

Mode of Action of Daptomycin, a Lipopeptide Antibiotic

by

Jawad Kadhum Muraih

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Jawad K. Muraih

Abstract

Daptomycin is a lipopeptide antibiotic that contains 13 amino acids and an N-terminally attached fatty acyl residue. The antibiotic kills Gram-positive bacteria by membrane depolarization. It has long been assumed that the mode of action of daptomycin involves the formation of oligomers on the bacterial cell membrane; however, at the outset of my studies, this had not been experimentally demonstrated.

In the work described in this thesis, I have used fluorescence energy transfer (FRET) between native daptomycin and an NBD-labeled daptomycin derivative to demonstrate that the antibiotic indeed forms oligomers on bacterial cell membranes. In a liposome model, oligomer formation depends on calcium and on phosphatidylglycerol (PG). The oligomer forms rapidly and is stable for a length of time longer than required for the bactericidal effect. Through variation of the ratio of FRET donor (native daptomycin) and acceptor (NBD-daptomycin), I have determined that the oligomer consists of approximately 6–7 molecules, or, depending on the structure of the oligomer, possibly up to twice that number.

Oligomer formation on liposomes and on bacterial membranes was confirmed using excimer fluorescence of a perylene-labeled daptomycin derivative. Excimer fluorescence was also used to demonstrate a stoichiometric interaction between daptomycin and PG.

It has previously been shown that the bactericidal activity of daptomycin requires calcium and correlates with the concentration of PG in the bacterial cell membrane; these requirements mirror those observed here for oligomer formation. Furthermore, membrane permeabilization is selective, and electron microscopy of bacterial membranes exposed to daptomycin has revealed no discontinuities or accretions of electron density. Both of these findings suggest formation of a small membrane lesion, which is compatible with the small size of the oligomer that was determined here. In conjunction with these previous findings, the experiments contained in my thesis strongly suggest that the oligomer is the bactericidal form of daptomycin.

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Dedication

To my parents, brothers and sisters

To my wife and children

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Chapter 1

Introduction

The work described in this thesis focuses on the antibiotic daptomycin, and in particular on the mechanism of its bactericidal action. Daptomycin is a cyclic lipopeptide antibiotic with activity against a wide spectrum of pathogenic Gram-positive bacteria. In 2003, daptomycin was approved by the U.S. Food and Drug Administration for the treatment of skin and skin structure infections (see Section 1.2.4 and reference [125]).

In this chapter, I will first present a brief general overview of antibiotics and bacterial resistance to them; this will serve to place the role and significance of daptomycin into context. I will then turn to daptomycin itself and discuss what was known about its mode of action at the outset of my studies. The chapter will conclude with a description of the research objectives of my thesis.

1.1 ANTIBIOTICS

One of the most valuable and successful achievements in medicine in the 20th century was the discovery and development of antibiotics for the treatment of bacterial infections. Antibiotics have saved the lives and improved the health of countless individuals. Shortly after

their introduction, they were considered by many the “magic bullets” that would spell the end of infectious diseases. However, these high hopes have not been fulfilled. Instead, bacterial resistance emerged and spread, which has compromised the effectiveness of an ever greater number of antibiotics, often to the point where those antibiotics have lost their utility for general clinical use. The inexorable progression of resistance has led to a situation in which some infections that once were believed to have been vanquished, have once more become difficult to treat. Therefore, fundamental and applied research on antibiotics must continue in order to improve their bactericidal activity and ensure their continued clinical effectiveness.

In general, antimicrobial drugs can be classified into two categories. The first one comprises synthetic drugs, and the second natural drugs that are synthesized by microorganisms. In addition, a number of semisynthetic drugs have been developed, which are chemical derivatives of the natural antibiotics; these derivatives usually have been optimized for more effective uptake and increased protection from bacterial resistance mechanisms. In particular, all β -lactam antibiotics in current clinical use are semisynthetic derivatives of natural compounds.

1.1.1 History

Throughout history, plant products were used empirically in the treatment of infectious diseases; however, neither the doctors nor their patients understood the modes of action of those therapeutic agents. In the earliest records from about 2600 BC from Mesopotamia, the use of plant oils obtained from *Cedrus* (cedar) and *Cupressus sempervirens* (cypress) species, *Glycyrrhiza glabra* (licorice), *Commiphora* species (myrrh), and *Papaver somniferum* (poppy juice) is described. These plants are still in use to date for the treatment of illnesses ranging from coughs and colds to parasitic infections and inflammation [29].

As described in the literature, many plants used for preparations of remedies were dried. However, fresh ingredients (green plants) occasionally were required. For extractions, simple or compound drugs were chopped, pounded, and pulverized, then dissolved in a liquid. Various liquids were used alone or in combination, including water, milk, beer, wine, sesame oil, olive

oil, and vinegar [8]. We do not know whether the choice of the solvent was made according to observed clinical effectiveness, or rather was governed by taste.

In ancient Egypt (~1500 BC), it was observed that molds are beneficial in the treatment of skin and wound infections; the Medical Papyri recommend the application of moldy bread crumbs and rags to such lesions [81]. Similar recommendations are found in the Chinese literature (Wu Shi Er Bing Fang) about the use of herbal drugs from around 1100 BC [29] and in the Indian Ayurvedic system, which dates from about 1000 BC. The latter system was the starting point for the primary text of Tibetan Medicine, Gyu-zhi (Four Tantras) [40]. In the ancient Western world, it was documented that the Greeks (~300 BC) further developed the use of herbal drugs by changing the characteristics of the used plants through cultivation [29].

While the prescriptions found in historical tradition were of course purely empirical and were not accompanied by any notion resembling the modern concept of antibiotics, they nevertheless indicated remarkable skills of observation and perception.

The modern, scientific era of antibacterial chemotherapy started in 1910. In this year, Paul Ehrlich introduced salvarsan (arsphenamine), which has bactericidal action against the spirochete *Treponema pallidum*, the cause of syphilis. The first synthetic drugs with activity against a broad spectrum of bacterial pathogens were the sulfonamides. The first sulfonamide drug, sulfamidochrysoidine, was synthesized in 1932 by two Bayer company scientists, Mietzsch and Klarer. This compound is also a red dye and went by the trade name of “Prontosil red” (Figure 1.1). In 1935, Gerhard Domagk observed that streptococcal infections in mice could be treated with this dye [36, 48, 50, 17]. Intriguingly, the compound had no antibacterial activity *in vitro*. This was subsequently explained by the observation that, in the metabolism of the animals’ or patients’ livers, the compound was reductively split into two components (Figure 1.1); one of these, sulfanilamide, carried the antibacterial activity [136, 44]).¹ Sulfanilamide is a competitive inhibitor of the enzyme dihydropteroate synthetase, which uses *p*-aminobenzoate as a substrate in the synthesis of dihydropteroate, an intermediate in the bacterial synthesis of

¹The need for metabolic activation also applies to arsphenamine; the native compound is not toxic to spirochetes [84].

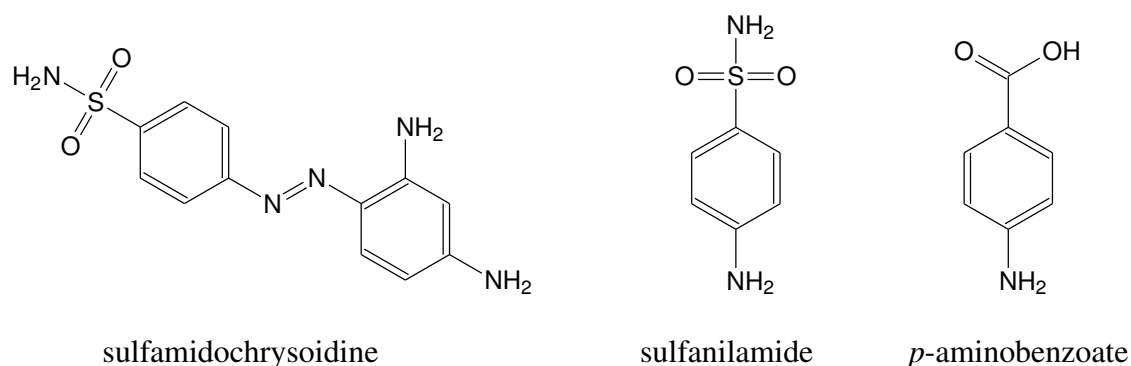


Figure 1.1 Chemical structures of sulfamidochrysoidine (“Prontosil red”) and its active metabolite sulfanilamide, which competes with *p*-aminobenzoate at the enzyme dihydropteroate synthetase (DHPS). Inhibition of DHPS suppresses bacterial synthesis of folic acid, which causes the antibacterial effect of sulfanilamide.

tetrahydrofolate. Deficiency of folate inhibits the bacteria because they are dependent on their own de novo folate synthesis. In contrast, mammals do not synthesize folic acid but instead are able to take it up from the environment, and are thus exempt from inhibition by sulfonamides [109]. Soon after introduction of sulfamidochrysoidine, other sulfonamide derivatives were developed in order to improve the potency and extend activity to a wider spectrum of bacterial species.

Penicillin was the first natural antibiotic to be discovered and isolated. It was obtained from *Penicillium notatum* in 1928 by Alexander Fleming [42]. However, he could not purify sufficient amounts for sustained clinical use. Then, in 1941, Chain and Florey demonstrated the therapeutic usefulness of penicillin [24]; however, they initially were also unable to produce enough penicillin for clinical use; large scale use did not start until 1943 [99].

Penicillin proved active against most Gram-positive bacteria and some Gram-negative ones, namely *Neisseria meningitidis* and *N. gonorrhoeae*. It inhibits muramyl-transpeptidase enzymes that catalyze the murein transpeptidation reaction. This prevents the cross-linking of polysaccharide chains of the bacterial cell wall peptidoglycan. As a result, the peptidoglycan becomes mechanically weakened and is no longer able to withstand the osmotic pressure of

the enclosed cell, which causes the cells to swell and burst [130].

Numerous other antibiotics were quickly discovered after penicillin came into use; among the major classes of antibiotics that have been used in humans, the majority were discovered within a time period of only around 10 years [50]. While penicillin itself is produced by a mold, the great majority of antibiotics are obtained from soil bacteria, in particular from member species of *Streptomyces* and related genera. In isolation, *Streptomyces* grows more slowly than for example *Bacillus* or *E. coli*. However, by producing antibiotics that kill off susceptible bacteria nearby, *Streptomyces* species are able to compete effectively in the soil; they are the most abundant kind of soil bacteria. The antibiotics synthesized by microorganisms can be used to control bacterial diseases in humans and animals [135].

In 1943, streptomycin was discovered by one of Waksman's students [118]. Like penicillin, streptomycin possesses bactericidal action; however, its mode of action is different and consists of the quick and irreversible inhibition of ribosomal protein synthesis [87, 32]. In that period, in 1939, R.J. Dubos discovered gramicidin [62], which is active against both Gram-positive bacteria, except for the Gram-positive bacilli, and selected Gram-negative organisms. Gramicidin causes transport of sodium and potassium ions across natural and artificial membranes [37]. Major antibiotics that were discovered shortly afterward include tetracycline and chloramphenicol, which both inhibit bacterial ribosomal protein biosynthesis and block bacterial growth [48]. In addition to the soil, antibiotic producer microorganisms have been isolated from other sources, such as wound infections, sewage and chicken throats [48]. It should be noted that many, even the majority of the discovered natural antibiotics are too toxic for therapeutic use in humans; examples are gramicidin and puromycin. However, some toxic antibiotics, such as actinomycin D and doxorubicin, can be used in the treatment of cancer.

1.1.2 Natural and synthetic antibiotics

Antibiotics can be synthesized by microorganisms or by artificial chemical methods. Some of the most common antibiotics are natural and used as isolated from the producing bacteria or

Table 1.1 Sources of some natural antibiotics

Source microorganism	Antibiotic
Bacteria	
<i>Streptomyces spp.</i>	Amphotericin B
	Chloramphenicol
	Erythromycin
	Neomycin
	Nystatin
	Rifampin
	Streptomycin
	Tetracyclines
	Vancomycin
	<i>Micromonospora spp.</i>
<i>Bacillus spp.</i>	Bacitracin
	Polymyxins
Fungi	
<i>Penicillium spp.</i>	Griseofulvin
	Penicillin
<i>Cephalosporium spp.</i>	Cephalosporins

fungi (Table 1.1). Other important chemotherapeutic antibiotics are entirely synthetic; among those listed in Table 1.2 are sulfonamides, trimethoprim, ciprofloxacin, isoniazid, and dapsone. Some natural antibiotics are also produced industrially by total synthesis; an example is chloramphenicol.

The effectiveness of antibiotics against pathogens is usually measured as the minimum inhibitory concentration (MIC), which is the lowest concentration of antibiotic that prevents the growth of a specific pathogen. In order for an antibiotic to be clinically effective, the MIC has to be attainable by therapeutic dosages. Using this criterion, many antibiotics have activity only against a narrow spectrum of pathogenic bacteria. On the other hand, broad-spectrum antibiotics have the ability to attack many different kinds of pathogens (Table 1.2). Many

Table 1.2 Mechanism of action of some common antibiotics.

