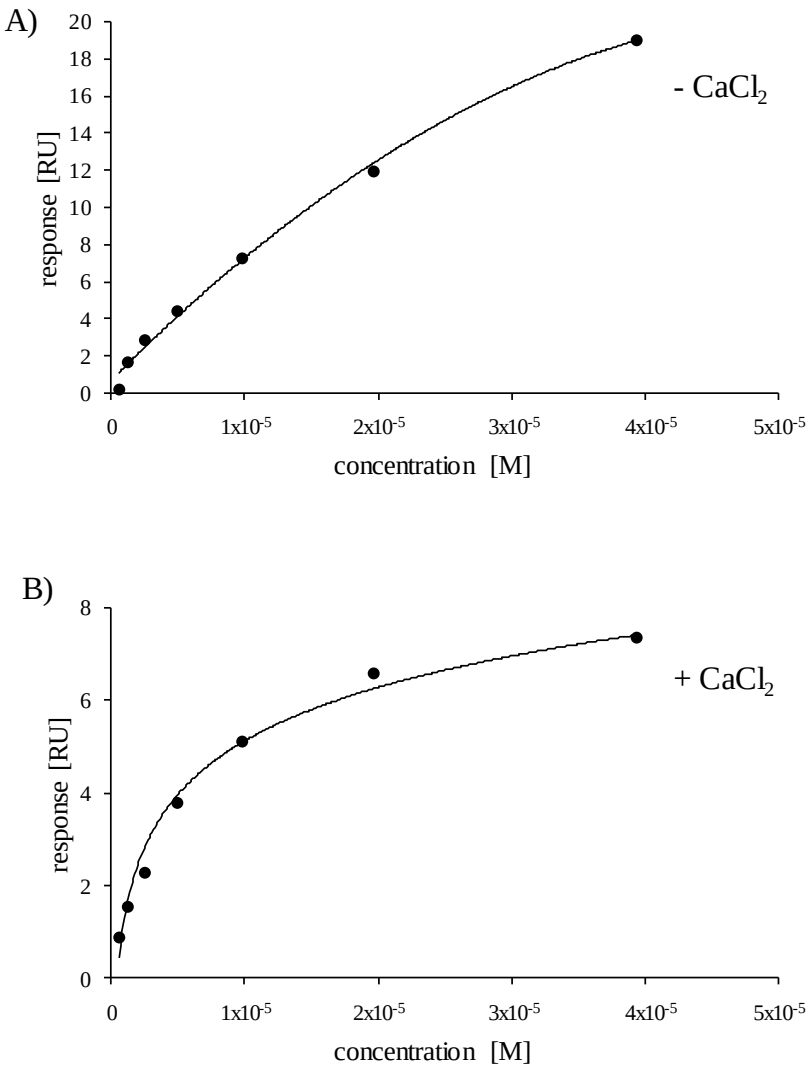



**Figure 4-17:** Steady-state analysis plots of binding of increasing concentrations TM (0 - 4x10$^{-5}$ M) to QoxA in the absence (A) and presence (B) of 5 mM $CaCl_2$.

Due to experimental limitations regarding the heterologous expression of ATP synthase, a functional assays on cell viability and a concurrent influence on the amount of ATP within cells treated with TM, TM-Dodec, TM-*N*-Oct or DAP, was performed using the luminescence-based BacTiter-Glo™ Microbial Cell Viability Kit (Promega). *S. aureus* Newman wildtype and the *cls2* disrupted mutant were treated with compounds at 2-fold MIC for 1 h, followed by addition of the BacTiter-Glo reagent and measurement of luminescence on a microplate reader (Infinite M200 Pro, Tecan). The recorded luminescence signal relies on the mono-oxygenation of luciferin, catalysed by firefly luciferase in the presence of $Mg^{2+}$ (supplied with assay reagent), molecular oxygen and ATP, with an increase of the luminescence signal proportional to the amount of available ATP. For all tested conditions

(wildtype and *cls2* mutant), no significant decrease of ATP within cells treated with TM, TM-Dodec, TM-*N*-Oct or DAP was observed (Figure 4-18).

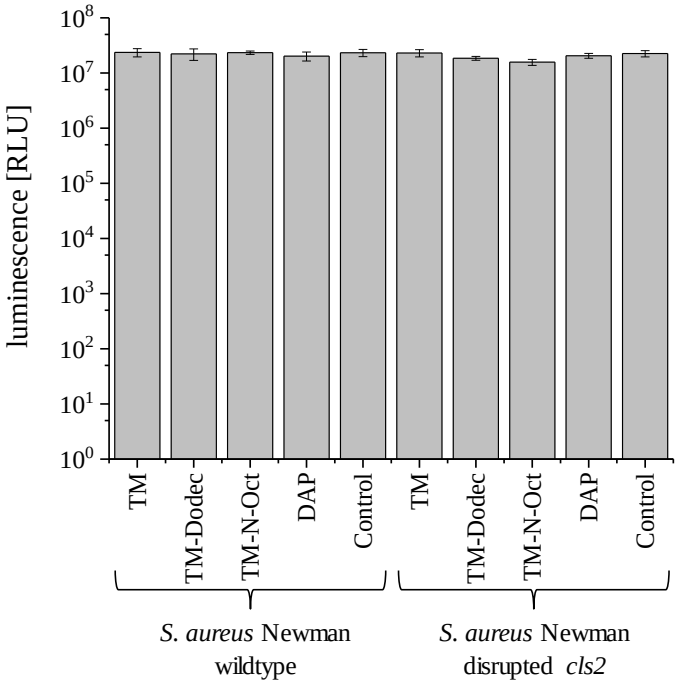



**Figure 4-18:** Cell viability based on ATP quantification of *S. aureus* Newman wildtype and disrupted *cls2* mutant over 1 h in the presence of 2-fold MIC of TM, TM-Dodec TM-*N*-Oct and DAP. Data are represented as mean ± standard deviation (SD).

### 4.1.4 ACTIVITY ON EUKARYOTIC CELLS

#### 4.1.4.1 CYTOTOXICITY

Cytotoxicity against human (U-2 OS: bone osteosarcoma) and murine (RAW264.7: leukemia macrophage; L929: subcutaneous connective tissue fibroblasts) eukaryotic cell lines was determined by a tetrazolium salt-based assay (MTT) after 5 d treatment of cells with TMs in serial dilution. The half-inhibitory concentration ($IC_{50}$) was determined by sigmoidal curve fitting using Origin 6.1G software.

Against human U-2 OS cells and murine macrophages (RAW 264.7), no *in vitro* cytotoxicity was observed for all TMs ($IC_{50}$: > 60 µg/mL, Table 4-1). Against murine L929 cells, both derivatives (TM-Dodec, TM-*N*-Oct) showed increased inhibitory effects compared to the parent compound TM ($IC_{50}$: > 100 µg/mL), but $IC_{50}$ values were still in a moderate range between 30 and 50 µg/mL.

**Table 4-17:** $IC_{50}$ values of TMs against human and murine cell lines.

| Cell line | $IC_{50}$ [µg/mL] | | |
|---|---|---|---|
| | TM | TM-Dodec | TM-*N*-Oct |
| human | | | |
| U-2 OS | > 100 | > 100 | > 100 |
| murine | | | |
| L929 | > 100 | 33.12 | 45.53 |
| RAW264.7 | > 60 | > 60 | > 60 |

### 4.1.4.2 MITOCHONDRIAL MEMBRANE POTENTIAL

As cardiolipin is not only found in the bacterial membrane but also in the inner mitochondrial membrane[297] of eukaryotic cells, effects of TMs on the mitochondrial membrane potential (MMP) were determined. U-2 OS cells were treated with TMs at 10 µg/mL for 18 h followed by a staining with tetramethyl rhodamine methyl ester (TMRM). The cationic, cell permeable fluorescence probe TMRM reacts sensitive towards changes of MMP, whereas a red-orange fluorescence indicates the presence of active mitochondria in living cells[298]. The relative MMP was calculated based on TMRM fluorescence intensity in the cytoplasm. Cells treated with 10 µg/mL TM, TM-Dodec or TM-*N*-Oct led to a negligible reduction of MMP (≤ 10 %) compared to untreated control cells (Figure 4-19), demonstrating no activity of TMs on the mitochondrial membrane potential of mammalian cells under these conditions and indicating an interaction with CL preferably in bacterial species.

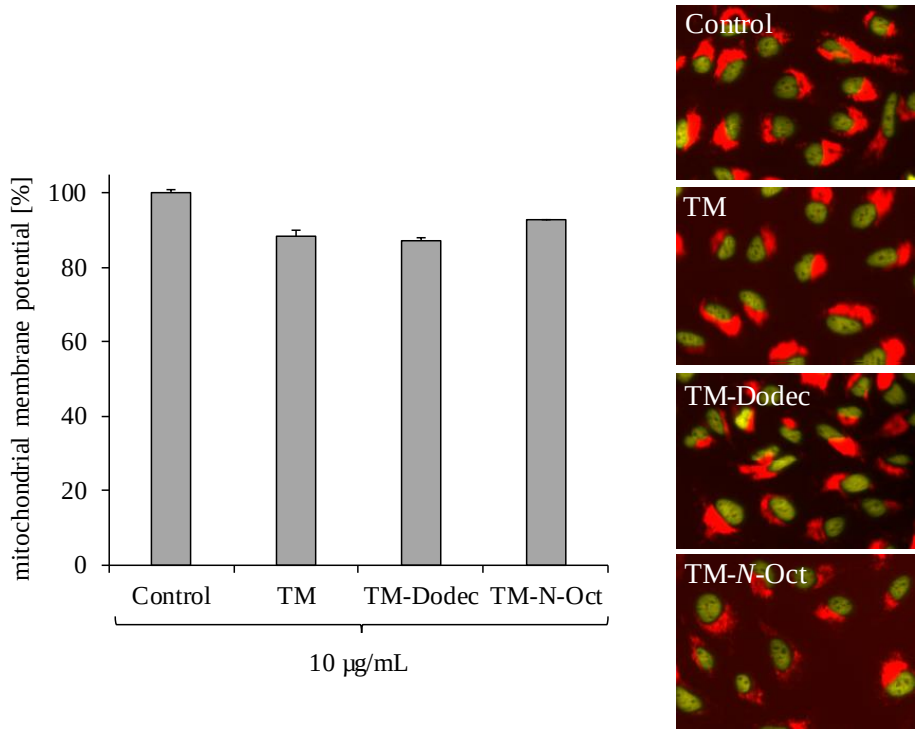


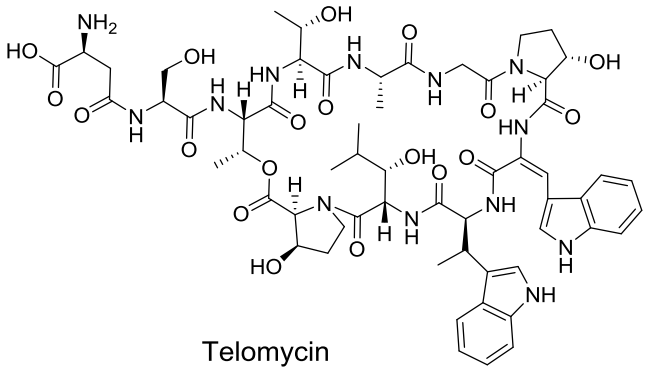

**Figure 4-19:** Determination of the mitochondrial membrane potential (MMP) in U-2 OS cells upon 18 h treatment with 10 µg/mL TM, TM-Dodec and TM-*N*-Oct. MMP was calculated based on TMRM fluorescence intensity in the cytoplasm. Cells were labelled with TMRM (mitochondria) and Hoechst33342 (nuclei). Data represent mean TMRM intensity ± standard deviation (SD) of three independent wells.