Antibiotic	Spectrum	Mechanism of action
Inhibitors of cell wall synthesis		
Penicillin	Mostly Gram+	Penicillins and cephalosporins inhibit transpeptidases that cross-link peptidoglycan chains in the bacterial cell wall
Ampicillin	Gram+, Gram-	
Carbenicillin	Gram+, Gram-	
Methicillin	Gram+	
Cephalosporins	Gram+, Gram-	
Vancomycin	Gram+	Binds directly to the D-Ala-D-Ala terminus of lipid II and inhibits the transpeptidation step
Bacitracin	Gram+	Sequesters lipid carrier (bactoprenol) that transports cell wall precursors across the plasma membrane
Inhibition of ribosomal protein synthesis		
Streptomycin	Gram+, Gram-, mycobacteria	Binds to 30S subunit, causes misreading of mRNA
Gentamicin	Gram-	Binds ribosome, causes misreading of mRNA
Chloramphenicol	Gram+, Gram-, rickettsiae and chlamydiae	Binds to 50S subunit, blocks peptide bond formation by peptidyl transferase

Table 1.2 – Continued

Antibiotic	Spectrum	Mechanism of action
Tetracyclines	Gram+, Gram-, rickettsiae and chlamydiae	Bind to 30S subunit, interfere with aminoacyl-tRNA binding
Erythromycin, clindamycin	Gram+, Gram-	Bind to 50S subunit and inhibit peptide chain elongation
Nucleic acid synthesis inhibitors		
Ciprofloxacin	Gram+, Gram-, rickettsiae, chlamydiae	Inhibit bacterial DNA gyrase, inhibit replication and transcription
Rifampin	Gram+, mycobacteria	Inhibit DNA-dependent RNA polymerase
Cell membrane disruption		
Polymyxin B	Gram-	Binds and dissociates lipopolysaccharide
Antimetabolites		
Sulfonamides	Gram+, Gram-	Inhibit folic acid synthesis by competing with p-aminobenzoate
Trimethoprim	Gram+, Gram-	Inhibition of dihydrofolate reductase
Dapsone	Mycobacteria	Interferes with folic acid synthesis
Isoniazid	Mycobacteria	Inhibits an enzyme in mycolic acid synthesis (katG)

broad-spectrum antibiotics are obtained through semisynthesis; examples are ampicillin and carbenicillin. Another use of semisynthesis is to combat bacterial resistance. For example,

the spectrum of the semisynthetic antibiotic methicillin resembles that of natural penicillin; however, the compound is stable toward β -lactamases, which are the most common resistance mechanism of its main target pathogen, *Staphylococcus aureus* [113].

1.1.3 Mechanisms of action of specific antibiotics

The action modes of antibiotics differ widely; Table 1.2 summarizes the mechanisms of some representative examples, including both natural and synthetic antibiotics.

Most of the antibiotics listed in Table 1.2 affect the synthesis of the cell wall. Such antibiotics typically have high therapeutic utility, since bacterial cell walls are composed of unique structures that are not found in human or other eukaryotic cells. Another major class of therapeutically useful antibiotics inhibit bacterial protein synthesis by binding to the prokaryotic ribosome. Many of these antibiotics bind to the ribosomal RNA rather than to proteins. Some of these antibiotics bind to the 30S subunit of the ribosome, whereas others attach to the 50S subunit. These antibiotics cause inhibition of protein synthesis in varying steps like aminoacyl-tRNA binding, peptide bond formation, mRNA reading, and translocation. The different activities of all these antibiotics have been instrumental in dissecting the function of the ribosome [113].

Many translation-inhibiting antibiotics affect selectively prokaryotic organisms because the structure of ribosomes is sufficiently different between them and humans. Thus, these compounds can be used to treat bacterial infections with minimal side effects to humans. However, puromycin, which is an aminonucleoside antibiotic obtained from *Streptomyces alboniger*, disrupts both prokaryotic and eukaryotic protein synthesis and therefore is useful only for *in vitro* research but not for therapy [111].

Antibacterial drugs that inhibit nucleic acid synthesis or damage cell membranes are usually less specific, and therefore more toxic, than the classes of antibiotics discussed above. This is because of the similarities in nucleic acid synthetic mechanisms and also of cell membrane structure between prokaryotes and eukaryotes. Good examples of DNA-damaging antibiotics

are doxorubicin and bleomycin [109]. On the other hand, quinolones such as ciprofloxacin are selective for inhibiting bacterial DNA gyrase, and are therefore widely used in medicine. Inhibition of gyrase is toxic since it interferes with DNA replication, repair, and transcription. Among membrane-damaging antibiotics, polymyxin binds to the lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria and disrupts both outer and inner bacterial plasma membranes. LPS has no similarity to human membrane lipids, and therefore polymyxin can be used in antibacterial therapy [113].

Antimetabolites block the functioning of metabolic pathways, often by competitively inhibiting biosynthetic enzymes. For example, trimethoprim inhibits folate reductase, which catalyzes formation of both dihydrofolate and tetrahydrofolate. Trimethoprim acts synergistically with sulfonamides to inhibit folic acid metabolism. As mentioned above, humans can not synthesize folic acid and they must obtain it in their diet, whereas the bacterial pathogens have to synthesize their own folic acid. However, folate reductase does occur in human metabolism, and its inhibition is highly toxic; indeed, folate reductase inhibitors such as methotrexate are used in tumour therapy and immunosuppressive therapy. Therefore, inhibitors of microbial folate reductase must be specific for the bacterial enzyme [109].

Another antimetabolite is isoniazid, which is used in the treatment of *Mycobacterium tuberculosis* infections (i.e., tuberculosis). Isoniazid reacts covalently with NAD. The adduct inhibits KatG, a mycobacterial catalase/peroxidase enzyme that is involved in the synthesis of mycolic acids, which are very long fatty acid residues found in the mycobacterial cell wall [113].

1.1.4 Resistance to antibiotics

As discussed in Section 1.1.2, microorganisms produce natural antibiotics. Those producer organisms need to protect themselves from their own toxic products, and therefore possess specific resistance mechanisms. The genes encoding these resistance mechanisms can, in many cases, spread between different bacterial strains by horizontal gene transfer and confer an-

timicrobial resistance to previously susceptible species, including pathogenic ones. Clinical application of antibiotics provides the selective pressure that causes such resistant strains to proliferate and spread. According to the literature, “indeed there is some evidence that at least some clinically relevant resistance genes have originated in environmental microbes” [146, 71]. The problem is compounded by the widespread use of antibiotics in farm animals, from which the resistant bacterial strains, or the genes, can then be transmitted to humans.

In addition to horizontal gene transfer, resistance can also arise by de novo mutations; this is obviously particularly important with synthetic antibiotics.

1.1.4.1 Resistance mechanisms. The biochemical mechanisms of resistance to antibiotics can be divided into the following categories [109, 88]:

(1) Primary resistance. The organisms may lack the structure that an antibiotic inhibits. For instance, *Mycoplasma* species lack the murein layer found in Gram-positive and Gram-negative bacteria and therefore are naturally resistant to penicillins. *Mycobacteria* resist many drugs because their cell peptidoglycan is surrounded by a dense lipid layer, which is impermeable to most drugs.

(2) Target site alteration. In this scenario, a change in the macromolecular target of the antibiotic—often by way of a point mutation—increases the ability of the target to discriminate between the inhibitory antibiotic and its physiological ligand. For example, point mutations in bacterial DNA topoisomerase II confer resistance to inhibitors such as ciprofloxacin, and base substitutions in the 23S rRNA decrease the affinity of the ribosomes for erythromycin or chloramphenicol.

(3) Compensatory changes in metabolism. Malaria parasites can develop resistance to the dihydrofolate reductase inhibitor pyrimethamine through compensatory overexpression of the enzyme [109]. In bacteria, resistance to sulfonamides may develop when they acquire the ability to take up folic acid from the environment, circumventing the pathway that is blocked by the sulfonamide drugs [88].

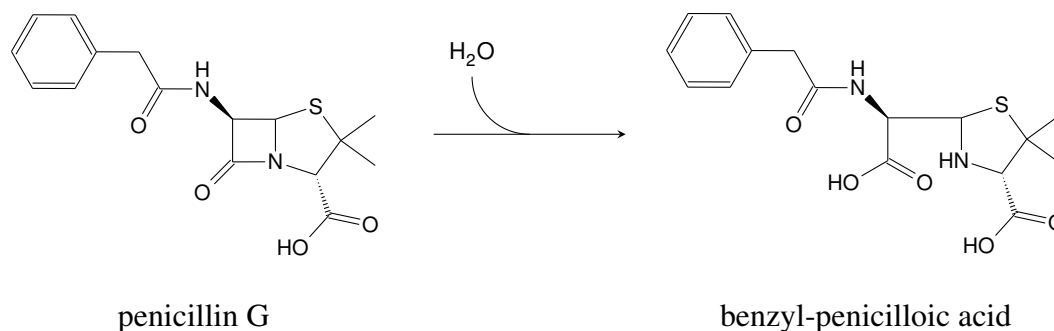


Figure 1.2 Structure of penicillin G and of benzyl-penicilloic acid, which results from its hydrolytic cleavage by β -lactamase.

(4) Inactivation of the antibiotic by chemical modifications. For example, many staphylococci produce β -lactamase, an enzyme that cleaves the β -lactam ring of most penicillins (Figure 1.2) and cephalosporins. Antibiotics also can be inactivated by addition of new groups to the chemical structure; this is common with aminoglycoside antibiotics, which may undergo enzymatic acetylation or phosphorylation.

(5) Mutation of transport proteins that mediate uptake of the antibiotic. For example, point mutations or deletion of porins may prevent permeation of antibiotics across the outer membranes of Gram-negative bacteria. The uptake of fosfomicin is mediated by the glycerolphosphate transporter; mutations of this non-essential transporter cause development of resistance and tend to occur rapidly.

(6) Organisms can resist antibiotics through active extrusion or efflux from the cell interior. This process is mediated by *efflux pumps*. The expression of efflux pumps is often increased by factor of >100 in multiresistant bacterial strains.

Some specific examples of bacterial resistance mechanisms that illustrate the above principles are listed in Table 1.3.

1.1.4.2 Antibiotic-resistant pathogens. There is no doubt that the therapeutic use of antibiotics generates a selective pressure that causes pathogenic bacteria to acquire resistance mechanisms through spontaneous mutation and gene transfer, and that the spreading of resis-

Table 1.3 Mechanisms of bacterial resistance to antibiotics (examples)

Resistance mechanism	Antibiotics	Microorganisms
Reduced membrane transport due to mutant porin proteins	Carbenicillin, cephalosporins	<i>Pseudomonas aeruginosa</i> , Enterobacteriaceae
Active efflux	Tetracyclines, chloramphenicol, erythromycin	<i>Staphylococcus aureus</i> , Enterobacteriaceae
Inactivation by β -Lactamases	Penicillins and cephalosporins	<i>Staphylococcus aureus</i> , <i>Neisseria meningitidis</i> , <i>E. coli</i>
Inactivation by phosphorylation, acetylation or methylation	Chloramphenicol, streptomycin	<i>Staphylococcus aureus</i> , Enterobacteriaceae
Mutations in ribosomal RNA and proteins	Streptomycin	<i>E. coli</i>
Mutant muramyl-transpeptidase	Methicillin and cephalosporins	Methicillin-resistant <i>S. aureus</i> (MRSA)
Mutant DNA gyrase	Ciprofloxacin	Enterobacteriaceae

tance jeopardizes the continued effectiveness of antibiotics. The problem is made worse by the widespread use of antibiotics in less compelling applications, such as shotgun use without clear medical indication, as well as medication of farm animals in order to increase meat yields. Over time, this has resulted in the emergence and spread of many highly resistant pathogenic bacteria worldwide.

Development of resistance among pathogenic bacteria was observed almost as soon as antibiotics had been discovered and introduced into clinical use. As an example, we can consider staphylococci, which are Gram-positive bacteria. They are divided into two major groups, coagulase-positive and the coagulase-negative staphylococci. Among the latter, the most prominent species is *Staphylococcus epidermidis*. This bacterium inhabits the surface of the skin and has low intrinsic pathogenicity, but is often encountered in opportunistic infections of catheters and implanted devices. The most pathogenic species is *Staphylococcus*

aureus, which is coagulase-positive and is commonly found on the skin and in the nasal passages of carriers [80]. *S. aureus* is a common cause of skin infections, abscesses, pneumonia, and septicemia.

At the outset of the antibiotic era, almost all staphylococci were susceptible to penicillin. However, shortly after introduction of penicillin, resistance became common, and was mostly caused by β -lactamases. In *S. aureus*, resistance to penicillin had a prevalence of less than 1% when penicillin first came into use; this figure increased to 14% in 1946, to 38% in 1947, and finally to more than 90% today [107, 50]. The emergence of β -lactamases in staphylococci was countered by the development of β -lactamase-resistant penicillins, such as methicillin, and of cephalosporins. In the early 1960s, *Staphylococcus aureus* strains then acquired resistance to these compounds [48]; this occurred through horizontal transfer of a gene encoding a different muramyl-transpeptidase or penicillin-binding protein. Methicillin-resistant *S. aureus* (MRSA) has since spread around the world and is now a major hospital pathogen.