## 4.2 DISCUSSION

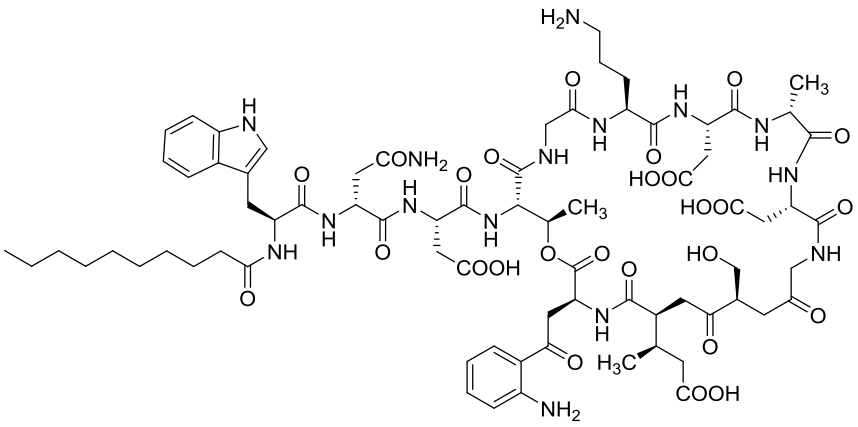
### 4.2.1 CALCIUM-DEPENDENT ANTIBACTERIAL ACTIVITY

The natural product telomycin (TM) is produced by *Streptomyces canus* and was already described in 1950.[145–147] It depicts a cyclic depsipeptide antibiotic with potent antibacterial activity against Gram-positive bacteria including multidrug-resistant (MDR) pathogens.[146,148] Only recently, the biosynthesis of TM was characterized in order to optimize production yields and to generate novel TM derivatives.[148] Through genetic engineering, enzymes involved in the biosynthetic machinery of the natural producer strain were characterised and several TM intermediates were identified. One key finding was the identification of naturally occurring lipopeptides as precursor for TM, which exhibited improved antibacterial activity compared to TM. These studies embossed the development of semi-synthetic lipopeptides, which aimed to provide chemically optimized telomycin derivatives with a main focus on the synthesis of acyl derivatives via modification at the *iso*-$^{1}$Asp terminus of the natural molecule. Two promising derivatives (TM-Dodec[148], TM-*N*-Oct) were generated through structural modifications by the addition of unlike linear acyl chains to the amino group and carboxylic acid group of *iso*-$^{1}$Asp, respectively (TM-Dodec: dodecanoic acid; TM-*N*-Oct: ethylamine octanoic acid; Figure 4-1). Their antibacterial effects in terms of minimal inhibitory concentrations were determined against a panel of Gram-positive bacteria and both derivatives display on average 16- to 128-fold improved potency compared to TM (Table 4-1). The cyclic structure of telomycin resembles the structure of daptomycin (DAP, Figure 4-20), a calcium-dependent antibiotic (CDA) used as last resort treatment for infections caused by MDR Gram-positive bacteria.[158,299,300] The lipopeptide DAP acts in a calcium-dependent manner on the cellular membrane which, in turn, implicates depolarization of the membrane potential, and finally induces cell death as part of a bactericidal mechanism of action.[151,301–303] However, the mechanism of action of DAP is still not completely understood but one important feature is its dependency on $Ca^{2+}$ ions.[304–306] So far, it seems like that the DAP-$Ca^{2+}$ complex is formed in order to obtain a more amphiphilic molecule, which enables DAP to interact with anionic phospholipids (primarily phosphatidylglycerol).[151,307] By inserting its lipid side chain, DAP binds to the cell membrane and due to interaction with a second calcium ion oligomerization takes place[308,309]; presumably due to conformational changes of the molecule.[301,308,310] This seems to trigger a deeper insertion of DAP into the membrane and leads to a rearrangement of lipid

domains, followed by impairing several cellular processes.[309] Based on structural similarities of DAP and TM, the antibacterial activity of TM and both derivatives were re-assessed against *S. aureus* Newman in the presence of different concentrations of calcium. Interestingly, a $Ca^{2+}$-dependent increase of TM's activity was indeed observed and TM, in the presence of $Ca^{2+}$, displayed a comparable potency to both derivatives (TM-*N*-Oct, TM-Dodec). Intriguingly, the activity of these modified derivatives did not rely on the presence of $Ca^{2+}$ (Table 4-2).



Telomycin



Daptomycin

**Figure 4-20:** Structure of calcium-dependent antibiotics (CDA) telomycin and daptomycin.

Since TM and DAP are structurally related and show similarities in antimicrobial activity, the effect of $Ca^{2+}$ on TM was assessed in NMR studies as previously reported for DAP.[304,306] The interaction of TM with $Ca^{2+}$ was studied in the presence of increasing amounts of a 0.1 M $CaCl_2$ solution by NMR. After the addition of $CaCl_2$, $^1H$ NMR spectra showed broadening of specific signals (Figure 4-4 A, black boxes) which indicates binding of TM to

$Ca^{2+}$. In order to determine if TM oligomerizes in the presence of $Ca^{2+}$ – as observed for DAP – or if conformational changes of TM occur, 2D ROESY NMR spectra were recorded (data not shown). Preliminary assessment of these data indicated that four amino acids, $^{3}$Thr, $^{4}$Thr, $^{2}$Ser and *iso*-$^{1}$Asp (Figure 4-4 D) seem to be mostly affected by the addition of $Ca^{2+}$. For instance, cross-peaks between *iso*-$^{1}$Asp - $^{4}$Thr and $^{2}$Ser - $^{4}$Thr disappeared when calcium was present. Moreover, our data led to the assumption that $Ca^{2+}$ binds between $^{3}$Thr and $^{4}$Thr (Figure 4-21), which could lead to the exposure of the side chain (*iso*-$^{1}$Asp, $^{2}$Ser) of TM. This presumed mechanism is crucial for TM's antibacterial activity as it is only biologically active in the presence of $Ca^{2+}$ ions. Regarding TM-Dodec and TM-*N*-Oct, binding to $Ca^{2+}$ was observed as indicated by broadening of certain $^{1}$H signals (Figure 4-4 B, C). However, no conformational changes were observed in 2D ROESY NMR spectra as all cross-peaks remained identical. This might explain why both derivatives are able to act calcium-independent. It is likely that their lipophilic side chains are *per se* exposed ('active conformation'), which might contribute to the mechanism of action, similar to DAP, whose lipid tail interacts with the cytoplasmic membrane.[151,301] Moreover, to prove the hypothesis on TM's conformational changes and eliminate the possibility of oligomerization, quantum mechanical calculations to generate 3D structures based on 2D-ROSEY experiments are in progress (Dr. Matthias Köck, HIPS-MINS). Besides $Ca^{2+}$, several monovalent and divalent ions were tested for their interaction with TM. Monovalent ions showed no interaction with the molecule at any concentration. In contrast, divalent ions seem to interact with TM as indicated by the broadening of some proton signals, with $Cu^{2+}$ exhibiting the strongest effect, followed by $Ba^{2+}$ and $Ca^{2+}$ (Figure 4-5, black boxes, Table 4-5). Regarding their influence on the biological activity of TM, only $Ca^{2+}$ and $Mg^{2+}$ were suitable for MIC determination, due to toxicity of $Ba^{2+}$ and $Cu^{2+}$ ions. Overall, only ions which showed interaction with TM and which are mainly involved in the physiology of the human body were of main interest (e.g. $Ca^{2+}$, $Mg^{2+}$, $Na^{+}$ or $Zn^{2+}$). At physiological concentrations, solely $Ca^{2+}$ revealed a positive influence on TM's activity and no changes were observed for $Mg^{2+}$ (Table 4-6).

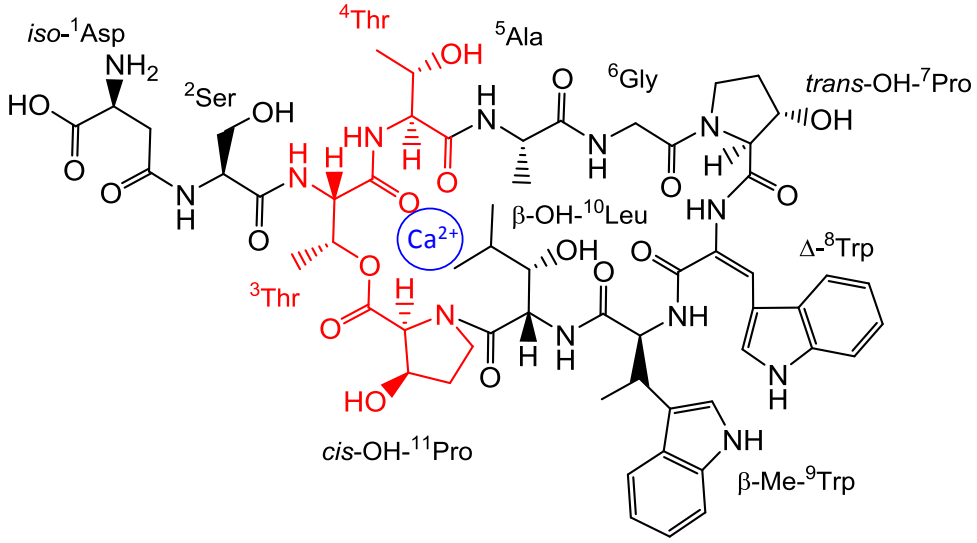



**Figure 4-21:** Proposed binding site of $Ca^{2+}$ between $^{3}$Thr and $^{4}$Thr of telomycin.

Due to these observed differences between natural and semi-synthetic TMs, and in order to assess if the antibacterial mechanism of action of TM-Dodec and TM-*N*-Oct is identical to that of $Ca^{2+}$-activated TM, co-resistance studies were performed (see chapter 4.2.2). Interestingly, both acyl derivatives exhibited only marginal or no co-resistance with TM as assessed by MIC testing with TM-resistant and cardiolipin (CL)-depleted mutant strains of *S. aureus* (Table 4-7). In conclusion, semi-synthetic TM derivatives most probably hit additional targets and their antibacterial effect does not exclusively rely on CL binding, which might also hold true for TM itself (see chapter 4.2.2).

Based on the above discussed findings, the characterisation of TMs was assessed in the presence of physiological $Ca^{2+}$ concentrations (1.25 mM).