With the spreading of MRSA, vancomycin became the drug of choice for treating infections by staphylococci with lacking or uncertain susceptibility to β -lactams [50]. Vancomycin is a glycopeptide antibiotic produced by *Streptomyces orientalis*. It contains a peptide linked to a disaccharide. The antibiotic blocks peptidoglycan synthesis by inhibiting the transpeptidation step that across-links adjacent peptidoglycan strands. It exercises this inhibition by binding to the substrate rather than the enzyme (Figure 1.3), which is unusual. The peptide portion of vancomycin binds to the D-alanine-D-alanine terminal sequence on the pentapeptide portion of peptidoglycan. For several decades, vancomycin was a reliable standby in the treatment of antibiotic-resistant staphylococcal and enterococcal infections. However, in 1997, vancomycin-resistant *Staphylococcus aureus* (VRSA) was first isolated in Japan [95]. This finding was rapidly followed by the isolation of VRSA strains in the U.S. and other countries [126]. VRSA emerged through horizontal gene transfer of the *vanA* gene from *Enterococcus faecalis* [25, 60, 35]. While vancomycin-resistant staphylococci and enterococci are not yet as widespread as MRSA, vancomycin is losing ground as the default drug for treating severe

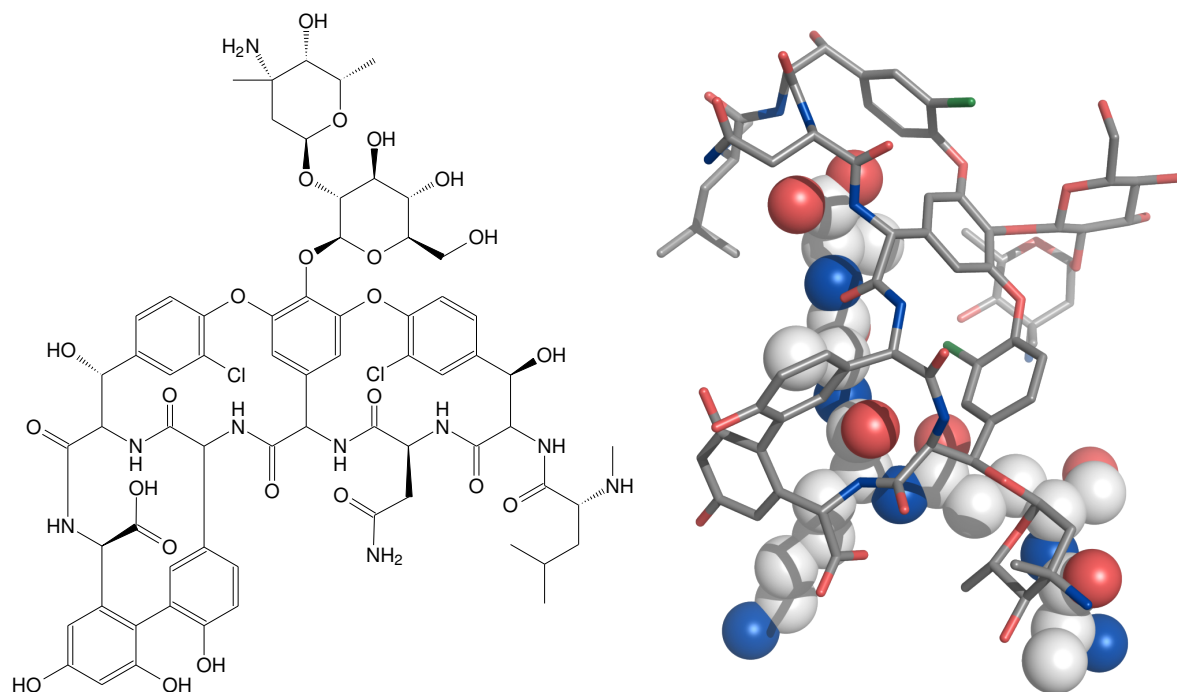


Figure 1.3 Structure of vancomycin (left) and of the related glycopeptide antibiotic A82846B, bound to its target pentapeptide moiety (shown as spheres) of lipid II [114]. Within the pentapeptide, the terminal D-alanine residue faces upward. This residue is cleaved by muramyl-transpeptidase; its blockade by glycopeptide antibiotics prevents transpeptidation and thus the cross-linking of murein. (Oxygen atoms are shown in red, nitrogens in blue, and chlorine in green).

Staphylococcus aureus infections. With the fading of vancomycin, other therapeutic options are required. Novel alternatives that have entered clinical trials or been approved for application are linezolid, ceftobiprole, and daptomycin.

Linezolid is a member of the oxazolidinone class of drugs (Figure 1.4). It has recently been recommended as an another choice of the treatment for some of these infections [121, 35]. Linezolid inhibits ribosomal protein synthesis at the stage of initiation [131].

Ceftobiprole is a novel broad-spectrum cephalosporin that is in phase III of clinical development [19]. Ceftobiprole is interesting, since it seems to be the first β -lactam designed to inhibit the penicillin-binding proteins (PBP) in MRSA [31], thus potentially restoring the usefulness of β -lactams against this important pathogen.

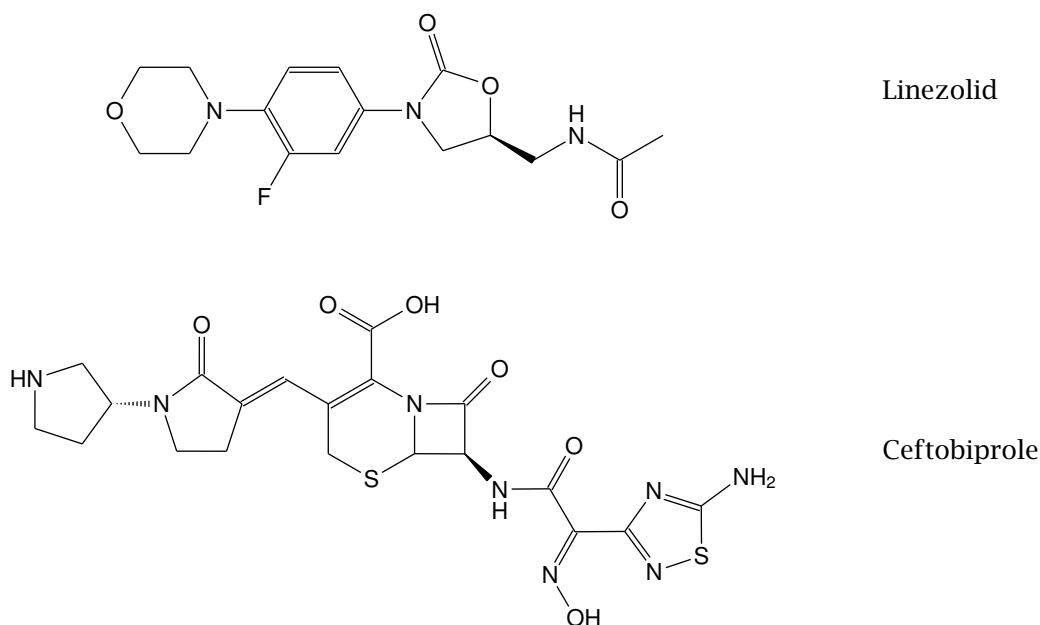


Figure 1.4 Chemical structures of linezolid and ceftobiprole. Linezolid is a synthetic antibiotic used for the treatment of serious infections caused by Gram-positive bacteria. Ceftobiprole is a novel broad-spectrum cephalosporin that is in phase III of clinical development.

Daptomycin is another antibiotic that has recently been introduced into clinical use against otherwise resistant Gram-positive pathogens [127]. Daptomycin has been widely adopted and is currently in widespread use in North America. A few cases of resistance have been reported to date [57, 85]. In addition to staphylococci, daptomycin is also used against enterococci, Gram-positive bacteria [96] that are intrinsically resistant to many antibiotics including cephalosporins, penicillin, and aminoglycosides [108]. *Enterococcus faecium* and *E. faecalis* are the clinically most important species [137, 140]. They are a frequent cause of urinary tract infections, bacteremia, and endocarditis, all of which can be difficult to treat due to resistance [96]. Traditionally, only ampicillin and vancomycin were available choices in the treatment of most cases [46]. As of 2000, resistance to ampicillin had become common, leaving vancomycin as the sole choice for treatment [65]. Vancomycin-resistant enterococci (VRE) were not considered as a clinical problem until recently. However, in the last few years, VRE infections have become much more frequent and emerged as a major health care problem.

Among streptococci, the major pathogens are *Streptococcus pneumoniae* or pneumococcus, *Streptococcus pyogenes*, and *Streptococcus agalactiae*. Infection with *S. pneumoniae* becomes manifest mostly as respiratory disease, which is responsible for 40,000 deaths yearly in the U.S. alone, however, this concerns mostly elderly patients [64, 106]. Around 5 million children in the world, less than 5 years old, die each year due to pneumococcal infection [64], which, reflects lack of access to proper nutrition and treatment more than antibiotic resistance.

Gram-negative bacteria such as gonococci and meningococci were susceptible to β -lactams such as penicillin G. However, penicillin G is destroyed by stomach acid. Penicillin V is similar to penicillin G, but it is more acid-resistant and can be given orally. Ampicillin can be administered orally and possesses a broad spectrum of activity against Gram-negative bacteria like *Haemophilus influenzae*, *Salmonella*, and *Shigella*. As is the case with staphylococci, resistance to penicillin and ampicillin due to formation of serine-type β -lactamases has become common. One option to overcome this resistance is through combination with β -lactamase inhibitors; another is the use of cephalosporins such as cefotaxim, which are quite stable to serine-type β -lactamases. However, in addition to the serine-type β -lactamases, Gram-negative bacteria also possess metalloenzyme β -lactamases, which cleave virtually every β -lactam antibiotic in existence [113].

In the last two decades, pathogens have been found that are resistant to almost all antibiotics, including all β -lactams and vancomycin. A crisis stage for antibiotics in general may be reached [82, 13, 63, 82] if we do not find a novel antibiotics to effectively combat these widely resistant pathogens. One novel family of antibacterial compounds are the cyclic lipopeptide antibiotics. Among these, daptomycin is already established in clinical practice, while others are at various stages of development [12].

1.1.5 Peptide antibiotics

Peptide antibiotics are widely considered to be potential therapeutic alternatives for infections caused by multidrug-resistant bacteria [55, 141]. Many antibiotic peptides exhibit a lethal

mode of action against pathogens (see reference [20] and Table 1.2), which in many cases is mediated by permeabilization of the bacterial cell membranes.

Peptide antibiotics can be divided into two structural categories, namely cyclic and acyclic molecules. Alamethicin and suzukacillin-A, which are produced by the fungus *Trichoderma viride*, are examples of linear peptide antibiotics[103, 74], whereas tachypleusins from *Tachypleus tridentatus* and gramicidin S from *Bacillus brevis* are cyclic peptides [6]. Figure 1.5 shows examples of linear and cyclic peptide antibiotics.

While the mechanisms of actions of peptide antibiotics are varying and not always fully understood, many of them attack and permeabilize the bacterial cell membranes. Antibiotic peptides are often cationic, and they bind to anionic lipids in the target membranes. Additionally, hydrophobic interactions contribute to the peptides' affinity for the membranes. Penetration of the head groups of the lipids unevenly distends and disrupts the membrane. Changes to the net charge of the system may contribute to membrane destabilization [93, 147]. An example of a synthetic cationic peptide that has entered clinical trials, for topical application in catheter infections, is omiganan pentahydrochloride [116]. Overall, the clinical application of such peptides is still at an early stage of development [54].

While membrane disruption through mixed electrostatic and hydrophobic interactions occurs with many antibiotic peptides, one should not assume that all peptide antibiotics share this same mode of action. For example, even lipopeptide molecules that are structurally as similar as daptomycin and amphomycin (see Figure 1.7) have significantly different modes of action.

1.1.6 Cyclic lipopeptide antibiotics

Cyclic lipopeptide antibiotics are divided into two structural classes. The first class comprises the cyclic lipodepsipeptides, which include at least one ester bond ($-\text{COOR}-$) instead of an amide bond ($-\text{CONHR}-$). An example is daptomycin (Figure 1.6), which is produced by *Streptomyces roseosporus*. Other cyclic depsipeptides that are structurally related to daptomycin are A54145, which is produced by *Streptomyces fradiae*, and the so-called calcium-

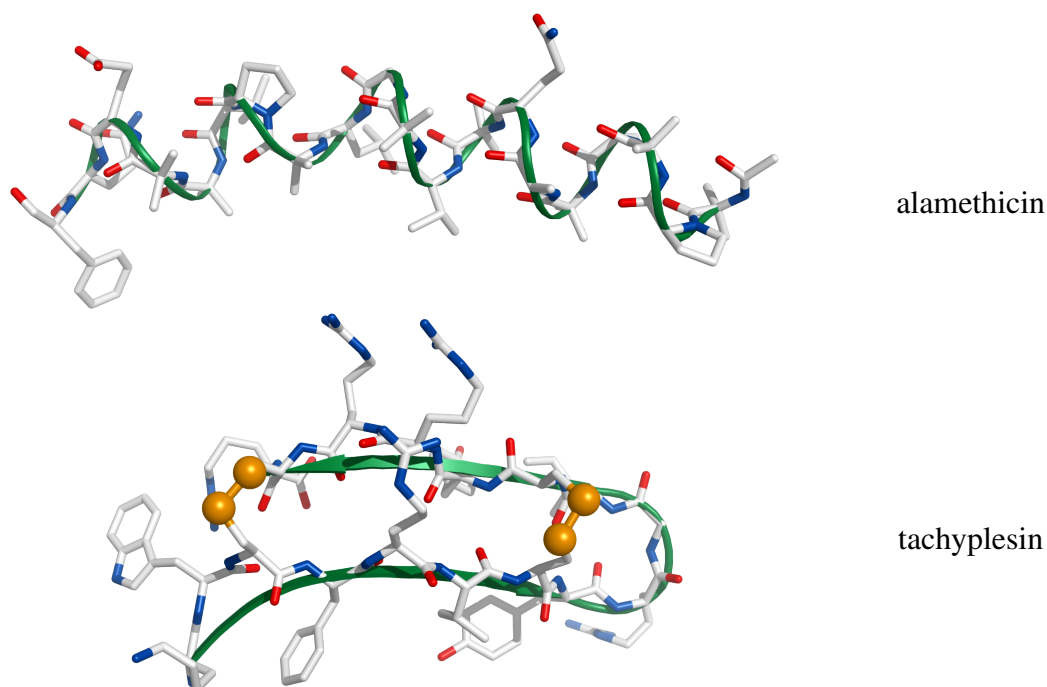


Figure 1.5 Crystal structures of alamethicin [45] and tachyplesin [76]. Alamethicin is a linear polypeptide ionophore antibiotic containing 20 residue containing residues of α -aminoisobutyric acid (Aib) and a C-terminal L-phenylalaninol (Phol) unit. It adopts an α -helical structure. Tachyplesin is a cyclic peptide antibiotic consisting of 17 amino acid residues with a carboxyl-terminal arginine alpha-amide. Tachyplesin I adopts a rather rigid conformation that consists mostly of β -structure and is stabilized by two disulfide bridges.

dependent antibiotic (CDA) from *Streptomyces coelicolor* A [73, 104]. Figure 1.7 shows the chemical structures of daptomycin and those related lipodepsipeptide antibiotics. Each of these antibiotics has one or more variable positions, and the figure shows one arbitrary example for each. All these cyclic depsipeptides have peptide rings closed by threonine, such that the ring-closing ester bond attaches to the OH group of the threonine side chain. Daptomycin and A54145 include three exocyclic amino acid residues, with the terminal amino group attached to fatty acyl tail. However, CDA contains only one exocyclic amino acid residue (serine) to which the fatty acyl tail is attached.