An intrinsic resistance pattern of Gram-negative bacteria was observed, as none of the tested Gram-negative pathogens, including a membrane-permeabilized *E. coli* TolC-efflux deficient strain, was susceptible towards TMs (Table 4-3). These results indicate that susceptibility towards TMs relies on structural differences of the cell envelope of Gram-negative compared to Gram-positive bacteria (Figure 4-22). The major difference is the presence of the outer membrane (OM) in Gram-negative bacteria which consists of a lipopolysaccharide (LPS) layer (outer leaflet) and phospholipids (inner leaflet) and is absent in Gram-positive bacteria.[311] It serves to protect the cell against external stress (e.g. toxic molecules) and depicts a barrier to several antibacterial agents such as the hydrophobic antibiotic rifampicin.[235,311] However, an increase of the permeability of the OM by

membrane sensitizing agents such as polymyxins enable these compounds to act on Gram-negative bacteria.[235,236] Nevertheless, regarding the susceptibility of the membrane-permeabilized *E. coli* TolC-efflux deficient strain towards TMs, structural changes of the cell envelope do not seem to be sufficient to cause inhibition of Gram-negative bacteria. Moreover, it was excluded that the resistance relies on efflux since the present *E. coli* strain depicts an TolC-efflux deficient mutant.[312] Thus, it was concluded that TMs might target a structure within the Gram-positive cell envelope. This was consolidated since an overall broad activity spectrum against Gram-positive bacteria was observed. However, *M. smegmatis* and *N. asteroids* were not susceptible, but against slow growing mycobacteria (*M. tuberculosis*, *M. bovis*), inhibition was detected. In general, differences in susceptibility of TMs against mycobacteria compared to other Gram-positive bacteria may also rely on a different composition of the cell envelope (Figure 4-22). Even though mycobacteria are classified as Gram-positive bacteria, their outer envelope comprises structural features of Gram-negative bacteria such as an OM, and in total, their organisation is much more complex. The main component of the OM is mycolic acid, which is covalently linked to arabinogalactan. Additionally, within different mycobacterial species the arrangement of the mycolic acids structure differs, which may account for differences in sensitivity against external agents as observed for TMs.[313]

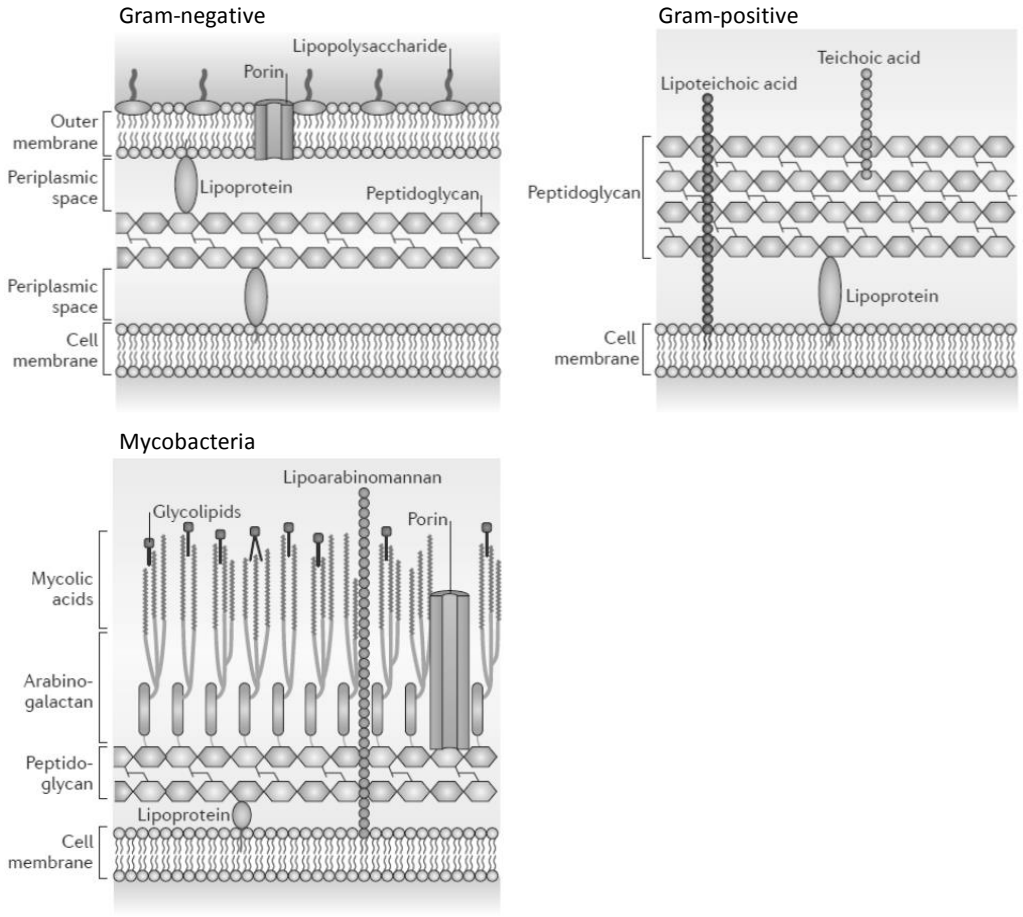



**Figure 4-22:** Cellular envelope of Gram-negative bacteria, Gram-positive bacteria and mycobacteria. Adapted from Brown et al..[52]

A comparison of activity spectra of all TMs revealed TM to be on average 2- to 8-fold less potent and by tendency, TM-*N*-Oct was slightly more active than TM-Dodec, in particular against Staphylococci. TM was virtually inactive against two resistant *S. aureus* strains (MDR, VISA/MRSA), but both derivatives were able to inhibit their growth. So far, this finding cannot be explained but it hints towards altered cell wall compositions of resistant strains, which hinder natural TM to enter the cell. Due to the emergence of hospital-acquired infections caused by pathogens such as Staphylococci, and a permanent increase of resistance mediated by these pathogens towards clinically used antibiotics, the effect of TMs against clinical *S. aureus* isolates was investigated. $MIC_{50}$ and $MIC_{90}$ values were determined and encouragingly, values were in the same range as observed with laboratory strains (low µg/mL range; Table 4-4). These results represent TMs and in particular both acyl-derivatives as promising source for the development of new drugs to target the emergence of nosocomial infections.

TMs exert a bactericidal effect on Gram-positive bacteria including *S. aureus* and vancomycin-resistant Enterococci (VRE).[146,148,160] Remarkably, despite a comparable activity pattern of TMs and DAP (Table 4-3), TM-Dodec exerts an even stronger bactericidal effect on *S. aureus* compared to the gold-standard antibiotic DAP.[148] Overall, structural similarities, as well as an overlap in the antibacterial spectrum and the $Ca^{2+}$-dependent mechanism of both, TM and DAP, hinted towards similar mechanisms of action. Thus, a general mechanistic comparison of TMs and DAP was performed. The potency of TM on the bacterial membrane potential was assessed by exposing *S. carnosus* DSM-20501 to 5-fold MIC of TM in the presence of 0-10 mM $CaCl_2$ over 2.5 h. A $Ca^{2+}$-dependent membrane depolarization was observed with an increase in depolarization proportional to the amount of present $CaCl_2$ (Figure 4-3). At $Ca^{2+}$ concentrations $\geq$ 0.25 mM a 80 % reduction of the MP was observed, indicating a calcium-dependent effect on the cellular membrane, similar to the mechanism observed for DAP.[314] Previous studies indicate that membrane depolarization by DAP occurs only as a consequence of cell membrane mechanisms which are mediated to trigger cell death.[309,315] However, since the mechanism of action of DAP is still controversially discussed, several models exist. Besides a membrane disrupting action by the formation of pores[316], studies described DAP to act as inhibitor of the biosynthesis of peptidoglycan (PG).[317,318] Additionally, it was suggested that the main target depicts the cell wall and not the cell membrane.[319] Recent studies confirmed that the inhibition of the cell wall synthesis counts to its action, a mechanism which is based on structural changes of the cell membrane by rearranging lipid domains[309]. This mechanism seems to impair several cellular processes and might account for the depolarization of the membrane, a process that could also be contemplated for TMs.

Due to TM's excellent activity on Gram-positive bacteria in broth, all derivatives were also assessed for their intracellular antibacterial activity. At concentrations as low as 4-fold MIC, TM, TM-Dodec and TM-*N*-Oct reduced the number of *S. aureus* cells by approximately 2-$\log_{10}$ (Figure 4-2). Again, both modified derivatives were more potent compared to the natural product, as TM-Dodec and TM-*N*-Oct were still effective in this screen at lower antibiotic concentrations (1-fold MIC), whereas TM showed only a negligible activity at this concentration. The ability to kill intracellular pathogens is an important property of antibiotics that are used to treat severe infections caused by some bacteria. *S. aureus* depicts a major pathogen which is responsible for community- and hospital-acquired infections[320–322] and due to the severe problem of increasing numbers of MDR strains, only limited

treatment options are available. Moreover, *S. aureus* was shown to overcome host-defending mechanisms, which leads to increased levels of the bacteria within the host. For instance, *S. aureus* is able to survive within macrophages[323–326] by overcoming phagocytosis, which is normally initiated as the host's innate immune response[327]. With regard to the surviving abilities of *S. aureus* within macrophages and the results gained upon treatment of infected cells with TMs (Figure 4-2), the potential use of the compounds to treat infections caused by *S. aureus*, including MDR strains and potentially other Gram-positive pathogens, was consolidated.

### 4.2.2 MECHANISM OF ACTION AND RESISTANCE MECHANISM

#### 4.2.2.1 GENETIC CHANGES IN RESISTANT MUTANTS

To evaluate putative resistance mechanisms and targets of TMs, *S. aureus* mutants were generated by stepwise increasing the antibiotic concentration and mutants finally tolerated concentrations of 100 µg/mL TM and TM-Dodec. Initially, these mutants developed at frequencies of $4 \times 10^{-6}$ for TM and $8.5 \times 10^{-9}$ for TM-Dodec when treated at the respective 4-fold MIC. Since the frequency of resistance for TM-Dodec was significantly lower than that for TM, the modified derivative might have additional bacterial targets or exhibit a different mechanism of action than TM. MIC testing confirmed the resistance phenotype of both sets of mutants against the respective telomycin derivative. Additionally, TM-Dodec-resistant mutants revealed cross-resistance to TM and TM-*N*-Oct ( Table 4-8), but TM-resistant mutants were still susceptible to both derivatives (Table 4-7). Against all other tested antibiotics, including DAP, no co-resistance was observed, which indicates that TMs might have a new target and exert novel mechanisms of action.

The mechanism underlying resistance was further investigated by genome analysis of resistant mutants in comparison to the wildtype *S. aureus* Newman. Besides the presence of several inconsistent mutations (Table 4-7, Table 4-8), all TM- and TM-Dodec resistant mutants revealed mutations within *cls2*, a gene encoding cardiolipin synthase 2 (Cls2), which catalyses the reversible transphosphatidylation of two phosphatidylglycerol (PG) molecules into CL (Figure 4-23).[292] In six mutants (Mt-T.4, Mt-T.7, Mt-T.8, Mt-T.9, Mt-D.1, Mt-D.3) the point mutation led to a premature stop codon within *cls2*. Additional mutations within a gene encoding a diacylglycerol kinase (DagK, most probably DgkB from Pfam00781 superfamily of soluble proteins) were identified in four TM-resistant mutants

(Mt-T.1, Mt-T.4, Mt-T.7, Mt-T.8, Mt-T.9). DagKs are involved in the biosynthesis of phospholipids by catalysing the phosphorylation of diacylglycerol (DAG) into phosphatidic acid (PA).[328,329] Furthermore, DAG is linked to the generation of lipoteichoic acid (LTA), since DAG is generated during the addition of glyercol-1-P groups from PG to the LTA.[328,330] Moreover, in all TM-Dodec-resistant mutants, mutations of a gene encoding a GntR-type family repressor (PmtR) of an ABC transporter[294] were present. Interestingly, in seven out of ten mutants the mutation occurred in the same amino acid (# 30), which led to a stop codon in all seven mutants. In summary, the resistance patterns of TM- and TM-Dodec-resistant mutants hint towards effects of TMs, which are dependent on cell envelope components (CL, DAG, LTA) Moreover, the genomic characterisation indicated that resistance towards acylated TM-derivatives might involve additional cellular changes (additional, common mutation in PmtR encoding gene) compared to TM (only *cls2* mutation common in all mutants).

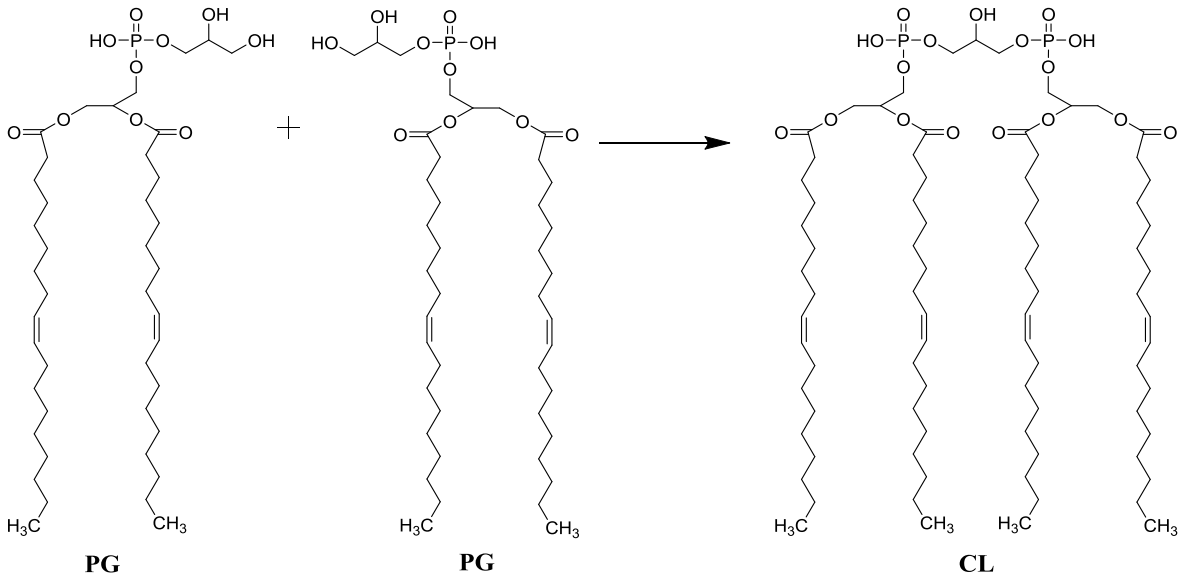



**Figure 4-23:** Transphosphatidylation of two phosphatidylglycerol (PG) molecules into cardiolipin (CL) Phospholipids are depicted as representatives with unsaturated 18-carbon acyl tails (typical length of synthetic phospholipids; natural occurring phospholipids can vary in the length/number of the acyl tail and the position/number of unsaturated bonds).[331]

Since all mutants generated under the pressure of TM and TM-Dodec had mutations of *cls2* in common, and all other identified mutations were either only present in one set of mutants or did not appear commonly, CL seems to contribute to the antibacterial effect of TMs. Thus, for a further characterization of the resistance mechanism and the mechanism of action the role of CL including the function of *cls2* was in the main focus.

#### 4.2.2.2 ROLE OF CARDIOLIPIN

The exact mechanism of action of TMs is not known and the compounds did not show an effect in RNA, DNA and protein synthesis reporter screens (data not shown; Dr. A. Müller, University Bonn). Instead, the induction of cell wall stress response was observed, which hints to a mechanism related to cell wall synthesis. Additionally, resistance studies (see previous chapter 4.2.2.1) hinted towards interaction of TMs with cell membrane components, especially with the phospholipid CL. CL is found in the bacterial and mitochondrial membrane and depicts an important phospholipid component of these domains[332,333]. In general, the relative amount of lipid types differs among different species (Table 4-18). This may account for differences in susceptibility of TMs against Gram-positive compared to Gram-negative bacteria. For instance, the content of CL is usually much higher in Gram-positive bacteria compared to other species (approx. 3- to 10-fold higher), whereas Gram-negative bacteria mostly contain phosphatidylethanolamine (PE), a phospholipid which is completely absent in some Gram-positive species. Nevertheless, TMs are also active against Gram-positive bacteria with a low CL content (*B. subtilis*). Thus, an interaction with CL does not seem be the main trigger for their bactericidal effect and it is likely that an additional target (protein) which is only present in Gram-positive bacteria, and presumably associated with the cell envelope accounts for the mechanism of action of TMs.

**Table 4-18:** Percentage of major phospholipids of total lipid content in Gram-positive, Gram-negative bacterial and mitochondrial membranes. Adapted from Epand and Epand[334], Osman et al.[335], and Schenkel and Bakovic[336]

| Species | % Total lipid | | |
|---|---|---|---|
| | CL | PG | PE |
| Gram-positive | | | |
| *S. aureus* | 42 | 85 | 0 |
| *S. pneumoniae* | 50 | 50 | 0 |
| *B. subtilis* | 4 | 70 | 12 |
| Gram-negative | | | |
| *E. coli* | 5 | 15 | 80 |
| *K. pneumoniae* | 6 | 5 | 82 |
| *P. aeruginosa* | 11 | 21 | 60 |
| Mitochondria | 15 | * | 30 |

*PG does not accumulate under normal conditions[335]; CL: cardiolipin; PG: phosphatidylglycerol; PE: phosphatidylethanolamine

Certainly, the interaction of TM and CL was also described by Johnston *et al.*, who described CL as the antibacterial target of TM.[161] Within their study, a localization of TM in CL containing compartments (septa, poles) was provided. Due to CL's structure (head groups

possess smaller cross section compared to cross section of lipid tails) it possess a high intrinsic curvature what leads to an enrichment of CL within these domains.[331,337,338] An accumulation of TMs in CL-rich compartments could be also confirmed here, not only for TM but also for the acylated derivatives. *B. subtilis* and *S. aureus* were incubated with fluorophore-tagged telomycin analogues and an accumulation of TM, TM-Dodec and TM-*N*-Oct tool compounds at septa and poles was observed (Figure 4-13). Furthermore, the fluorescence pattern of intracellular TM derivatives overlaps with fluorescence of NAO, a red fluorescent indicator for CL (Figure 4-14).[296]

To investigate the interaction of TMs with potential other lipid binding partners of the cell membrane, binding of alkyne-coupled TM derivatives (TM-pentyne; TM-decyne, mimicking TM-Dodec) to membrane lipids and lipoteichoic acid (LTA) was determined. Direct binding of TMs was exclusively observed for CL in a CL concentration-dependent manner (max. binding in the presence of 64 pmol CL; Figure 4-12) and all other lipids as well as LTA did not show interaction at all (Figure 4-11). Furthermore, the binding capacity of TM (TM-pentyne) was shown be improved in the presence of high $Ca^{2+}$ concentration (10 mM). Remarkably, the binding of CL to TM is in general dependent on calcium, since $Ca^{2+}$ depletion by the addition of EDTA prevented binding at any tested CL concentration. Even though, binding of TMs to CL was proven this phospholipid is rather unlikely to be the antibacterial target of TMs. A disruption of *cls* and a thereof resulting CL-depletion was not lethal (Figure 4-6) and proved that CL is not directly involved in the bactericidal effect of TMs. Moreover, no direct correlation between the bacterial CL-content and the antibacterial activity of TMs was observed (e.g. *B. subtilis vs. E. coli*, Table 4-3, Table 4-18), what furthermore hints towards interaction of TMs with other – so far unknown – target molecules.

Since CL is not only present in bacteria but also in the inner mitochondrial membrane[297], effects of TMs compared to DAP on the mitochondrial membrane potential (MMP) were analysed. Due to CL binding there is a potential risk that TMs induce adverse effects *in vivo*, which are characterized by e.g. cardiotoxicity.[339] To exclude such side effects TM's toxicity was evaluated in a cell culture model. Human bone osteosarcoma cells (U-2 OS) were treated with TM, TM-Dodec, TM-*N*-Oct and DAP at 10 µg/mL and the MMP was determined after 18 h. For all compounds, only a negligible reduction of the MMP (≤ 10 %) compared to untreated control cells was observed (Figure 4-19). Since TMs have no major effect on MMP of eukaryotic cells it is rather unlikely that their interaction with (bacterial) CL is problematic with regards to *in vivo* toxicity, particularly the induction of cardiotoxic effects due to

disrupted mitochondrial function[339]. Moreover, TMs in general do not show cytotoxic effects on eukaryotic cell lines[148] (Table 4-17). However, these findings need to be confirmed *in vivo,* ideally in a well-monitored rodent model.

As already mentioned, the reversible transphosphatidylation of PG molecules into CL is catalysed by a cardiolipin synthase (Cls)[292]. In *S. aureus* species, the synthesis of CL is mediated by two distinct Cls genes: *cls1* and *cls2*.[293] Both synthases are linked to CL accumulation, but under normal growth conditions, *cls2* encodes the major synthase Cls2, whereas *cls1* encodes the alternative enzyme Cls1, which can compensate for a loss of functional Cls2 under conditions of osmotic or pH stress.[292,293] However, a loss of functional Cls2 is associated with a decrease of CL under normal growth conditions.[293] Based on this, lipids from *S. aureus* Newman wildtype and from *cls1* and *cls2* disrupted *S. aureus* mutants were extracted and analysed by thin-layer chromatography (TLC). Spots detected by TLC indicated that a disruption of *cls2* led to a decline in the appearance of CL, in comparison to CL accumulation in *S. aureus* Newman wildtype and *cls1* disrupted mutant (Figure 4-7). Moreover, the accumulation of PG in *cls2* disrupted mutant appeared to be slightly higher, in comparison to wildtype and *cls1* disrupted mutant. This further underlines that the generation of CL is hampered in mutants bearing disrupted *cls2*, indicated by no conversion of PG into CL. This confirms that mutations of *cls2* lead to a changed phospholipid composition what in turn contributes to antibiotic resistance and hints towards a CL-related mechanism of TMs. Moreover, the characterization of both *cls* disrupted *S. aureus* Newman mutants confirmed that only the inactivation of *cls2* is involved in decreased susceptibility to TMs (Table 4-11). MIC determination revealed that a disruption of *cls2* affected the activity of TM significantly more (MIC: > 64 µg/mL) than of both semi-synthetic derivatives (only 4- to 8-fold less active). This indicates that the activity of natural TM indirectly or directly highly relies on the presence of CL but does not prove CL to be the antibacterial target. Even more, a depletion of CL is not sufficient to cause a complete resistance towards both acylated derivatives, hinting towards a CL-independent effect of both derivatives, again supporting the assumption of an interaction with other target(s). Interestingly, inactivation of neither *cls1* nor *cls2* did affect the activity of DAP, even though mutations of *cls* in *Staphylococcus* or clinical *Enterococcus* (VRE) isolates were previously associated with resistance to DAP.[64,65,340] Certainly, not only *cls* but also other genes were simultaneously affected within DAP-resistant mutants. Those genes were identified to be responsible for structural changes of the cell envelope. Interestingly, genes responsible for those changes

differed within different bacterial species (VRE *vs.* Staphylococci). Within VRE, *liaF* and *gdpD* were mainly associated with resistance to DAP[65], whereas *mprF* was mostly affected within *S. aureus* isolates.[64] Those genes have in common, that they are activated as a response to antibacterial agents, which results in changes in the composition of the cellular membrane. This is probably mediated based on a simultaneous mutations of other genes involved in phospholipid synthesis, such as *cls2* or *pgsA* (encoding PG synthase PgsA).[341] Mutations of *mprF* (encoding lysyl-PG synthase) were associated with a decreased generation of PG and an increased conversion of this phospholipid into lysyl-PG (LPG). This leads to changes of the membrane charge and hinders cationic antimicrobial peptides (CAMPs) to enter the cell.[64,342–344] In this context, one TM-resistant mutant (Mt-T.3) yielded a mutation within the lysyltransferase flippase domain of MprF. However no decreased sensitivity towards DAP was observed, indicating that this mutation did not lead to structural changes of the membrane as observed within DAP-resistant mutants, and does not seem to contribute to a resistance mechanism against TM. Regarding structural changes based on mutations of *liaF* or *gdpD*, none of the mutants yielded additional mutations within those genes. In general, mechanisms that led to resistance towards TMs seem to differ from mechanisms observed in DAP-resistant bacteria, since no cross-resistance to DAP was observed. However, with regard to the affected genes (Table 4-9, Table 4-10) in TM- and TM-Dodec-resistant mutants, it seems that the so far unknown mechanism is likewise associated to the cell membrane, in particular to CL, and that there are some genes mutated which are also found to be affected in DAP-resistant mutants.