The second structural class of lipopeptide antibiotics are the true cyclic lipopeptides, or

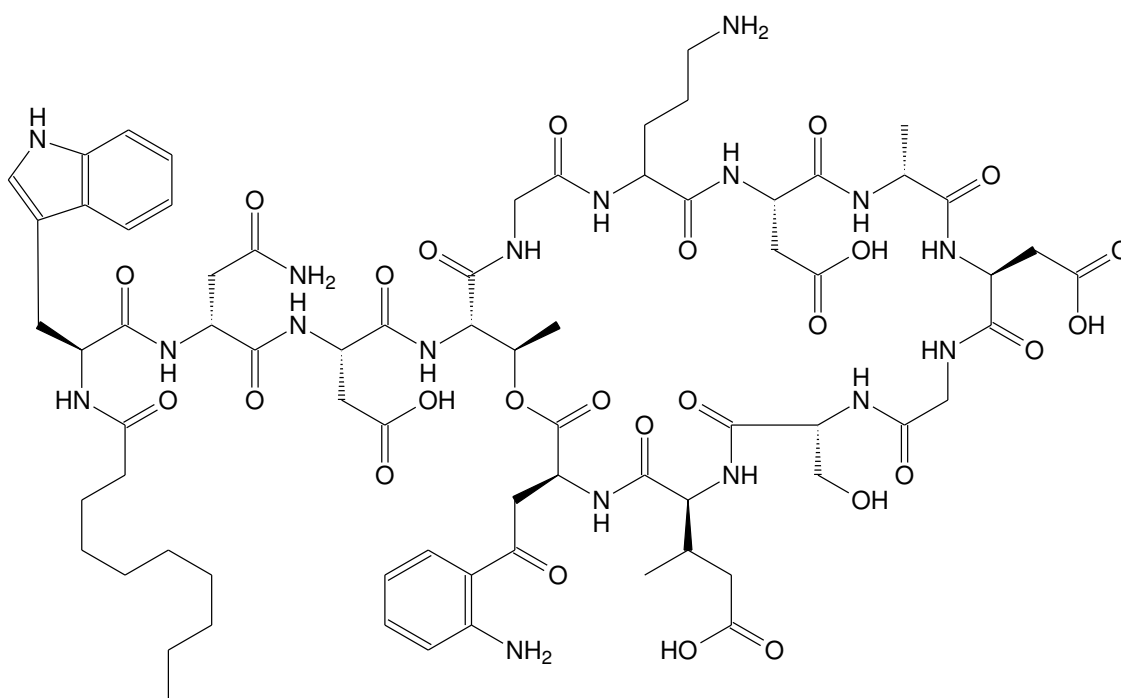


Figure 1.6 Structure of daptomycin, which is an acidic cyclic lipopeptide antibiotic containing 13 amino acids. The ten C-terminal amino acids form the cycle; the three N-terminal residues are exocyclic. A decanoyl residue is attached to the N-terminal tryptophan.

lactams. Examples are amphotycin, which was discovered over sixty years ago [58] and is produced by *Streptomyces canus*, friulimicin from *Actinoplanes friuliensis*, and laspartomycin from *Streptomyces viridochromogenes var. komebensis* [73]. Friulimicin and laspartomycin are also shown in Figure 1.7.

The lipopeptides amphotycin, friulimycin and laspartomycin are similar to the cyclic lipodepsipeptides daptomycin, A54145 and CDA with respect to their acidic nature. Both groups contain non-standard amino acids, D-amino acids, and C- and N-methylated residues [122]; for instance, daptomycin contains the non-standard amino acids kynurenine, ornithine, and 3-methylglutamate in addition to D-alanine, D-serine, and D-asparagine. Some positions of amino acids in the daptomycin molecule are conserved, including aspartic acid residues Asp⁷ and Asp⁹. Moreover, D- and L-amino acids occupy homologous positions. These traits are

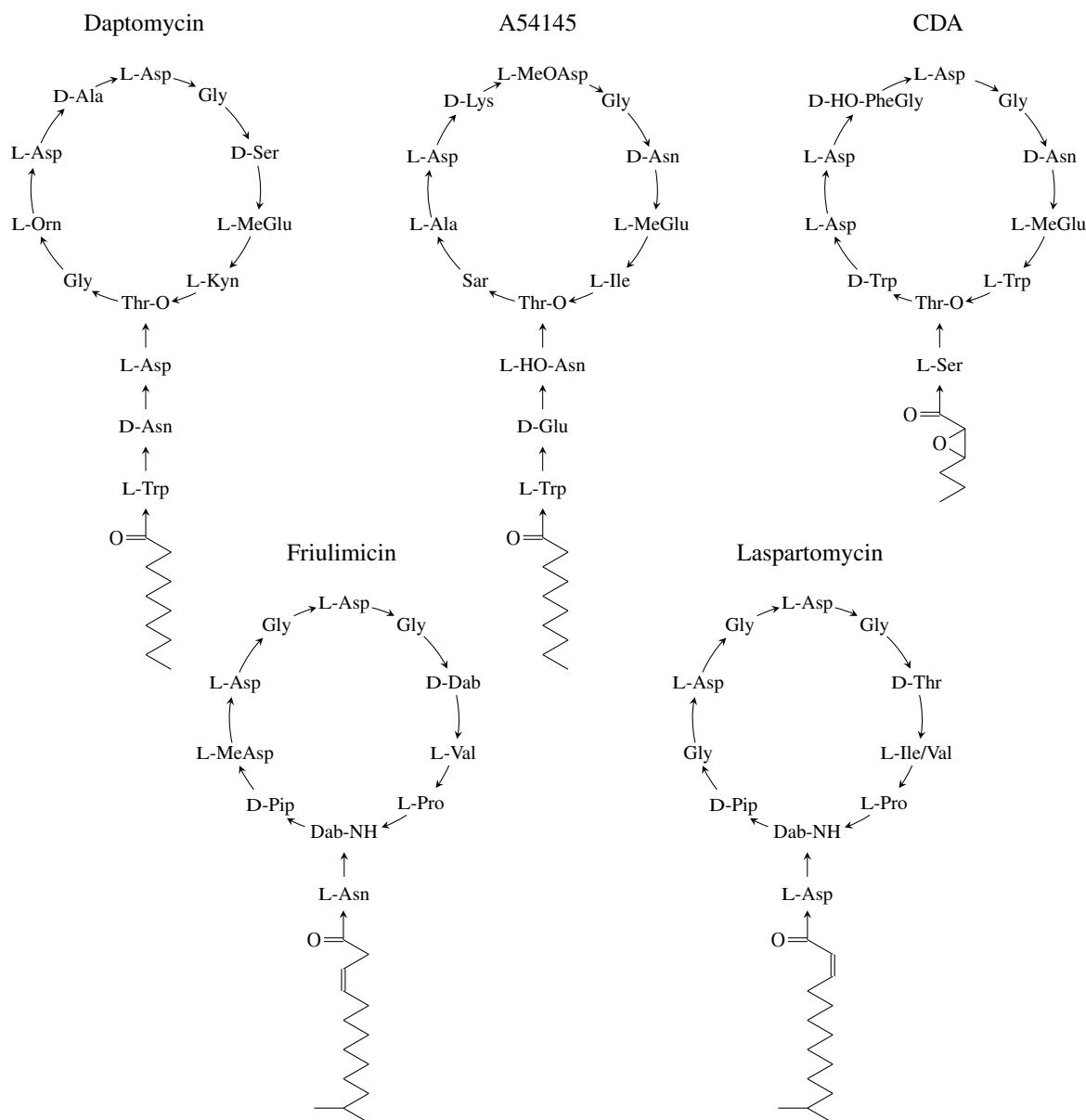


Figure 1.7 Chemical structures of daptomycin and of related lipopeptides and lipodepsipeptides. Amphomycin resembles friulimicin, save for an aspartate residue that replaces asparagine in the exocyclic position. The first three are cyclic depsipeptides, since the peptide ring is closed by an ester bond. Daptomycin and A54145 include three exocyclic amino acid residues with terminal amino group attached to lipid tail. In contrast, CDA contains one exocyclic amino acid residue (Ser) linked to a shorter lipid tail. No ester bond is present in amphomycin, friulimicin, and laspartomycin, which therefore are true cyclic lipopeptides. Each of the antibiotics has one or more variable positions, and here shows one arbitrary example for each. Dab=2,4-diaminobutyric acid, Pip=Pipecolic acid, MeOGlu=Methoxy-glutamate, MeGlu=Methylglutamate, MeOAsp=Methoxy-aspartate.

conserved between the daptomycin group and the amphomycin group, even though these antibiotics differ in their modes of action (see below). The conserved positions are important for antibiotic activity [73].

Despite these lipopeptide antibiotics sharing characteristic structural features with daptomycin such as a lipopeptide moiety with a cyclic decapeptide core and a fatty acid tail attached to the exocyclic amino terminus, they vary in their mechanisms of action. In particular, amphomycin, friulimycin, and laspartomycin inhibit the biosynthesis of membrane-bound peptidoglycan precursor of Gram-positive cell walls by binding to the substrate undecaprenylphosphate, the universal carbohydrate carrier involved in several biosynthetic pathways. In contrast, daptomycin and A54145 do not detectably interfere with this pathway at minimum inhibitory concentrations [119]. The only well-documented target molecule for daptomycin is phosphatidylglycerol; this will be discussed in more detail in Section 1.2.3 and Chapter 2. For CDA, no target has been characterized at the molecular level.

1.2 DAPTOMYCIN

Daptomycin is an acidic cyclic lipopeptide antibiotic that contains 13 amino acids, with a decanoyl residue attached to the N-terminal amino group (Figure 1.6; [5, 22]). In early reports, the molecule was referred to as LY146032. It is a member of a complex of cyclic lipopeptides (A21978C) that share the same peptide moiety but vary with respect to the length of the N-terminal fatty acyl tail [34]. All these lipopeptide antibiotics are obtained from *Streptomyces roseosporus* [5].

Daptomycin was discovered by researchers at Eli Lilly and Company in the early 1980s. In the late 1980s and early 1990s, Lilly conducted clinical trials for use of the drug in infections caused by Gram-positive organisms. These early clinical trials were discontinued because high-dose therapy caused adverse effects on skeletal muscle, including myalgia and potential myositis [38]. The worldwide rights to daptomycin were licensed to Cubist Pharmaceuticals in

1997, and new clinical trials with modified dosage regimens were begun in 1999. After FDA approval in 2003, the drug came to be marketed under trade name Cubicin®.

1.2.1 Physicochemical properties

The mainly acidic nature of daptomycin and the negative net charge that prevails at neutral pH explain its aqueous solubility. However, the decanoyl tail and the presence of several hydrophobic amino acid residues cause an overall amphipathic behaviour. The amphipathicity of daptomycin is useful for purification by reversed-phase HPLC. In aqueous solution, daptomycin self-associates to form micelles. ¹H NMR studies at pH 6 suggest that molecular aggregation takes place at concentrations in the millimolar range [9, 68]. This aggregation makes it difficult to study the conformation of monomeric daptomycin in solution. NMR measurements of daptomycin at 0.8 mM in the presence of excess calcium showed a binding stoichiometry of 1:1 [9].

1.2.2 Biosynthesis

Daptomycin is naturally synthesized and assembled within *Streptomyces roseosporus* cells by a system of three non-ribosomal peptide synthetases (NRPSs), named DptA, DptBC, and DptD [98, 89, 11]. Each of these synthetases contains multiple modules, each of which mediates the recognition, activation and incorporation of one specific amino acid into the oligopeptide chain [41]. Each module contains at least three catalytic domains: a C domain for condensation, an A domain for activation (or adenylation), and a T domain for thiolation.