Under normal growth conditions, *S. aureus* undergoes a change in the phospholipid composition from logarithmic- to stationary-growth phase. During logarithmic growth, the major cell membrane component is PG, which is converted into CL during cellular growth. This in turn leads to a decrease of PG with a concurrent increase of CL as phospholipid in stationary-phase (30-40% of cell membrane constitutes of CL in stationary-phase).[345] Considering CL as an important cellular component during bacterial growth leads to the assumption that inactivated *cls* genes might interrupt cell growth. Certainly, as already mentioned, Cls1 is able to compensate the loss of functional Cls2 under conditions of osmotic or pH stress.[292,293,346] To investigate whether a *cls2* disrupted mutant is able to compensate the loss of Cls2 by Cls1 activation under stress conditions, expressed by a regain in susceptibility towards TMs, *S. aureus* Newman wildtype and the *cls2* disrupted mutant were incubated under different stress conditions, followed by lipid extraction and MIC

determination. Against wildtype *S. aureus* Newman, no changes on susceptibility towards all TMs were observed (Table 4-12) at any test condition. Regarding *cls2* disrupted mutant, only low pH conditions (pH 2) led to a slight improvement of activity to TM. However, against DAP, wildtype as well as *cls2* disrupted mutant showed 16- and 8-fold increased susceptibility, respectively, under growth conditions at pH 2. Other stress-inducing conditions (4 M NaCl and pH 4) did not or only marginally influence the susceptibility towards DAP. Since DAP not only showed increased activity under low pH conditions (pH 2) against *cls2* mutant but also against the wildtype, it can be excluded, that the observed effect relies on *cls* mutations. It rather seems that pH 2 led in general to changes that might rely on a changed phospholipid composition, which increased susceptibility to DAP but not to TMs. Acidic pH conditions can lead to an increase of LPG production in *S. aureus*.[347,348] However, as already mentioned, the increase of LPG leads to resistance towards DAP and not to increased sensitivity.[64,342] Based on this, it is likely that pH 2 conditions induced a different change in cell membrane composition. To prove this assumption the amount of all lipid species present under stress conditions needs to be determined in comparison to normal growth conditions. In the course of this study, phospholipids of *S. aureus* Newman and *cls2* disrupted mutant incubated under stress conditions were extracted but analysis of LC-MS data could not be analysed properly since only trace amounts of lipids (partly below limit of detection) were detected. The HPLC method was validated using a CL-standard, however the amount of extracted was not sufficient to be detected. For a concise evaluation of phospholipids and to quantify CL methods (TLC, LC-MS) need to be optimized, a careful repeat of the experiments is needed, and extractions efficiency must be improved.

In conclusion, the presence of CL is a major factor for natural TM to be active against bacteria. As shown by susceptibility studies performed with the CL-depleted *S. aureus* mutant (*cls2* disrupted mutant; Table 4-11), the absence of CL is already sufficient to cause high-level resistance towards natural TM (≥ 64-fold decreased sensitivity compared to wildtype) and it does not seem that further cellular changes are needed. Nevertheless, in five out of seven TM-resistant mutants SNPs were identified in the same gene encoding a diacylglycerol kinase, an enzyme that is involved in the synthesis of phospholipids. As already mentioned, DagKs catalyse the phosphorylation of DAG into PA[328,329], which yields in further steps the generation of PG. DAG in turn is generated by the addition of glyercol-1-P groups from PG to LTA, a mechanism that functions in a circulating way, since DAG is re-introduced into the biosynthetic pathway that yields PG (Figure 4-24).[328,330] A

dysfunction of DagK leads to a lethal accumulation of DAG within bacteria.[328,330] However, it seems that the mutations found in TM-resistant mutants did not lead an increased accumulation of DAG, or at least was not lethal, since mutants did not display a fitness cost compared to the wildtype (Figure 4-8 A). However, to get further insights into the mechanism underlying mutations of DagK, the presence of all phospholipids included in the biosynthetic pathway of PG/LTA production needs to be analysed. Nonetheless, this indicates that malfunctioning DagK, does not seem to contribute significantly to resistance against TM, and that CL is the major key for natural TM to perform its action in the first place.

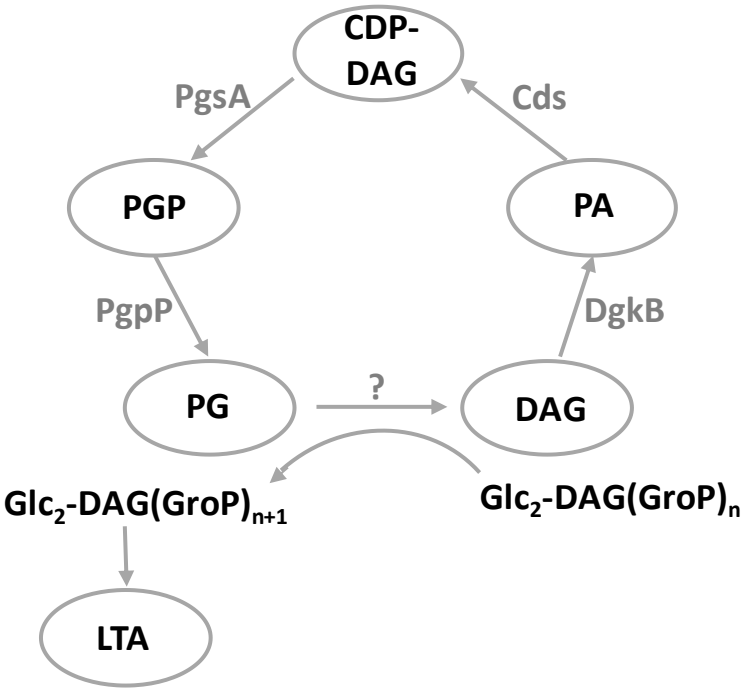



**Figure 4-24:** Role of diacylglycerol kinases in the biosynthetic pathway of PG and LTA. An unknown enzyme (?) catalyses the transfer of glycerol-1-P groups from PG to the LTA glycolipid anchor diglucosyldiacylglycerol ($Glc_2DAG$) yielding LTA and DAG, whereas DAG is re-introduced into the PG biosynthesis cycle. DgkB catalyses the phosphorylation of DAG into PA leading in further steps to the generation of PG. LTA: lipoteichoic acid, DAG: diacylglycerol, PA: phosphatidic acid, CDP-DAG: cytidine diphosphate diacylglycerol, PGP: phosphatidylglycerol phosphate, PG: phosphatidylglycerol, DgkB: DAG kinase, Cds: CDP-DAG synthase, PgsA: PGP synthase, PgpP: PGP phosphatase. Adapted from Jerga et al.[330]

In contrast, the depletion of CL (*cls2* disrupted mutant; Table 4-11) did not lead to complete resistance towards both acylated derivatives (TM-Dodec: 8-fold decreased activity, TM-*N*-Oct: 4-fold decreased activity). In all TM-Dodec-resistant mutants, a second gene besides *cls2* was mutated. This additional mutation within all TM-Dodec-resistant mutants thus contributes to high-level resistance towards TMs. The affected gene was recently identified to encode a repressor (PmtR) of a phenol-soluble modulin (PSM) toxin export system

(belonging to the family ABC transporter).[294] PSMs depict a family of staphylococcal toxins that are involved in the pathogenesis of *S. aureus*[349–351] and moreover, they are involved in regulating their own efflux. To avoid self-mediated lethality by PSMs, *Staphylococci* have established a self-regulating efflux system whose expression is induced by specific PSM subtypes (mainly low-level cytolytic PSMs, e.g. PSMα4).[294] The system underlying PSM secretion (Pmt; an ATP-binding cassette transporter) is encoded by four genes (*pmtA*, *pmtB*, *pmtC*, *pmtD*) arranged on a single operon (*pmt*).[295,350] The presence of Pmt was described to be essential for bacterial survival, as a deletion led to a lethal accumulation of PSMs within *S. aureus*.[350] The regulator PmtR is encoded by *pmtR* which is located upstream the *pmt* gene cluster [294] and was characterized as a member of YtrA subfamily of helix-turn-helix GntR-type transcriptional regulators.[352,353] YtrA acts as repressor of the *ytrABCDEF* operon of *B. subtilis*, which is required for induction in bacterial responses to cell wall (lipid II) targeting antibiotics.[354] Since PmtR depicts a homologue of YtrA, the lipid II targeting antibiotic vancomycin[355] was included in co-resistance studies of TM-Dodec-resistant mutants. However, no decrease in sensitivity was observed when tested against TM-Dodec-resistant mutants compared to wildtype *S. aureus* (Table 4-8). Those results support the assumption that TM-Dodec seems to exhibit a novel mechanism of action by targeting a molecule different to that of known cell wall targeting antibacterial agents such as DAP and vancomycin. Regarding its regulatory function, Otto and co-workers described that no other genes but the *pmt* cluster is regulated by PmtR and no other substrate than PSMs were described in the context of this export system.[294] In the absence of intracellular PSMs, PmtR prevents a permanent expression of the efflux system by binding to the *pmt* promoter (PmtR-*pmt* complex) upstream *pmtR*. The production of PSMs, controlled by the accessory gene regulator (*arg*) quorum-sensing system[356], leads to the transcription of the efflux system by the binding of PSM to PmtR, what leads to the disruption of the PmtR-*pmt* complex and expression of the efflux system. The observed mutations of *pmtR* in TM-Dodec-resistant mutants are inactivating (deletions) and thus, lead to the absence of PmtR and a permanently expressed efflux pump could be present. Fitness studies of TM-Dodec-resistant mutants revealed a retarded growth compared to the wildtype *S. aureus* Newman (Figure 4-8 B), indicating a PmtR-related growth defect. Additionally, a comparison to the fitness of TM-resistant (Figure 4-8 A) and *cls* disrupted mutants (Figure 4-6) confirmed that the additional mutations within *pmtR* are responsible for the retarded growth as *cls2* mutations are fitness neutral. In this context, Joo et al. described that a permanent and wasteful production of Pmt (*pmtR*-knockout mutant) is reflected in slight but significant bacterial growth deficiencies[294]

This consolidates the assumption of a permanent expressed export system within TM-Dodec-resistant mutants. Moreover, PSMs are related to biofilm formation in *S. aureus*. On the one hand, PSMs are involved in structuring the 3-dimensionality of biofilms and on the other hand they facilitate a controlled detachment of the biofilm which is crucial for the dissemination of cells during infection.[295,357–359] The characterisation of TM-Dodec-resistant mutants revealed, that the observed growth defects were accompanied by an impaired biofilm formation after 24 h incubation in all mutants in comparison to *S. aureus* Newman wildtype (Figure 4-9). After additional 24 h of incubation (48 h time-point), a further growth of the biofilm of the wildtype was observed, but for all mutants no changes or a degradation of their biofilm was observed. Assuming an increased expression of the efflux pump it might be possible that the unusual increased export of PSM leads to an uncontrolled and early detachment of the biofilms.

Taking together the facts that mutations of *pmtR* were only present in TM-Dodec-resistant mutants and that those mutants showed an overall retarded fitness (growth and biofilm) compared to TM-resistant mutants (only *cls2* mutations), lead to the conclusion, that the observed growth defects rely on mechanisms mediated by dysfunctional PmtR. Even though at the current stage there is no prove, it seems that the activity on the mutant strains of acylated TMs is hampered due to an upregulation of the efflux pump Pmt. Additionally, although so far only PSMs are described as substrates of Pmt, it might be possible that resistance towards TMs is mediated by efflux of the compounds. However, to get further insight into the proposed mechanism it needs to be assessed whether the mutations in *pmtR* indeed lead to increased expression of Pmt and whether TMs are substrates of this specific transporter. A PmtR knockout mutant is currently generated and studies in comparison to the wildtype strain are planned. Those include qPCR to investigate the expression levels of PmtR/Pmt and activity studies (including cross-resistance towards TM). Moreover, it needs to be investigated if merely the loss of function of PmtR is sufficient to cause resistance against TM-Dodec or if it only contributes to growth defects and if a concurrent CL deficiency is needed in any case for high-level resistance. The impact of the described mutations in *cls2* and *pmtR* on transcriptome level is currently analysed by means of RNA sequencing experiments (performed in cooperation with Dr. A. Westermann/Prof Dr. J. Vogel, HIRI, Würzburg).

In summary, TM and both derivatives seem to act by interfering with the bacterial cell envelope. However, even though binding of acylated TM to CL was proven, and an accumulation of both TM-derivatives within CL containing compartments was shown, the overall data set ($Ca^{2+}$-independency, susceptibility of *cls2* mutants, *in vitro* resistance development) still hints towards a mechanism in which CL only plays an indirect role. So far, the binding site of TMs on CL is not known but it seems likely that TMs bind *via* the cyclic peptide core structure, which is identical in natural TM and acylated derivatives. The binding of TM to CL is presumably needed to enable the compound to interact with the cell (most probably with the cell membrane; CL as "anchor" for TM), whereas acylated derivatives are able to interfere with the cells independent of the presence of CL. They presumably interact with the membrane by inserting their exposed lipophilic side chain (comparable to DAP), which is slightly less effective in the absence of CL. Natural TM first needs to undergo a conformational change ($Ca^{2+}$-dependent) to resemble the 3D structure of acylated TMs and its membrane interaction fully relies on the presence of CL. In any case, the interaction with cell membrane seems to be only the first step of the mechanism of action and it is likely that the bactericidal effect of TMs relies on the interaction with a common additional target, which might be found in the cell wall.

### 4.2.3 TARGET IDENTIFICATION

Given that the interaction of CL with TMs mainly affects the activity of natural TM, and that no complete resistance against TM-Dodec was observed when CL was depleted, it is likely, that an additional (protein) target(s) account for the bactericidal mechanisms mediated by TMs. Moreover, the depletion of CL is not lethal (Figure 4-6), further indicating CL not representing the main antibacterial target for TMs.

Since *in vitro* resistance development only hinted towards mechanisms mediated in association with the cell membrane, but no specific target molecule was identified, a protein pull down was performed. *B. subtilis* served as BSL1 model organism and semi-synthetic TM- and TM-*N*-Oct-biotin-tagged derivatives served as tool compounds to perform protein pull-down experiments. To identify proteins that interacted with TMs, proteins were separated by SDS-PAGE, followed by mass-spectrometry analysis. Since the success rate of identifying trypsin digested proteins by mass-spectrometry is dependent on the intensity of proteins, only proteins that appeared in the presence of 200 µg TM-*N*-Oct were chosen for further analysis (Figure 4-15 A) by MADLI-TOF/MS and LC-MS/MS. Four different

proteins were successfully identified as binding partners in the pull-down experiment: αβ-subunit of ATP synthase, a putative lipoprotein YcdA, subunit II (QoxA) of the quinol oxidase, and flagellin (Figure 4-15 B). Flagellin depicts the protein component of the bacterial flagellum which is highly abundant in flagellated bacteria.[360] The flagellum is needed for the bacterial movement and is found in Gram-positive as well as Gram-negative bacteria, such as *E. coli* or *Pseudomonas* spp., but is absent in Staphyloccoci.[361,362] Binding of an antibiotic to flagellin might probably hinder bacterial movement but not cause bacterial killing. Furthermore, TMs depict an intrinsic resistance pattern to Gram-negative bacteria (flagellin containing bacteria) but strong inhibitory activities against Staphylococci (flagellin missing bacteria), what led to the exclusion of this protein to be involved in TMs mechanism of action. Moreover, the protein mass detected by protein separation on SDS-PAGE (33 kDa) differs slightly from the actual mass of the intact protein (38 kDa), whereas masses for all other identified protein are in accordance to theoretical masses. However, the fishing of flagellin (or part of it) might have happened in association with YcdA, a putative lipoprotein that was previously characterized to be involved in the movement of *B. subtilis* but it is not essential for cellular growth.[363] Besides a correlation to the swarming motility, the protein is uncharacterized and was only found in *B. subtilis* with no homologues in *S. aureus*. Nevertheless, direct binding of TMs to YcdA, which was heterologously expressed in *E. coli*, was analysed by SPR. Binding to TM, TM-Dodec, TM-*N*-Oct compared to DAP was monitored in the presence of increasing compound concentrations. Additionally, the effect of $Ca^{2+}$ on the binding affinities was analysed. In accordance to protein fishing, binding to YcdA was only observed for TM-*N*-Oct, which bound in a concentration-dependent manner with moderate affinity in the micro-molar range (Table 4-16; Figure 4-16). Addition of 5 mM $CaCl_2$ only led to a minor increase of binding affinity (approx. 1.5-fold) in comparison to binding in the absence of $Ca^{2+}$. For both cases (± $Ca^{2+}$) a slow dissociation ($4.3x10^{-3}$ s) was observed. Since the involvement of YcdA in TM's mechanism of action is so far unknown, the generation of an *ycdA* knockout mutant (wildtype *B. subtilis* DSM-10) is planned to further characterize its involvement in the mechanism of action of TMs. Besides YcdA, binding of TMs and DAP to QoxA was also analysed. So far, solely binding of TM to QoxA was measured with a rather low binding affinity (50 µM; Table 4-16; Figure 4-16). However, binding affinity was increased approximately 9-fold (6 µM) when $Ca^{2+}$ was present. Even though the potential mechanism underlying the binding of TM to QoxA still needs to be analysed, a $Ca^{2+}$-dependent mechanism of TM was again determined. QoxA depicts subunit II of the terminal oxidase cytochrome $aa_3$ and is involved in transferring

electrons to subunit I of the oxidase. Cytochrome *aa3* was characterized to be most probably the major oxidase of the branched respiratory chain of *B. subtilis*.[364] The aerobic growth of *B. subtilis* is branched into a cytochrome *c* oxidase and a quinol oxidase branch, whereas cytochrome $aa_3$ is found in the latter one. Cytochrome $aa_3$ belongs to the family of proton pumping heme-copper oxidases and consists of subunit I (QoxB), II (QoxA), III (QoxC) and IV (QoxD) which are encoded by *qoxB*, *qoxA*, *qoxC* and *qoxD*, respectively.[365–368] Additionally, two other *bd*-type cytochrome terminal oxidases are found within the quinol branch, cytochrome *bd* (encoded by *cydAB*) and cytochrome *bb'* oxidase (encoded by *ythAB*), both of which are not associated with a proton pumping function.[364,369,370] The cytochrome *c* branch differs in the type of terminal enzyme as it terminates in a cytochrome $caa_3$ oxidase, which also belongs to the family of proton-pumping heme-copper oxidases and consists of subunits I-IV (CtaD, CtaC, CtaE, CtaF), encoded by *ctaDCEF*.[364,368,371] Oxidases belonging to the heme-copper family share the presence of a subunit that is homologue to subunit I of the mitochondrial cytochrome *c* oxidase (QoxB and CtaD for *B. subtilis*). Those subunits depict the active sites and contain a heme-copper centre consisting of two hemes (O, A or B) and copper ($Cu_B$).[372,373] Besides *E. coli*[373],*B. subtilis* depicts a species whose respiratory system is well characterized, in contrast to *Staphylococci* among which only little is known.[374] Studies in *S. aureus* alternated between the presence of two and three respiratory branches[375,376], but recent studies hinted more towards the two branched system by a *bd*-type oxidase (encoded by *cydAB*)[377–379] and a heme-copper oxidase (encoded by *qoxABCD* operon).[380,381] Certainly, the *qoxABCD* operon found in *S. aureus* encodes four subunits that are related to $caa_3$-type oxidases found in *B. subtilis* and refers rather to a cytochrome *c* related oxidase, than to a quinol one. In general, the aerobic respiration in different bacteria relies on a common feature, the presence of terminal oxidases, which all consists of homologues of subunit I-IV. They are involved in the electron transport during the last step of the respiratory chain (Figure 4-25) by transferring electrons to molecular oxygen what leads to a reduction of oxygen to water[371,382]. This process includes the oxidation of quinol, either directly by quinol oxidases (quinol branch) or via the $bc_1$ complex in interaction with membrane bound cytochromes (cytochrome branch, Figure 4-25). Quinols (menaquinol or ubiquinol) needed to proceed those steps, are generated through the oxidation of quinones by three different reductases, succinate (SDH), type-2 NADH or glycerol-3-phosphate reductase.[383–385]

The essentiality of the QoxA within the respiratory chain is not known, since previous studies focused either on the function of the whole *qoxABCD* operon (complete loss of the

*qox* operon) or on subunits other than subunit II.[364,366] Based on this, it is not clear if QoxA represents an antibacterial target and if an interaction of TM with QoxA is able to trigger a collapse of the whole respiratory chain, especially since alternative terminal oxidases are available.[364]

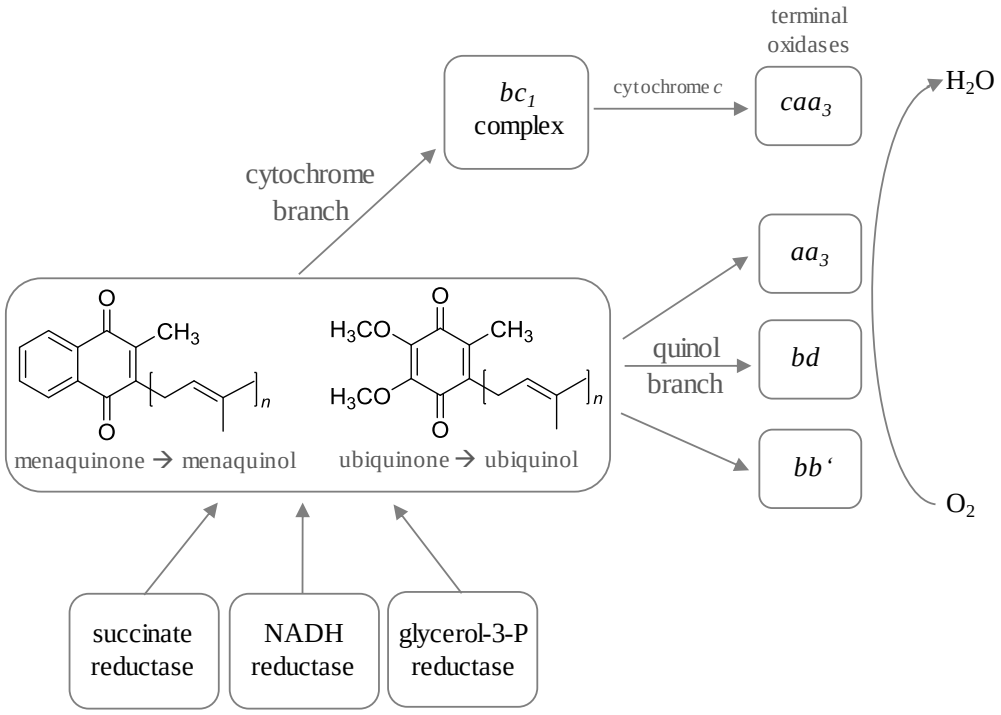



**Figure 4-25:** Branched aerobic respiratory chain (quinol and cytochrome branch) terminating in one of four terminal oxidases. Electrons are donated by the oxidation of menaquinones (mostly distributed among Gram-positive and anaerobic Gram-negative bacteria[383–385]) or ubiquinones (predominantly found among aerobic Gram-negative bacteria[384,386]). *n*: 1-12 (isoprene subunits). Adapted from Winstedt et al.[364]