The roles of the A and the T domain in the activation of an amino acid are illustrated in Figure 1.8A. Once attached to the T domain, the residue awaits condensation to a preceding residue by its cognate C domain (Figure 1.8B). The overall process starts off with the attachment of the fatty acyl residue to the N-terminal tryptophan [98, 143]; it is supplied by the DptF protein (Figure 1.8C), which in turn acquires it after adenylation of the fatty acid by DptE (not shown). Like other acyl carrier proteins, DptF contains a 4'-phosphopantetheine moiety.

Peptide synthesis proceeds in the sequence from DptA via DptBC to DptD; the C-terminal thioesterase (TE) domain in DptD forms the lactone bond between threonine and kynurenine and releases the molecule [73, 51]. Epimerase (E) domains in DptA and DptBC cause epimerization of the initially bound L-amino acid residues to the D-forms before incorporation; this applies to D-Asn², D-Ala⁸, and D-Ser¹¹.

Non-proteinogenic amino acids such as ornithine⁶, methylglutamate¹², and kynurine¹³ are located within the 10-amino acids macrolactone ring; these residues significantly contribute to the bioactivity of daptomycin [105, 51].

As is the case with daptomycin, the peptide synthetases for A54145 have been molecularly characterized, and a number of recombinant hybrids have been created in order to elucidate structure and function and to create improved derivatives [104, 105]. This will be discussed below.

1.2.3 Mode of action

The antibacterial activity of daptomycin is Ca²⁺-dependent. The antibacterial potency reaches its maximum at a Ca²⁺ concentration of 1.2 mM, which is the same as the concentration of free calcium in human extracellular fluid [14].

In early studies, Allen *et al.* [5] suggested that daptomycin can inhibit peptidoglycan biosynthesis in *Staphylococcus aureus*. Daptomycin also caused leakage of K⁺ ions, thus causing dissipation of the membrane potential in *B. megaterium* and *S. aureus*. These researchers suggested that the cytoplasmic membrane may be the target for daptomycin and the dissipation of membrane potential might result from its interference with the uptake of precursors for peptidoglycan biosynthesis. However, all these observations were made at daptomycin concentrations several times higher than the minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation; these are probably not attainable *in vivo*. At MIC concentrations in the presence of Ca²⁺, daptomycin inhibited regeneration of cells from protoplasts in *Enterococcus*

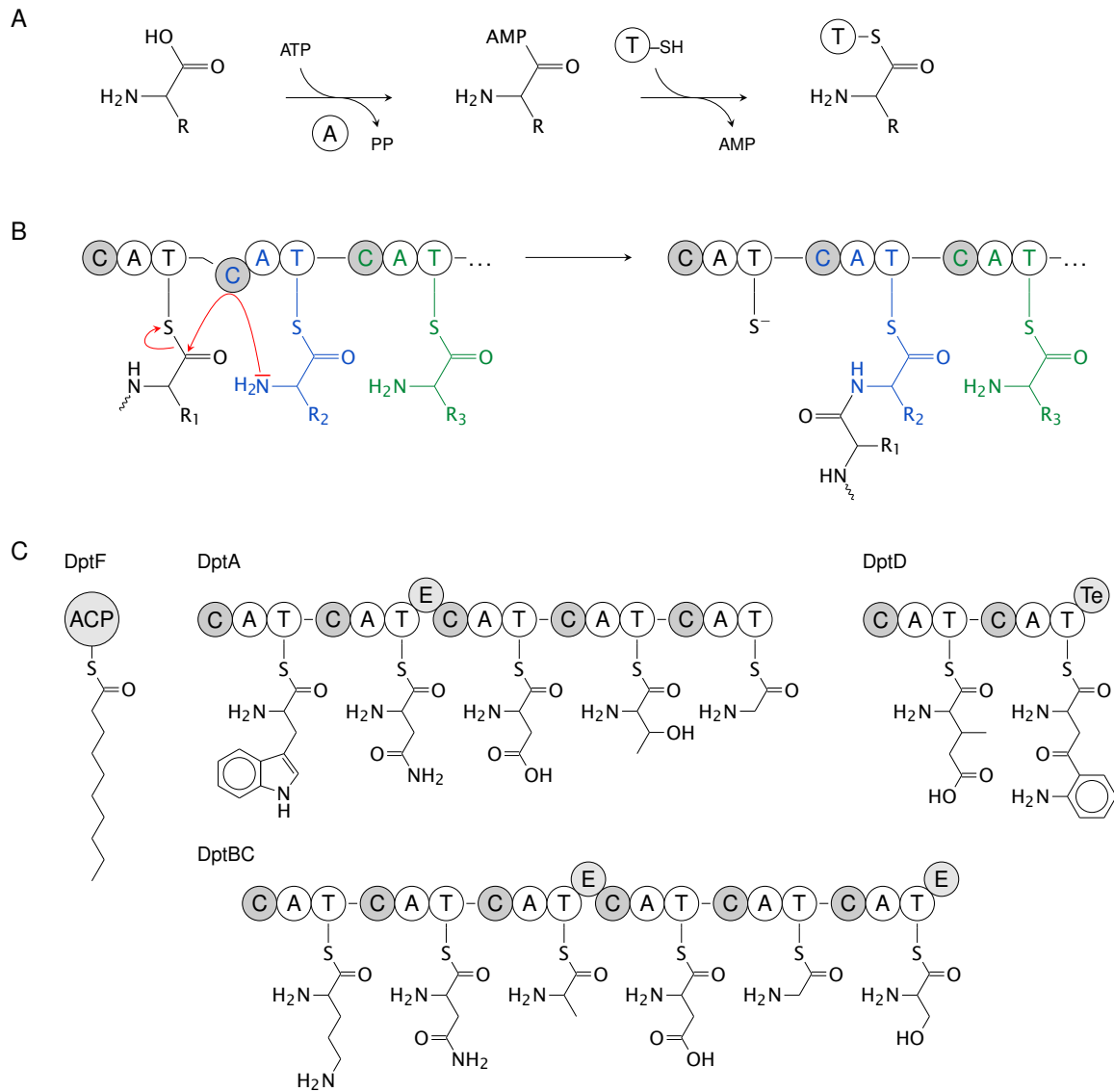


Figure 1.8 Biosynthesis of daptomycin by the Dpt non-ribosomal peptide synthesis system. For each amino acid to be incorporated, the Dpt system contains a module with a C domain, an A domain, a T domain, and an optional E domain, which mediate condensation, activation, thiolation, and epimerization, respectively. **A:** ATP-dependent activation and thiolation of an amino acid by the A and the T domain of the cognate module. **B:** A C domain attaches its cognate amino acid to the C-terminus of the peptide that is linked to preceding T domain. **C:** Organization of the Dpt system. The amino acid-conferring modules are distributed between three proteins (DptA, DptBC, and DptD). The DptF module supplies the activated fatty acid to DptA, where it is attached to the N-terminal tryptophan. The terminal thioesterase (Te) domain of DptD cyclizes and cleaves the lipopeptide.

faecium, however, vancomycin did not [18]. The same authors also observed an inhibition of lipoteichoic acid (LTA) biosynthesis and proposed this pathway as the target of daptomycin [22, 18].

The proposed direct effects on macromolecular synthesis suggested by the studies cited above could not be confirmed in more recent experiments with used concentrations of daptomycin similar to MIC [119]. At low concentrations, daptomycin still causes rapid depolarization of the bacterial cell membrane [125]. Depolarization will interfere with substrate transport, which may cause secondary disruption of macromolecular synthesis. In this context, it is also worth noting that daptomycin is active against bacteria in the stationary phase [92]. Stationary bacteria are not killed by β -lactams, whose established mode of action is to inhibit murein synthesis; non-dividing bacteria do not vigorously synthesize peptidoglycan. The effect of daptomycin on resting bacteria therefore strongly suggests a different mechanism.

As the examples of gramicidin and valinomycin show, membrane depolarization is sufficient for bactericidal action, and thus the observation of depolarization in daptomycin-exposed cells [125] would account for the bactericidal activity of daptomycin and additional effects may contribute. L-forms of *Bacillus subtilis*, which are cell wall-deficient bacteria that can grow and proliferate in osmotically stabilizing media, showed hypersensitivity to daptomycin [145]. This provides solid evidence that the target for daptomycin is the membrane; however, most recently, a new study has suggested that daptomycin may cause the redistribution and functional disruption of membrane-associated enzymes, which are required in cell wall synthesis and cell division, thereby affecting cell wall morphology and septation. This conclusion was based on the colocalization of membrane-bound labeled daptomycin with the essential cell division protein DivIVA, which correlated with cell wall and membrane defects that led to changes in cell morphology [112]. Aberrant septation was also noted using electron microscopy [27]. Of note, that study showed no discontinuities or deformities in the lipid bilayer as such. Daptomycin caused leakage of calcein from the bacterial cells but no entry of the DNA-intercalating fluorescent dye ToPro3.

In combination, the above findings suggest formation of small, discrete membrane lesions by daptomycin. It has been proposed that such membrane lesions consist of daptomycin oligomers that form after calcium-dependent membrane binding and insertion of individual daptomycin molecules (Figure 1.9 [125]). Alternatively, it has been proposed that daptomycin needs to undergo calcium-dependent oligomerization in solution in order to become competent for membrane insertion (Figure 1.10). This hypothesis was based on the observation of daptomycin oligomers or micelles in solution by NMR. This method was also used to characterize the residues involved in the binding of calcium. In solution, daptomycin and calcium bind stoichiometrically [9]. In the apo (calcium-free) structure, the four acidic residues, Asp³, Asp⁷, Asp⁹ and MeGlu¹² are not close enough to form a single Ca²⁺ binding site. Another study [68] suggested that one Ca²⁺ binding site may be formed by Asp³ and Asp⁷; a second Ca²⁺ binding site may be involved in a second conformational change that depends on the presence of negatively charged lipid head groups. However, this second conformational change has not been characterized structurally [68, 61].

Due to the limited sensitivity of the method, the NMR experiments were performed at concentrations far in excess of the MIC. They do therefore not prove that oligomerization or micelle formation in solution indeed occurs at MIC.

Some studies on the antimicrobial activity of derivatives of daptomycin, addressed by determining the minimum inhibitory concentration (MIC) in the presence of calcium, confirmed that the methyl-group in methylglutamate (MeGlu¹²) and the Kyn¹³ residue are vital for activity [51]. To determine whether the acidic residues in daptomycin affect its bactericidal potency, the researchers substituted all acidic side chains in daptomycin to neutral ones—Asp to Asn, and Glu to Gln—and measured the MIC. Substitution of Asp⁷ and Asp⁹ led to complete loss of bioactivity, whereas there were no significant effects for Asp³ and MeGlu¹² on the bactericidal activity, suggesting that only conserved positions of Asp⁷ and Asp⁹ are essential for cation binding [51, 115]. The substitution of MeGlu¹² with Glu in the daptomycin molecule structure led to a 7-fold increase in the MIC compared with the native compound and was in

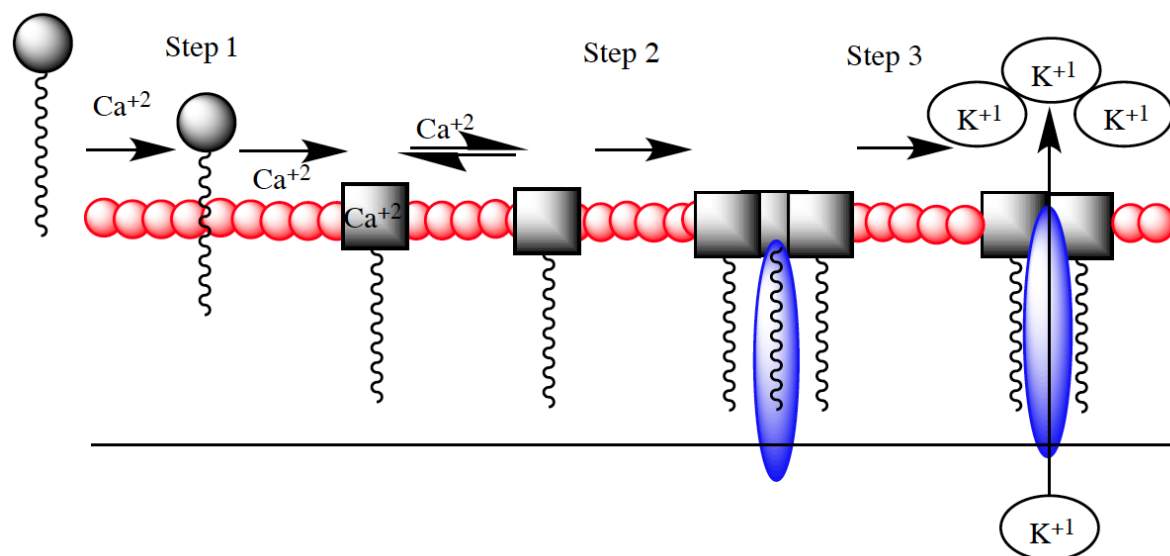


Figure 1.9 Hypothetical mechanism of daptomycin action by membrane depolarization. In the first step, daptomycin binds to the cytoplasmic membrane in a calcium-dependent manner. It then oligomerizes and inserts into the membrane, forming a membrane defect that releases potassium ions and causes rapid cell death [125, 127].

full agreement with the results of Nguyen *et al.*, likewise, the substitution of Kyn¹³ with Trp increased the MIC [105].

Resistance to daptomycin is rarely reported from the Gram-positive bacteria. The only well-characterized mechanism consists in the increased conversion of PG to lysyl-PG, which causes partial resistance [52, 53].