The electron transfer within the respiratory chain is coupled to a proton transport across the cell membrane what mediates the generation of a proton gradient that provides energy which is further processed by the $F_0F_1$-ATPase to synthesize ATP[387,388]. Among all organisms that involve ATP in their metabolisms, structural related $F_0F_1$-ATP synthases are found[388,389]. They consist of two domains: the extrinsic membrane part $F_1$ and the membrane embedded part $F_0$ (Figure 4-26). Both domains contain several subunits of which highly identical ($\geq$ 80%) homologues in ATP-consuming species exists (Figure 4-27). Domain $F_1$ is composed of subunit α, β, γ, δ and ε and involved in the synthesis of ATP. $F_0$ consists of subunit a, b and c and depicts a transmembrane proton channel. Mitochondrial ATP synthases harbour additional subunits within both domains ($F_1$: d, $F_6$; $F_0$: e, f, g, A6L, DAPIT), of which the membrane bound subunits do not have a function within the

generation of ATP.[389] The common stoichiometry among all enzymes is $\alpha_3\beta_3\delta\gamma\varepsilon ab_2c_{8\text{-}15}$ with the main difference in the number of c-subunits. The typical ATP synthase found within bacteria is encoded on a single operon *atpIBEFHAGDC*. Subunits of domain $F_1$ are encoded by *atpA* (α), *atpD* (β), *atpG* (γ), *atpH* (δ) and *atpC* (ε) and subunits of $F_0$ by *atpB* (a), *atpE* (b) and *atpF* (c). The protein encoded by *atpI* depicts a protein with unknown function.[390]

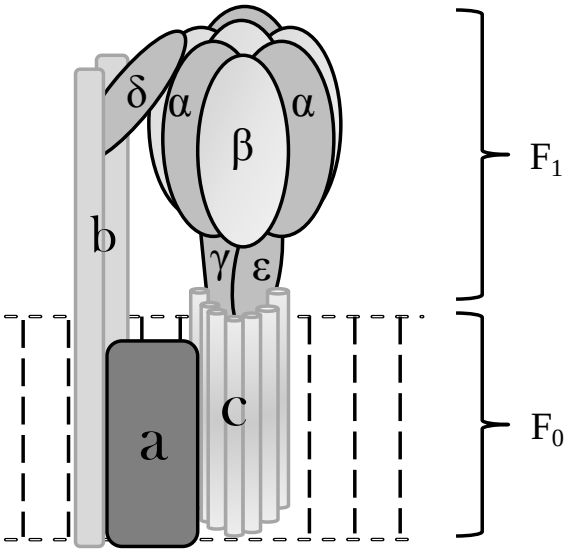



**Figure 4-26:** Schematic representation of the $F_0F_1$-ATP synthase. Membrane bound domain $F_0$ consists of subunit a, b and c, whereas the extrinsic part $F_1$ consists of subunits α, β, γ, δ and ε. Adapted from Balemans et al.[391]

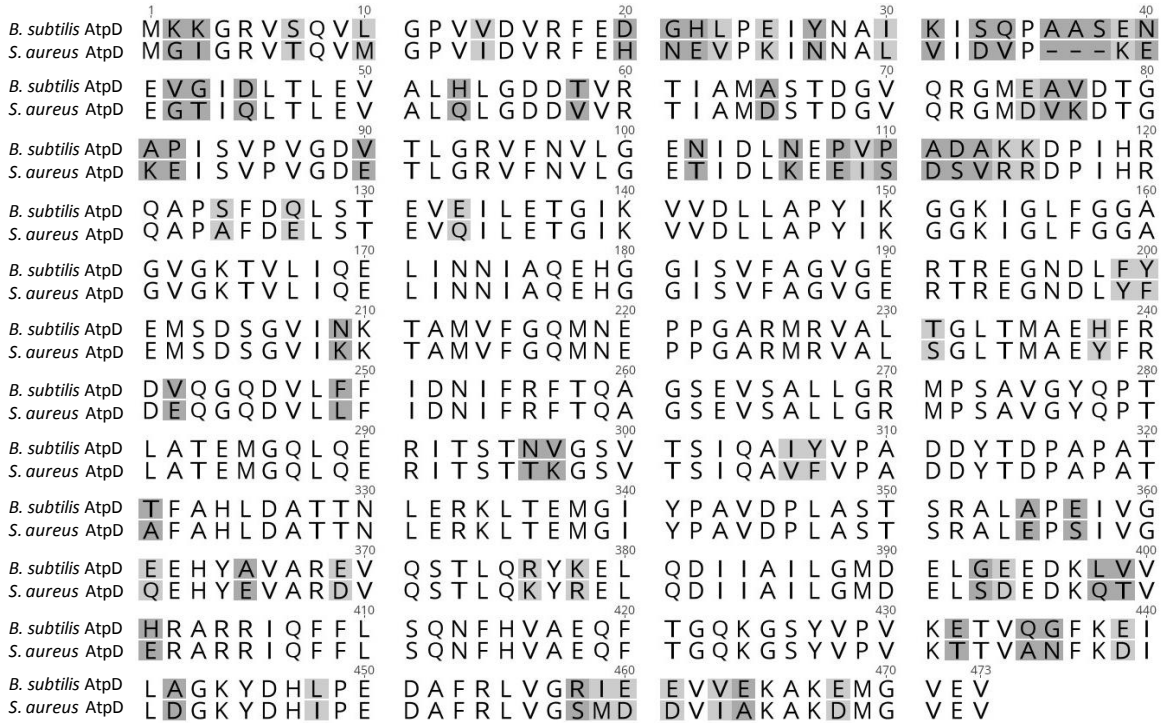



**Figure 4-27:** Exemplary alignment of amino acid sequences of subunit β (AtpD) from *B. subtilis* and *S. aureus*. Both share 87 % identity.

With regard to bacterial species, the ATP synthase depicts a potent antibacterial drug target. Serval natural and synthetic compounds are known to act antibacterial by inhibiting the ATP synthase (e.g. bedaquiline).[391,392] In this context, subunit αβ of the ATP synthase of *B. subtilis* was identified as potential target of TMs by protein fishing. Studies to analyse the binding of TMs to ATP synthase were restricted due to experimental limitations in the heterologous expression of ATP synthases. Thus, a functional assay on cell viability and a concurrent influence on the amount of ATP was performed. *S. aureus* Newman and *cls2* disrupted mutant were treated with TM, TM-Dodec, TM-*N*-Oct and DAP at 2-fold MIC and cell viability was assessed based on the amount of ATP within the cells. The treatment with any of the compounds did not decrease the amount of ATP (Figure 4-18) what leads to the conclusion that TMs do not directly interfere with the ATP synthase by interrupting its function. Previous studies performed with *S. aureus* transposon mutants revealed that *atpA*, *atpD* and *atpG* are not essential in *S. aureus* and that the transcript of *atpE* (subunit b of $F_0$ domain) plays an important role in the function of the ATP synthase.[391,393] This leads to the assumption, that a direct inference of TMs with subunits αβ might not be involved in the mechanism of action. However, to analyse sensitivity of bacteria with disrupted ATP synthase to TMs, an activity-based screen of *S. aureus* transposon mutants [obtained from

Nebraska Transposon Mutant Library] yielding transposon mutations in genes encoding several subunits of the ATP synthase (*atpA*, *atpB*, *atpD*, *atpG*, *atpH*) will be performed. Moreover, to analyse if disrupted subunits of cytochrome $caa_3$ oxidase contribute to a decrease in susceptibility of *S. aureus* towards TMs, mutants yielding transposon mutations in subunit I, II and III (*qoxB*, *qoxA*, *qoxC*) will additionally be tested.

So far, with regard to the results gained from the characterization of the proteins identified as potential target proteins, no direct impact on the mechanism of TMs could be revealed. Since YcdA is only present in *B. subtilis* and binding was solely observed for TM-*N*-Oct, a function as global target molecule can be excluded. Regarding QoxA, it is not clear if interactions with subunit II may induce lethality, especially since subunit I represents the active side of the oxidase. Regarding the ATP synthase as potential target of TMs, it was already mentioned, that subunit αβ might not be involved in the mechanism of action since they are not essential for the function of the synthase. Nevertheless, a reason why those proteins have been fished might be a connection between respiratory chain complexes and cardiolipin. The individual parts of the respiratory chain of bacteria and mitochondria are able to associate into supercomplexes[371,394], whose maintenance is associated with CL.[395–399] The formation of supercomplexes contributes to the stability of individual respiratory complexes and their function, e.g. the electron transport through the respiratory chain.[371,400–404] Previous studies in yeast as well as bacteria (*B. subtilis* and *E. coli*) revealed the presence of several supercomplexes, such as $bc_1$-$caa_3$[394,405–407] or $bc_1$-$caa_3$ with the ATP synthase, SDH-$aa_3$ and a putative association of $aa_3$ with the ATP synthase.[371] Moreover, the absence of CL was shown to lead to the dissociation of supercomplexes (e.g. $bc_1$-$caa_3$) and a thereof dependent decrease in the activity of the complexes.[394,402,408,409] Taking together the facts that CL is needed to maintain the respiratory chain and that TMs were shown to bind to CL, leads to the assumption, that proteins identified by target fishing rather interacted with CL than with TM. It seems as if TMs primarily bound to CL, which was still associated with proteins involved in respiration and energy metabolism.

So far, no direct prove is available that the above drawn hypothesis depicts an explanation why those proteins have been fished, but the present analysis strongly hints towards a CL-mediated binding.

In summary, the general comparison of TM to TM-Dodec and TM-*N*-Oct strongly indicated that both derivatives act calcium-independent and that their antibacterial effects do not

exclusively rely on the binding to CL. This furthermore indicates an interaction with an additional target, which might be common for all TMs to mediate a mechanism of action beyond membrane activity (presumably cell wall-associated). However, so far no distinct proteins targets could be assigned and alternative experiments to identify additional targets need to be performed. Moreover, since preliminary data revealed induction of cell wall stress by TMs, more specific screens could be performed to characterize the impact on the cell wall synthesis in detail (including studies on lipid II binding).

## 4.3 SUMMARY AND OUTLOOK

The cyclic depsipeptide TM was already described in 1950 by Bristol-Myers Company (New York, USA) and revealed promising antibacterial activities against Gram-positive bacteria including multidrug-resistant (MDR) pathogens. However, over the last 60 years no further investigation was described until the characterisation of the biosynthesis of the natural product. Based on the knowledge gained from those studies, semi-synthetic derivatives were generated, aiming to provide SAR-improved compounds. In the course of the present study, the two acylated derivatives TM-Dodec and TM-*N*-Oct were described to show promising antibacterial activities, including inhibition of MDR pathogens and clinical isolates, with overall improved activities compared to natural TM. However, the activities of TM and both derivatives are restricted to Gram-positive bacteria and Gram-negative pathogens revealed intrinsic resistance. Moreover, a calcium-dependent activity of TM could be revealed, whereas both derivatives were shown to act calcium-independent. Studies on the interaction of $Ca^{2+}$ with TM indicated that the molecule presumably needs to undergo conformational changes mediated by the binding to $Ca^{2+}$ to perform its action. In contrast, both derivatives seem to act without undergoing conformational changes.

Regarding the mechanism of action, similarities in activity to the cell membrane targeting calcium-dependent antibiotic DAP were observed. TMs exert a bactericidal effect, which was in previous studies shown to be even stronger for TM-Dodec compared to DAP. Moreover, a calcium-dependent membrane depolarization for TM was observed, which hints towards a membrane-related mechanism of action, similar to that of DAP. Studies on the resistance mechanism consolidated this assumption, since the depletion of cardiolipin, a major component of the cellular membrane, was found to be involved in the generation of resistance towards TM and TM-Dodec. Mutations or the absence of the gene (*cls2*) encoding for the synthase (cardiolipin synthase 2), which catalyses the generation of CL were shown to lead to decreased sensitivity of *S. aureus* Newman towards TMs. However, this effect was more pronounced towards TM, compared to TM-Dodec and TM-*N*-Oct. Moreover, studies on the binding of TMs to membrane lipids revealed an exclusively binding of TMs to CL, but not to other lipids or membrane components. The intracellular localization of TMs was additionally shown to happen in CL-rich domains. Nevertheless, all together this study revealed that CL is solely needed for TM but not required for both derivatives to act on the bacteria. It is possible, that the absence of the lipid side chain within the structure of natural TM hinders its interaction with the bacterial cell and that the binding to $Ca^{2+}$ and CL enables

the molecule to interact with the cell membrane. In contrast, both derivatives seem to be able to interfere with the cells by inserting their acyl chain into the membrane, similar as observed for DAP.

Some proteins (YcdA, QoxA, αβ-subunit of ATP synthase) were identified as potential binding partners in a protein pull-down experiments and binding could be confirmed to the lipoprotein YcdA (TM-*N*-Oct) and to subunit II (QoxA) of the terminal oxidase $aa_3$ (TM). However, at the current stage, it cannot be excluded that interaction with these proteins is associated with the presence of CL in the protein preparations. Previous studies showed that subunits of the respiratory chain (e.g. terminal oxidases, ATP synthase) are able to form respiratory chain supercomplexes whose stability and thereof based activity relies on the presence of CL. It might be possible that the binding of TMs to CL leads to a disruption of the respiratory chain complexes and a collapse of the cellular metabolism. To further investigate this assumption, a repeat of the target fishing experiment needs to be done to show if the same protein pattern will be detected from lysates of CL depleted *S. aureus* mutants. Although studies on the cell viability already showed no effect of TMs on the ATP amount, further studies to exclude a potential concentration- and time-dependent effect should be done. Additionally, a comparative binding study in the presence and absence of CL, with the proteins which showed interaction to TMs, could be performed.

Nevertheless, it is not clear if binding to CL depicts the starting point of TMs mechanisms of action and which cellular processes are affected by this mechanism. So far, studies strongly point towards a target protein exclusively present in Gram-positive bacteria, which might be found associated with the cell wall. In a primary step, TMs seem to interact with the membrane, whereas the presence of CL is crucial for natural TM but both semi-synthetic TM derivatives are able to interfere CL-independent. After insertion or binding to the cell membrane, TMs seems to interact with the so far unknown target enzyme, which in further steps causes a collapse of the bacterial metabolism (presumably linked to a hampered cell wall synthesis) and finally cell death (comparable to DAP). In addition, if CL would depict the main target of TMs, it is not clear why those compounds do not exert cytotoxic effects and inhibition of Gram-negative bacteria even though CL is also present within those types of cells, what further indicates the interaction with an additional common target for TMs.

In summary, studies performed to characterize the mechanism of action of TMs revealed an interference with the cellular membrane, but the exact mechanism of action still needs to be further investigated. Moreover, two derivatives bearing lipid side chains revealed improved activities compared to the parent molecule TM and a mechanism of action beyond CL-binding. However, further semi-synthetic optimisations (antimicrobial activity; physicochemical properties) with regard to further steps in the direction of preclinical development (ADME, PK/PD studies) need to be performed.

# 5 CONCLUSION AND RELEVANCE

The introduction of new antibiotic drugs in the clinics is accompanied by a fast development of resistance – a severe problem that exists almost since the beginning of the antibiotic era in the early 1900s.[1] In addition, over the last decades, resistance to antibiotics exacerbated due to the emergence of multidrug-resistant (MDR) bacterial species and a decline in the development of novel antibiotics. To avoid the advent and progression of the so-called "pre-antibiotic" era, the development of new antibiotics showing resistance-breaking properties by acting on new target molecules and exhibiting novel mechanisms of action is urgently needed.[5,21]

Up to day, the main and most promising sources for the development of new effective lead structures are still natural products (NPs) derived from microorganisms (e.g. bacteria, fungi).[72] During the "Golden Era" of antibiotic discovery (1930s-1960s) a wide variety of compound classes have been characterized (e.g. penicillins, tetracyclines), but only antibiotics with favourable pharmacological properties were further pursued and developed into drugs. Thus, a number of neglected NP scaffolds exist, which are already known for decades and which exhibit pronounced antibacterial activity, but viable drug development procedures were not available at the time of their discovery. Due to today's advanced knowledge in terms of e.g. chemical derivatization and synthesis and genetic engineering, at least some of those scaffolds are promising starting materials to develop new antibacterial drugs. However, to overcome hurdles in the drug development pipeline, such as unfavourable pharmaceutical and pharmacokinetic properties, it is of utmost importance to perform a detailed *in vitro* and *in vivo* profiling of such scaffolds and to further improve them. Crucial steps during preclinical development are the identification of the target molecule and the elucidation of the molecule's mechanism of action. These data can help to inform the modification of a scaffold to improve on-target activity, to overcome resistance, and to widen the antibacterial spectrum. Further, derivatization programs aim at optimizing pharmaceutical properties to eventually achieve optimal *in vivo* efficacy.

In the course of this study, NPs and respective derivatives of two different compound classes – chelocardins[107,108,139] and telomycins[145,148] – were characterized to elucidate their mechanism of action/resistance and to identify their target molecule(s). Both represent potent examples of underexploited compound classes derived from actinobacteria, an important source of NPs, with inhibitory activity against clinically relevant bacteria. Chelocardins are active against Gram-negative and Gram-positive pathogens and the

compounds possess resistance-breaking properties. This holds also true for telomycins as they act against MDR bacteria and display no cross-resistance to standard antibiotics; however, telomycins solely inhibit Gram-positive bacteria. Chelocardin and its more potent analogue amidochelocardin displayed *in vitro* inhibition of the ribosome without displaying cross-resistance with related tetracyclines. Their main target appeared to be related to bacterial membranes and efflux-mediated resistance in Gram-negative bacteria occurred only at very low frequency. Two new telomycin derivatives that act, in contrast to the original NP, independent of the presence of cardiolipin and calcium were in-depth characterized and it could be revealed that telomycins presumably act through a new mechanism targeting an enzyme involved in cell wall synthesis. Even though the main target molecule for both antibiotic families was not identified, the overall characterization revealed important insights into chelocardin's and telomycin's mechanisms of action and resistance mechanisms. These results contribute to a further optimisation of both compound classes and to the development of new potent lead structures, which can be forwarded into the development pipeline to provide new effective antibacterial drugs that can overcome resistance.

In summary, this work highlights that the re-assessment of neglected compound classes is a promising, alternative pathway to provide new effective compounds and that NPs still play an important role in drug development. The two compound classes that were characterised within this study are now in preclinical development with the overall goals to introduce new antibiotics into the pipeline, to fight against life-threatening bacterial infections, and to combat resistance.

# 6 APPENDIX

## 6.1 ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-binding cassette |
| ACN | Acetonitrile |
| ADMET | Absorption, distribution, metabolism, excretion, toxicity |
| AMR | Antimicrobial-resistance |
| Apr | Apramycin |
| APS | Ammonium Persulfate |
| ATP | Adenosine triphosphate |
| bp | Base pair |
| CAMHB | Cation-adjusted Müller-Hinton broth |
| CAMP | Cationic antimicrobial peptide |
| CCCP | Carbonyl cyanide 3- chlorophenylhydrazone |
| CDA | Calcium-dependent antibiotic |
| CDCHD | 2-carboxamindo-2-deacetyl-CHD, amidochelocardin |
| CFU | Colony forming unit |
| CHD | Chelocardin |
| CIP | Ciprofloxacin |
| CL | Cardiolipin |
| CLS | Cardiolipin synthase |
| cpm | Counts per minute |
| Da | Dalton |
| DAP | Daptomycin |
| dd | Double-distilled |
| DiOC2(3) | 3,3‘-diethyloxacarbocyanin iodide |
| DMEM | Dulbecco’s modified eagle medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| e.g. | *exempli gratia* |
| ESBL | Extended-spectrum β-lactamases |
| FBS | Foetal bovine serum |
| FCS | Foetal calf serum |
| FDA | Food and Drug Administration |
| FS | Frameshift |
| GBS | Group B *Streptococcus* |
| HGT | Horizontal gene transfer |
| HRP | Horseradish peroxidase |
| Hyg | Hygromycin |
| iChip | Isolation chip |
| $IC_{50}$ | Half maximal inhibitory concentration |
| $k_a$ | Association rate |

| | |
|---|---|
| KAN | Kanamycin |
| kb | Kilobase pair |
| $k_d$ | Dissociation rate |
| $K_D$ | Dissociation constant |
| kDa | Kilodalton |
| KPΔramR | *K. pneumoniae* DSM-30104 ramR knockout mutant |
| LB | Luria-Bertani broth |
| LC-MS | Liquid chromatography mass spectrometry |
| LPG | Lysyl-phosphatidylglycerol |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| MALDI-TOF | Matrix-assisted laser desorption/ionization-time of flight |
| Mar | Multiple antibiotic resistance |
| MATE | Multidrug and toxic compound extrusion |
| MBC | Minimum bactericidal concentration |
| MIC | Minimal inhibitory concentrations |
| MDR | Multidrug-resistant |
| MFS | Major facilitator superfamily |
| MFP | Membrane fusion protein |
| MHB | Müller Hinton Broth |
| MMS | Macromolecule synthesis |
| MOI | Multiplicity of infection |
| MS | Mass spectrometry |
| MTT | Thiazolyl blue tetrazolium bromide |
| MP | Membrane potential |
| MRSA | Methicillin-resistant *Staphyloccocus aureus* |
| Mt | Mutant |
| MQ | Milli-Q-water |
| MW | Molecular weight |
| M7H9 | Middlebrook 7H9 medium |
| NAO | Acridine orange 10-nonyl bromide |
| NCE | New chemical entities |
| n.d. | Not determined |
| NMR | Nuclear magnetic resonance |
| NP | Natural product |
| NPN | 1-*N*-phenylnaphtylamine |
| $OD_{600}$ | Optical density at 600 nm |
| OM | Outer membrane |
| Omp | Outer membrane porin |
| OTC | Oxytetracycline |
| PAβN | Phenylalanine arginine β-naphthylamide dihydrochloride |
| PBP | Penicillin-binding proteins |
| PBS | Phosphate-buffered saline |
| PC | Phosphatidylcholine |

| | |
|---|---|
| PCR | Polymerase chain reaction |
| PE | Phosphatidylethanolamine |
| PG | Phosphatidylglycerol |
| PI | Propidium iodide |
| PMB | Polymyxin B |
| PMBN | Polymyxin B nonapeptide |
| qPCR | Real-time polymerase chain reaction |
| R&D | Research and development |
| $R_{max}$ | Theoretical maximal resonance units |
| RNA | Ribonucleic acid |
| RIF | Rifampicin |
| $RIF^R$ | Rifampicin-resistant |
| RND | Resistance-nodulation-division |
| ROESY | Rotating-frame Overhauser SpectroscoPY |
| RPMI | Roswell Park Memorial Institute medium |
| rRNA | Ribosomal ribonucleic acid |
| RU | Resonance units |
| SAR | Structure-activity relationship |
| SD | Standard deviation |
| SDS | Sodium dodecyl sulphate |
| SDH | Succinate reductase |
| SNP | Single-nucleotide polymorphism |
| SPR | Surface Plasmon resonance |
| TCs | Tetracyclines |
| TET | Tetracycline |
| TEMED | Tetramethyl ethylenediamine |
| TLC | Thin-layer chromatography |
| TMRM | Tetramethyl rhodamine methyl ester |
| TSB | Tryptic Soy Broth |
| TM | Telomycin |
| TM-Dodec | Acylated telomycin derivative (dodecanoic acid added) |
| TM-*N*-Oct | Acylated telomycin derivative (ethylamine octanoic acid added) |
| UPEC | Uropathogenic *Escherichia coli* |
| UTI | Urinary tract infection |
| VISA | Vancomycin intermediate *Staphyloccocus aureus* |
| VRE | Vancomycin-resistant Enterococci |
| *vs*. | Versus |
| *v*/*v* | Volume-to-volume |
| *w*/*v* | Weight-in-volume |
| WHO | World Health Organization |
| Wt | Wildtype |

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