Gram-negative bacteria are not susceptible to daptomycin. For example, daptomycin has a MIC of 128 $\mu\text{g/ml}$ against *E. coli* [104], compared with about 1 $\mu\text{g/ml}$ against Gram-positive bacteria. However, *E. coli* becomes susceptible to daptomycin when the outer membrane is stripped away (J. Silverman, personal communication), indicating that daptomycin does not depend on targets specific to the cytoplasmic membranes of Gram-positive bacteria.

In sum, the evidence suggests an important role for membrane depolarization and permeabilization for the bactericidal effect of daptomycin on both proliferating and resting cells. While the formation of membrane-associated oligomers has been assumed to be involved, no

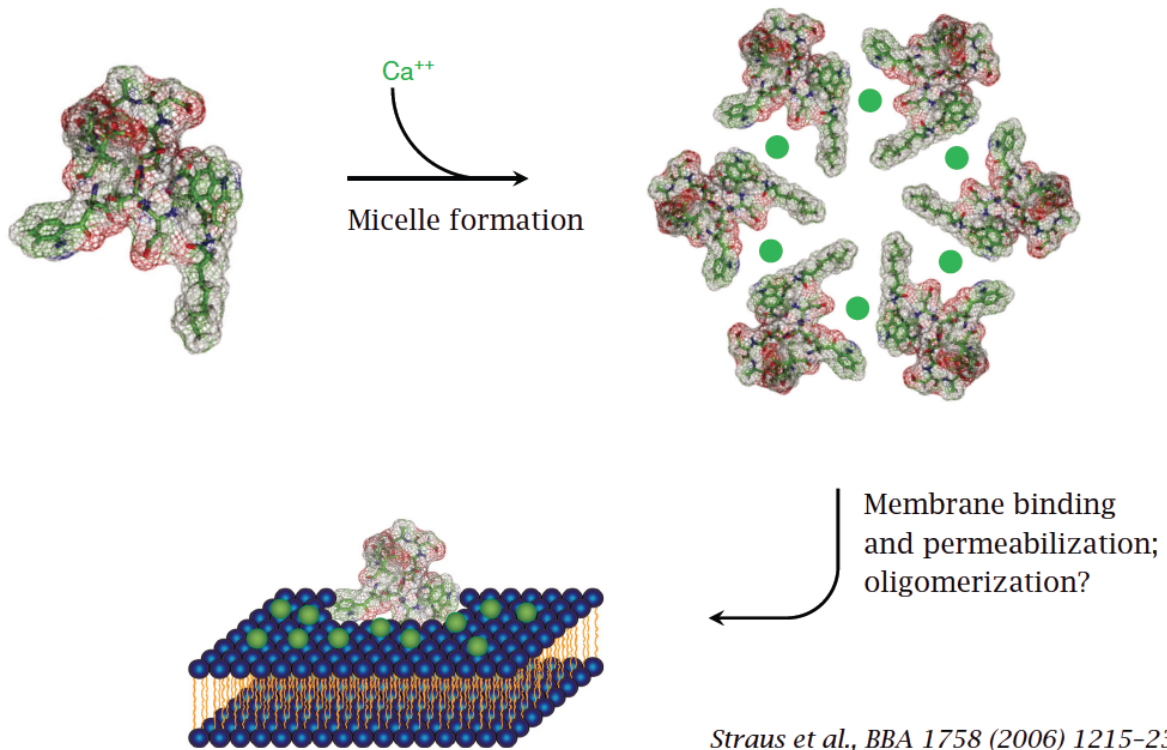


Figure 1.10 Proposed model of the action of daptomycin in solution based on NMR studies: (a) without calcium, daptomycin adopts a structure that is reasonably well defined but not highly amphiphilic. (b) At a molar ratio 1:1 calcium to daptomycin, the lipopeptide aggregates to form a micelle or oligomer with 14–16 subunits. (c) When daptomycin oligomers approach the bacterial membrane, they dissociate, and daptomycin inserts into the bilayer. Finally, bacterial cells are killed by membrane depolarization or some other membrane-associated event [128]. Figure adapted from reference [128].

experimental evidence was available in the literature prior to the work presented in this thesis.

1.2.4 Spectrum of Activity and Clinical Use

Daptomycin is active against a broad spectrum of Gram-positive organisms such as staphylococci and enterococci, irrespective of their resistance or susceptibility to β -lactams and vancomycin; therefore, daptomycin may be used in infections that may otherwise be difficult to treat [129, 27].

In 2003, the US Food and Drug Administration (FDA) approved daptomycin for the treatment of complicated skin and soft-tissue infections caused by various Gram-positive bacteria, making it the first lipopeptide antibiotic to be approved for use in humans. The bactericidal activity of daptomycin is concentration-dependent. The dosage regimen is 4 mg/kg intravenously once per day [7, 3, 30]. Daptomycin has also been used at 6 mg/kg to treat bacteremia due to vancomycin-resistant enterococci and right-sided infective endocarditis caused by *Staphylococcus aureus*, including methicillin-resistant strains (MRSA) [7, 23, 108].

Advantageous characteristics of daptomycin include a long half-life of 8–9 hours [91], which allows for once-daily dosing, as well as excellent antimicrobial activity against bacteria with reduced glycopeptide susceptibility [1, 117]. Trials assessing the efficacy of daptomycin in the treatment of complicated urinary tract infections are ongoing [139, 15]. Dose adjustment is necessary in renal dysfunction to avoid adverse effects. The primary toxicity of daptomycin is reversible dose-related myalgia and muscle weakness.

Pneumonia should not be treated by daptomycin due to the fact that daptomycin is inhibited by pulmonary surfactant [124]. The ability to decrease the inhibition recorded by pulmonary surfactant while maintaining the excellent antibacterial activity and low toxicity of daptomycin, that would potentially offer a clinical candidate with extended indications, including *S. pneumoniae* pneumonia [104]. A54145E, which is similar to daptomycin in containing MeGlu¹² and in its antibacterial activity, is less inhibited by surfactant [104]; however, it is more toxic than daptomycin [28]. These observations have motivated efforts to create hybrids of the two lipopeptides that would combine the favourable traits of both, as discussed in Section 1.2.5.

Bacterial resistance to daptomycin has been reported, although it remains rare [69]. Canton *et al.* demonstrated daptomycin non-susceptibility (defined by the Clinical and Laboratory Standards Institute [CLSI] as an MIC of $\geq 4 \mu\text{g/ml}$ for enterococci [23]) as daptomycin resistance. In general, for tested strains of staphylococci and streptococci, the MIC (minimum inhibitory concentration) inhibiting 90% (MIC₉₀) was from 0.25 to 0.5 $\mu\text{g/ml}$ and from 2 to 4 $\mu\text{g/ml}$ for enterococcal isolates [129]. Isolates of *Streptococcus pyogenes*, *S. agalactiae*, *S. dysgalac-*

tiae subsp. *equisimilis* and *viridans* streptococci are also susceptible to daptomycin *in vitro* [70].

1.2.5 Structural Variation of Daptomycin

The generation of new derivatives of daptomycin is interesting both with respect to improving activity and for elucidating the functional roles of individual amino acid residues. Such variants can be obtained 1) by semisynthesis, 2) through recombinant modification of the peptide synthetase systems, and 3) using chemoenzymatic methods, that is, enzyme-assisted semisynthesis.

Semisynthesis is essentially chemical modification of the native molecule, which can be more or less elaborate. The most straightforward application is the modification of the single free amino group of daptomycin, which is part of its ornithine residue. A large number of N-acylated analogues, even ones carrying substantial substituents such as an extra tryptophan, showed generally fairly well preserved activity, often equivalent to native daptomycin *in vitro* and *in vivo* [59]. However, this approach has so far not resulted in improved variants.

An example of the chemoenzymatic approach is the replacement of the native N-terminally attached fatty acyl residue. Daptomycin belongs to the A21978C complex of acidic lipopeptide antibiotics [10, 104, 73], whose members share the same peptide portion but differ with respect to the fatty acyl residue. This acyl portion displays a significant effect on the antibacterial activity of the A21978C variants [33] and therefore is a logical target for further experimental variation.

Figure 1.11 illustrates the steps of synthesis of acyl variants of daptomycin *in vitro*. In the first step, the ornithine residue is protected with t-Boc. The fatty acid side chains of A21978C can then be removed by incubation with *Actinoplanes utahensis* [33], or with the purified *Actinoplanes utahensis* deacylase enzyme obtained from a recombinant strain of *Streptomyces lividans* [75]. New analogs of A21978C can be synthesized by chemical reacylation of the N-terminus through activation of varying acyl esters and subsequent deprotection [33, 98].

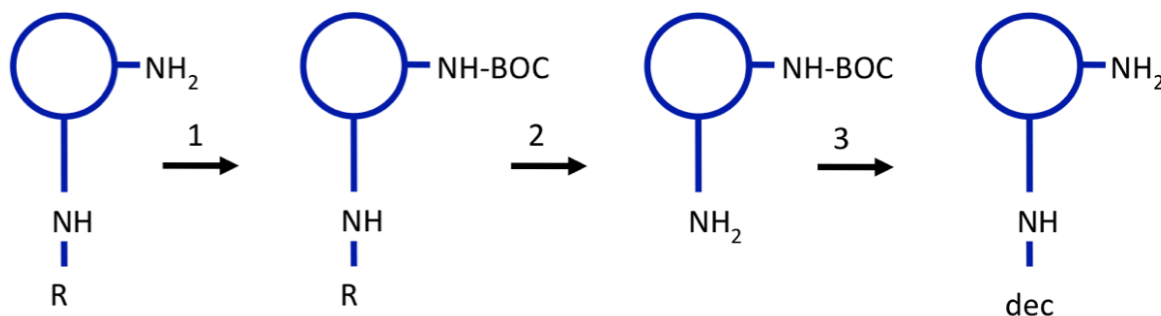


Figure 1.11 Chemoenzymatic synthesis of daptomycin fatty acyl variants. Step (1), protection of side chain amino group of ornithine residue in A27978C complex via tert-BOC. Step (2), enzymatic deacylation with *Actinoplanes utahensis* deacylase. Step (3), reacylation of the free N-terminal amino group with fatty acyl succinimidyl-ester and deprotection.

A series of fatty acyl derivatives were prepared and evaluated for both *in vitro* and *in vivo* against *Staphylococcus aureus* and *Streptococcus pyogenes*; however, none of these compounds, except daptomycin, could pass the clinical trials [98]. Fatty acyl derivatives have also been prepared from A54145.

An application of the chemoenzymatic approach concerns the use of a homologous thioesterase to create cyclic daptomycin variants from linear peptide precursors. The thioesterase (Te) domain is usually part of the C-terminal module in the ultimate peptide synthetase enzyme. It has been shown, however, that the independently expressed TE domain of the Dpt system (see section 1.2.2), or those of homologous non-ribosomal peptide synthetases, can catalyze the cyclization of synthetic linear thioester precursors and of variants thereof. This has been exploited to generate several daptomycin derivatives [73, 51].

Another chemoenzymatic study focused on the creation of daptomycin and A54145 hybrid molecules through enzymatic cyclization of the linear thioesters by the TE domains from A54145 and daptomycin NRPSs [73]. Structure-activity relationship (SAR) studies were carried out with a set of acidic lipopeptide variants of daptomycin, CDA, and A54145, containing an alternative macrolactam. Hybrid molecules including an exocyclic daptomycin peptide and

endocyclic A54145 peptide cores exhibited similar MICs, thus illustrating the potential for constructing structural hybrids[115].

Several studies have created hybrids of daptomycin and A54145 using recombinant DNA techniques. By replacing the entire DptD synthetase (see Section 1.2.2) with the homologous enzymes from the producer strains of A54145 or CDA, hybrid lipopeptides were created that contained Ile¹³ or Trp¹³ instead of *Kyn*¹³ [97, 26, 105]. A subsequent study reported hybrid lipopeptides of daptomycin and A54145 with modifications by exchanging individual amino acid residues at various positions throughout the peptide sequence [104]. The goal of that study was to identify variants that would combine the favourably low toxicity of daptomycin with the resistance to inhibition by lung surfactant displayed by A54145. Reportedly, one variant with the desired profile was indeed obtained, although ultimately none of the variants has so far entered clinical trials.

It is worth noting that, in the latter study, variants differed in biosynthetic yield by up to almost three orders of magnitude. It seems likely that individual modules have evolved toward cooperation with specific neighbor modules, and not all substitutions are therefore tolerated equally well. The recombinant engineering approach therefore is not as general and straightforward as it might appear at first sight.

1.3 RESEARCH OBJECTIVES

As it has been pointed out in the foregoing, that the only target for daptomycin supported by solid evidence is the membrane; there is no unequivocal evidence for any damage to the cell that does not follow from membrane depolarization. Therefore, studies with model membranes seem promising and relevant. I wanted to address the following open questions about the characterization of mechanism of action of daptomycin on artificial and bacterial membranes:

1. Oligomerization of daptomycin molecules on membranes has been postulated, but no experimental evidence had been reported at the outset of this study. Therefore, the decision to determine whether or not oligomer formation occurs on liposomes and on bacterial cells has been made.
2. If daptomycin can oligomerize on membranes, what are the roles of specific lipids in the daptomycin binding and oligomer formation, and how does the lipid composition correlate with daptomycin bactericidal activity?
3. Oligomerization of daptomycin in solution had been shown by NMR at concentrations far above the MIC; therefore, the functional significance of these oligomers is uncertain. We tried to determine whether or not daptomycin forms oligomers in solution at physiological concentrations.

Our studies were conducted using techniques that monitor fluorescence, which is significantly more sensitive than NMR and permits daptomycin molecules to be observed under conditions and concentrations similar to MIC on both model liposomes and bacterial cells. This approach enabled us to gain novel insights into the mode of action of daptomycin.

Chapter 2

Oligomerization of daptomycin on membranes

2.1 INTRODUCTION

Daptomycin is a lipopeptide antibiotic produced by *Streptomyces roseosporus*. It is a cyclic molecule with a decanoyl fatty acid side chain attached to the exocyclic, N-terminal single tryptophan residue [10]. It contains thirteen amino acid residues, some of which are non-standard ones, including a kynurenine and an ornithine (Figure 2.1). Daptomycin possesses rapid bactericidal activity against a broad spectrum of Gram-positive pathogens [133, 132]. It is active against difficult pathogens such as vancomycin-resistant staphylococci and enterococci [83]. Daptomycin is approved for clinical use in the treatment of *Staphylococcus aureus*, including MRSA, in bacteremia, right-sided endocarditis, and complicated skin and skin structure infections.

Most of the results presented in this chapter have been published in: “Oligomerization of daptomycin on membranes”, by Muraih, J.K., Pearson, A., Silverman, J. and Palmer, M. (2011), *Biochim Biophys Acta* 1808:1154-60.

Authors’ contributions: The preparation of Alexa Fluor 350-daptomycin, as described in Section 2.2.2, and the test of its antimicrobial activity were performed by A. Pearson. All other experiments were performed by the thesis author.

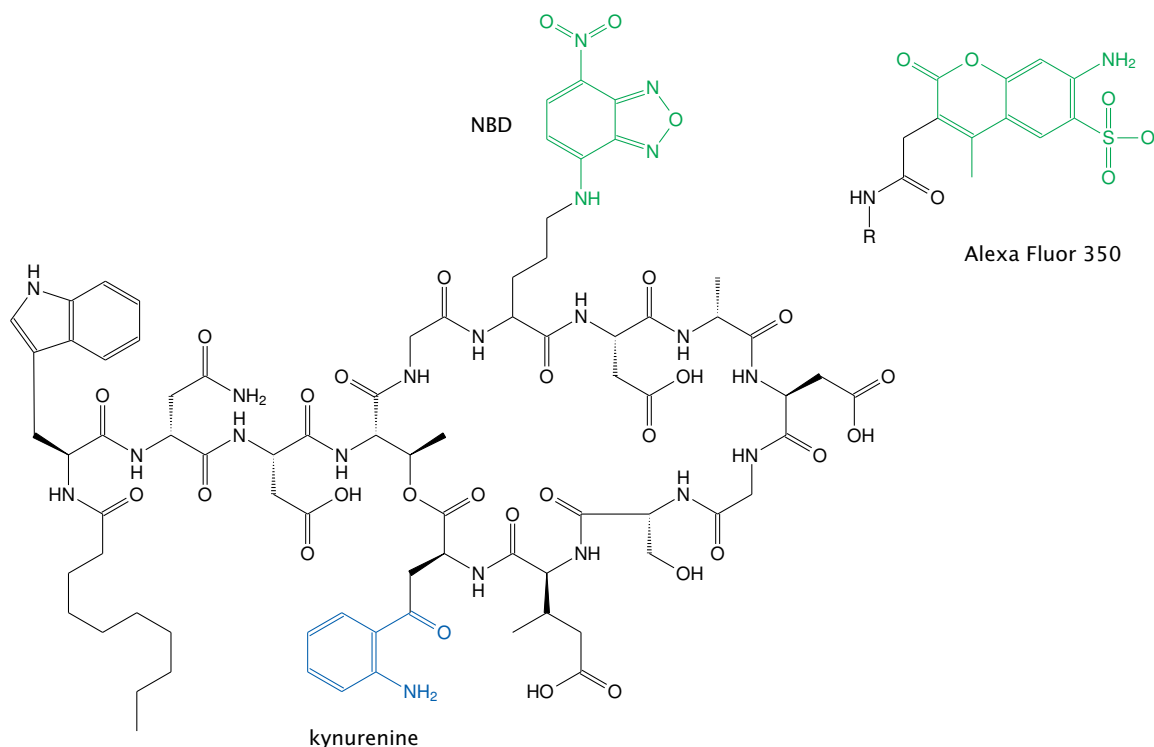


Figure 2.1 Structures of fluorescently labeled daptomycin derivatives used in this study (NBD-daptomycin and Alexa Fluor 350-daptomycin). The decanoyl residue is attached to the exocyclic N-terminal tryptophan residue. The ornithine residue carries the sole free amino group, which is free in daptomycin but labeled with the fluorophores NBD or Alexa Fluor 350 in the two derivatives. The kynurenine residue possesses intrinsic fluorescence.

The bactericidal activity of daptomycin is mediated by membrane permeabilization and depolarization [125]. It is calcium-dependent and applies to both resting and proliferating cells [92]. Susceptibility of bacteria to daptomycin is correlated with the membrane content of phosphatidylglycerol; it is decreased by mutations that promote the conversion of phosphatidylglycerol to lysylphosphatidylglycerol [47]. On bacterial cell membranes, fluorescently labeled daptomycin localizes to phosphatidylglycerol-rich membrane areas [52].

Membrane-permeabilizing proteins and peptides commonly form oligomeric structures on the target membrane. Daptomycin oligomers have been detected in solution at relatively high (0.75 mM) concentrations, and the formation of oligomers prior to membrane binding has been interpreted as an integral step in daptomycin activity [9]. However, there is as yet no

experimental evidence of daptomycin oligomers on the lipid membranes.

In the present study, we have examined the oligomerization of daptomycin both in solution and on membranes. To this end, we used daptomycin derivatives fluorescently labeled with NBD (7-nitro-2,1,3-benzoxadiazol) and with Alexa Fluor 350. Oligomerization was detected through fluorescence resonance energy transfer (FRET) as well as through the self-quenching of NBD fluorescence. We detected oligomerization on liposome membranes containing phosphatidylglycerol, combined with either phosphatidylcholine (PC) or phosphatidylethanolamine (PE) plus cardiolipin (CL), as well as on membrane vesicles prepared from *Bacillus subtilis* (ATCC 6633) cells. In contrast, no oligomerization was observed with liposomes containing phosphatidylcholine alone, nor in solution at daptomycin concentrations similar to those required for antibacterial activity. The requirements that we find for oligomerization correlate with those for membrane permeabilization and bacterial susceptibility [10, 47], suggesting that the membrane lesion is caused by the daptomycin oligomer.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of NBD-daptomycin

Unlabeled daptomycin was supplied by Cubist Pharmaceuticals Inc. (Lexington, MA, USA). NBD-Cl (4-Chloro,7-nitro-2,1,3-benzoxadiazol, or 7-chloro-4-nitrobenzofurazan) was bought from Fluka (Fluka, St. Louis, MO, USA). All other chemicals were obtained from Bioshop (Burlington, ON) and were of analytical grade. Daptomycin (0.6 mM) was dissolved in 50 mM sodium borate buffer (pH 8) containing 20 mM EDTA. NBD-Cl (25 mM) was dissolved in acetonitrile. 300 μ l of daptomycin solution and 100 μ l of NDB-Cl solution were mixed in a reaction vial and incubated at 60 °C for 2h. The mixture was then cooled in an ice water bath for 2 minutes, and finally acidified by adding 400 μ l of acetic acid (50 mM) to stop the labeling reaction.

An HPLC system (Waters 625 LC) was used to purify the labeled daptomycin on a reversed-phase column (Agilent, Eclipse XDB-C8, 150 x 4.6 mm). The mobile phase was 20 mM ammonium acetate (pH 5.5) with an acetonitrile gradient from 30 to 40% and a flow rate of 1 mL/min [90, 134]. Fractions were examined by spectrophotometry, spectrofluorometry (see below), and mass spectrometry using a Micromass Q-TOF Ultima GLOBAL mass spectrometer. The molecular weight of the purified NBD-labeled daptomycin determined by mass-spectrometry was 1783.7 Da. This corresponds to the expected molecular weight of 1784 Da for the stoichiometric adduct (Figure 2.1). To determine the concentration, we assumed a molar extinction coefficient at 476 nm of 24,000 as previously determined for the NBD-adduct of alanine [79].

2.2.2 Preparation of Alexa Fluor 350-daptomycin

Daptomycin trifluoroacetate salt (20.34 mg, 0.013 mmol) was dissolved in DMF at 0 °C. Alexa Fluor-350 succinimide (Molecular Probes, 5 mg, 0.013 mmol) was added and stirred for 5 min. Hünig's base (N,N-diisopropylethylamine) was added and the reaction mixture was stirred for 60 min. The reaction solution was poured into a flask containing cold diethylether (35 mL), which led to precipitation. The precipitate was collected by centrifugation, washed with diethylether and again recovered by centrifugation. The material was dried under high vacuum and then purified via HPLC.

2.2.3 Preparation of large unilamellar vesicles (LUV) and of bacterial membrane vesicles

All phospholipid compounds—1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-[phospho-(1-glycerol)] (DMPG), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoethanol (POPE), 1,2-dioleoyl-sn-glycerol-3-phospho-(1-rac-glycerol) (DOPG), and 1,3-bis[1,2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (cardiolipin; TOCL)—were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

The required amounts of DMPC and DMPG, or of POPE, DOPG and cardiolipin, were weighed, dissolved in chloroform and transferred to a round-bottom flask. The solvent was evaporated with nitrogen and the resulting lipid films further dried under vacuum for three hours. The lipids were then suspended in HEPES buffer (20 mM, pH 7.4) containing 150 mM sodium chloride. Finally, the suspension was extruded 10-15 times through 100 nm polycarbonate filters, using a nitrogen-pressurized extrusion device [94]. The liposomes were employed in the fluorescence experiments at a final concentration of 250 μM of lipid.

Bacterial membranes were prepared from *Bacillus subtilis*, grown in LB broth, according to the procedure described by Konings et al. [72], except that the Potter homogenization was omitted, and the membranes were treated with a probe sonicator on ice for 2 minutes instead. Again as in the same reference [72], the membrane vesicles obtained were quantitated by assaying for membrane protein using the Lowry method.

2.2.4 Antibacterial activity tests for daptomycin and NBD-daptomycin

2.2.4.1 Disc diffusion. A disk diffusion test was used primarily to determine antibacterial activity of unlabeled daptomycin and its derivative NBD-daptomycin. Petri dishes containing LB-Agar, supplemented with calcium (5 mM), were streaked with a liquid culture of *Bacillus subtilis*. Paper disks were cut from Whatman grade 1 filter paper with a hole punch, soaked in daptomycin or NBD-daptomycin dissolved at various concentrations, blotted dry on a sheet of filter paper, and placed on top of the inoculated agar plates. The plates were incubated overnight at 37 °C, and the zones of inhibition measured with a ruler.

2.2.4.2 Broth dilution. A broth dilution is more accurate than the disc diffusion. NBD-daptomycin and unlabeled daptomycin were tested for antibacterial activity by broth dilution. LB broth was supplemented with calcium (5 mM) and daptomycin and NBD-daptomycin, respectively, at concentrations of 10, 5, 3, 2, 1.5, and 1 $\mu\text{g/ml}$. Growth controls without antibiotic were also included. Each tube was inoculated with *Bacillus subtilis* ATCC 6633 and incubated overnight at 37 °C. Growth inhibition was evaluated visually by turbidity.

2.2.5 Fluorescence studies

Emission spectra and time-based scans were acquired on a QuantaMaster 4 spectrofluorimeter (Photon Technology International, London, ON). Kynurenine fluorescence was excited at 365 nm and the emission recorded from 400 to 600 nm. NBD fluorescence was excited at 465 nm and the emission recorded from 490 to 600 nm. Alexa Fluor 350 was excited at 350 nm and emission recorded from 400 to 600 nm. Bandpasses for excitation and emission were between 2 and 5 nm. The steady state spectra shown were acquired at 37 °C; spectra acquired at room temperature were virtually indistinguishable.

The concentrations of calcium, where present, were 200 mM with PC liposomes and 5 mM in all other cases. For the calculation of the Förster radius [43] for kynurenine and NBD on PC/PG membranes, the quantum yield of kynurenine (0.039) was obtained by comparison to a quinine sulfate standard. The spectral overlap integral for donor emission and acceptor absorbance was determined from the absorption spectrum of NBD-daptomycin (see Figure 2.2) and the emission spectrum of unlabeled daptomycin on DMPC/DMPG membranes in the presence of calcium, with correction for the wavelength-dependent sensitivity of the spectrofluorimeter. For the orientation factor κ^2 , a value of $2/3$ was used, which applies in the commonly assumed case of randomly distributed orientations of donor and acceptor [43].

Fluorescence lifetime measurements were performed on a FluoTime 100 Lifetime spectrometer using a P-C-370 diode laser light source (PicoQuant, Berlin, Germany). The lifetime instrument did not have a thermostatted cell holder, and therefore lifetime measurements were performed at room temperature. Kynurenine emission was isolated using a 460 ± 10 nm band pass filter (Melles-Griot, Brossard, QC). Experimental decays were numerically fit with three exponentials, with resulting χ^2 values typically below 1.2. From these three components, average lifetimes were calculated according to the equation:

$$\langle \tau \rangle = \frac{\sum \alpha_i \tau_i}{\sum \alpha_i} \quad (2.1)$$

where α_i represents the amplitude at time zero and τ_i the lifetime of the i^{th} component.

2.2.6 Numerical fitting

Numerical fits were performed with Gnuplot. The effect of Ca^{++} on the fluorescence of daptomycin and NBD-daptomycin on membranes (Figure 2.4) was fit with the Hill equation, as follows:

$$\text{Relative fluorescence increase} = \frac{[\text{Ca}^{2+}]^n}{[\text{Ca}^{2+}]^n + K^n} \quad (2.2)$$

with K and n as variable parameters. The kinetics of the decrease of daptomycin fluorescence in the presence of EDTA (Figure 2.8) was fit with a triple-exponential model, as follows:

$$\text{Fluorescence intensity} = \text{baseline} + a e^{-\frac{t}{\tau_a}} + b e^{-\frac{t}{\tau_b}} + c e^{-\frac{t}{\tau_c}} \quad (2.3)$$

with *baseline* and all pre-exponentials (a , b , c) and lifetimes (τ_a , τ_b , τ_c) as variable parameters.

2.3 RESULTS

2.3.1 Characterization of NBD-daptomycin

The absorption and emission spectra of purified NBD-daptomycin are shown in Figure 2.2C. The main absorbance peak of NBD-daptomycin occupies a very similar spectral position as the kynurenine fluorescence emission peak of unlabeled daptomycin (Figure 2.2A). This spectral overlap indicates that fluorescence energy transfer (FRET) will occur from kynurenine to NBD. Indeed, when the kynurenine residue in NBD-daptomycin is excited (at 365 nm), the only fluorescence peak observed is that typical of NBD, indicating that virtually all photons absorbed by kynurenine are transferred to NBD through FRET.

The antibacterial activity against *Bacillus subtilis* of NBD-daptomycin was identical to that of unmodified daptomycin (MIC = 3 $\mu\text{g/ml}$). Alexa Fluor 350-daptomycin was found to be 8-16 fold less active than unlabeled daptomycin in standard broth dilution assays. In a disk diffusion test for activity also against *Bacillus subtilis*, NBD-daptomycin at 81 $\mu\text{g/ml}$ produced a zone of inhibition similar to that of unlabeled daptomycin at 100 $\mu\text{g/ml}$ (16 mm across).

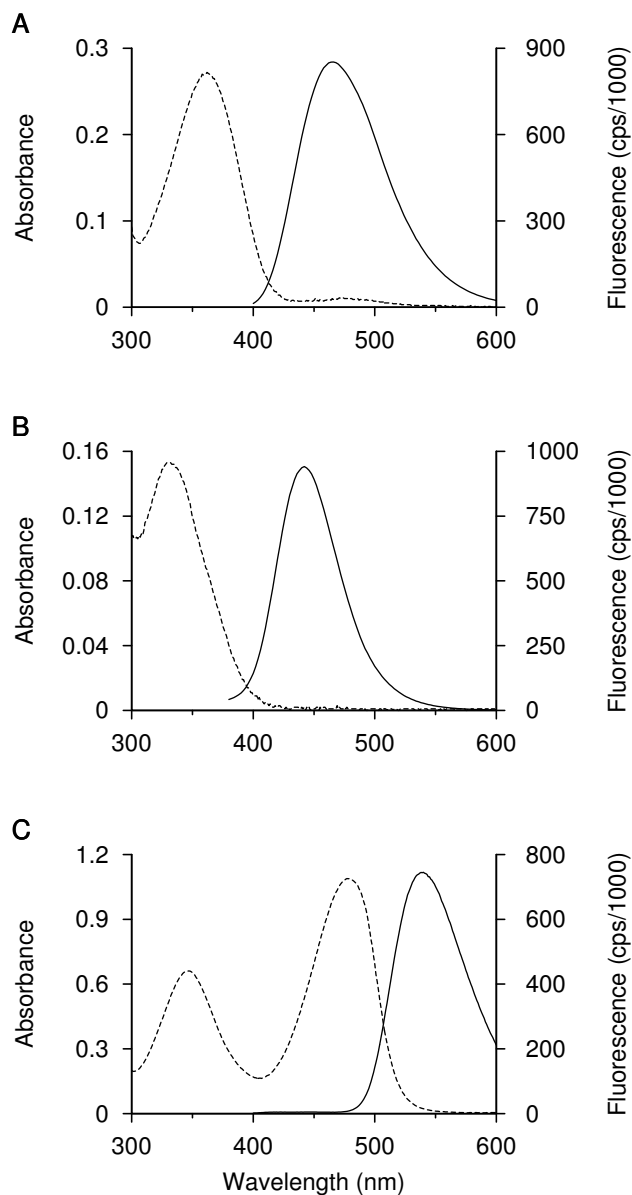


Figure 2.2 Absorbance (dashed lines) and fluorescence emission (solid lines) spectra of (A) daptomycin, (B) Alexa Fluor 350-daptomycin and (C) NBD-daptomycin. The kynurenine residue in daptomycin causes the absorbance peak around 360 nm and the emission peak at 460 nm. The absorbance and emission peaks of Alexa Fluor 350-daptomycin occur in similar positions. NBD-daptomycin has an absorbance peak at 475 nm that overlaps the emission peaks of the other two; this overlap causes fluorescence resonance energy transfer (FRET) from daptomycin and Alexa Fluor 350-daptomycin to NBD-daptomycin. Direct excitation at 475 nm allows the observation of NBD fluorescence alone.

The results indicate that NBD-daptomycin retains antibacterial activity very similar to that of unmodified daptomycin, and therefore that it should be a valid model to study the interaction of daptomycin with membranes.

2.3.2 Calcium-dependent membrane interaction of daptomycin and NBD-daptomycin

It has previously been reported that the kynurenine fluorescence of daptomycin increases in intensity and undergoes a blue shift upon interaction with calcium and negatively charged phospholipid membranes, composed of phosphatidylcholine and phosphatidylglycerol [77]. Such changes are observed with many fluorophores upon transition from a more polar environment to a less polar one, such as the hydrophobic interior of a lipid bilayer. A similar change in the fluorescence signal also occurs on neutral (PC) membranes (Figure 2.3A). However, the gain in intensity is less, and the position of the fluorescence peak is different from that observed on PC/PG membranes, suggesting a different environment of the kynurenine residue and, hence, a different conformation of the daptomycin molecule [68, 77]. Also note that with PC alone the fluorescence change occurs only at a far higher concentration of calcium.

NBD-daptomycin also gains in fluorescence intensity in the presence of membranes and calcium (Figure 2.3B). Here the fluorescence gain appears larger on PC membranes than on PC/PG membranes, although the opposite is actually true after NBD self-quenching on PC/PG membranes is accounted for (compare Figure 2.6, below). The spectral difference indicates that the labeled residue (ornithine), like the kynurenine residue, senses different environments on the two types of membranes.

Figure 2.4 shows the response of the kynurenine fluorescence of daptomycin and of the NBD fluorescence of NBD-daptomycin, respectively, to increasing Ca^{2+} concentrations, on DMPC/DMPG membranes and DMPC membranes. On both membranes, the NBD fluorescence and the kynurenine fluorescence respond at similar calcium concentrations, further suggesting that labeling with NBD does not significantly perturb the function of daptomycin.

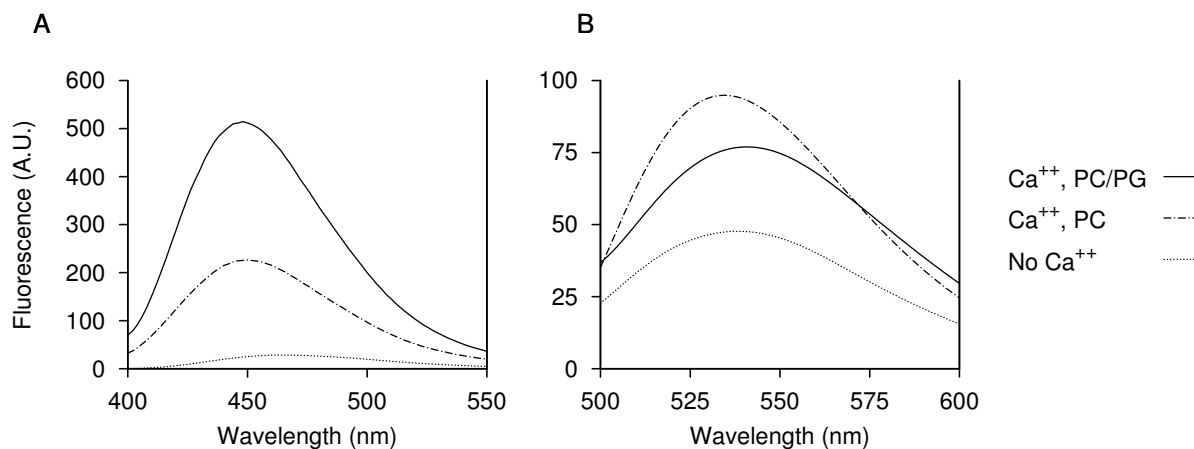


Figure 2.3 Fluorescence emission spectra of daptomycin ($4.8 \mu\text{M}$) (A), and of NBD-daptomycin ($0.91 \mu\text{M}$) on liposome membranes (B), in the absence and presence of calcium. The liposomes consisted of phosphatidylcholine and phosphatidylglycerol (DMPC/DMPG) or phosphatidylcholine only (DMPC) at saturating calcium concentrations. The excitation wavelength was 365 nm with native daptomycin and 465 nm with NBD-daptomycin. The two samples without calcium are normalized to the same maximum intensity and represented as dotted lines; the two samples containing calcium on PC/PG (solid lines) and PC only (dashed lines) were scaled accordingly, so that the relative gains in intensity upon addition of calcium and lipids can be compared.

2.3.3 Detection of daptomycin oligomerization on membranes by FRET

The efficiency of FRET is distance-dependent, and it can approach 100% if the distance between the donor and an acceptor is well below the Förster radius (R_0). For kynurenine in unlabeled daptomycin as the donor, bound to DMPC/DMPG membranes, and NBD-daptomycin as the acceptor, we estimated R_0 as 2.7 nm (see Methods section). Since the daptomycin molecule is smaller than that, formation of hybrid oligomers from daptomycin and NBD-daptomycin should cause FRET from the unlabeled to the labeled species. However, some degree of FRET should also occur without oligomerization, due to the proximity of some donors and acceptors when both are randomly distributed in the membrane plane [144].

To distinguish between these two effects, we measured FRET at different ratios of daptomycin to NBD-daptomycin, while keeping the absolute concentration of NBD-daptomycin constant. The rationale of this experiment is as follows: If donors (daptomycin) and acceptors

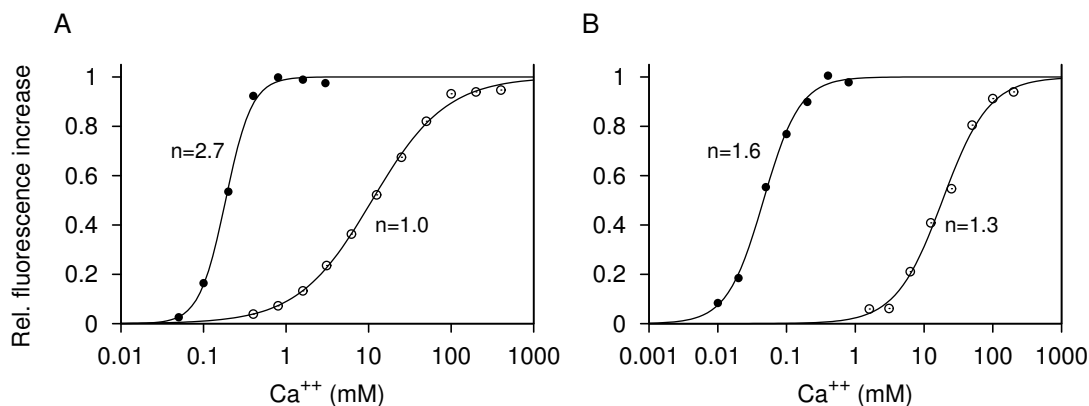


Figure 2.4 Increase in fluorescence intensity of kynurenine of daptomycin (A) and of NBD of NBD-daptomycin (B) in response to increasing calcium concentrations, incubated with DMPC/DMPG membranes (filled circles) and with PC membranes (empty circles), respectively. The solid lines are least squares fits to the Hill equation; the respective fitted values of the Hill coefficient (n) are stated next to each curve.

(NBD-daptomycin) remain monomeric and separate, the efficiency of FRET, as measured by the reduction of donor emission, varies only with the acceptor concentration but not the donor concentration [144]; hence, the extent of FRET should not vary between the samples in our experiment. On the other hand, if donor and acceptor form hybrid oligomers, the probability for a donor to end up within close proximity to an acceptor molecule will increase with the relative amount of acceptors present. Therefore, at a fixed concentration of NBD-daptomycin, the efficiency of FRET should increase with decreasing concentration of unlabeled daptomycin.

FRET can be observed both with time-resolved (Figure 2.5A) and with steady-state fluorescence measurements (Figure 2.5B). In the time-resolved experiment, an increase in FRET corresponds to a decrease in the donor's excited state lifetime. Figure 2.5A shows that, on DMPC membranes, the lifetime is almost the same at donor-acceptor ratios of 16:1 and 1:1, indicating no change in FRET and therefore no oligomerization. In contrast, on PC/PG membranes, the same change in the donor to acceptor ratio causes a fourfold reduction in the kynurenine lifetime, indicating that oligomerization occurs on these membranes.

Also shown for comparison are the lifetimes of unlabeled daptomycin alone. On both PC

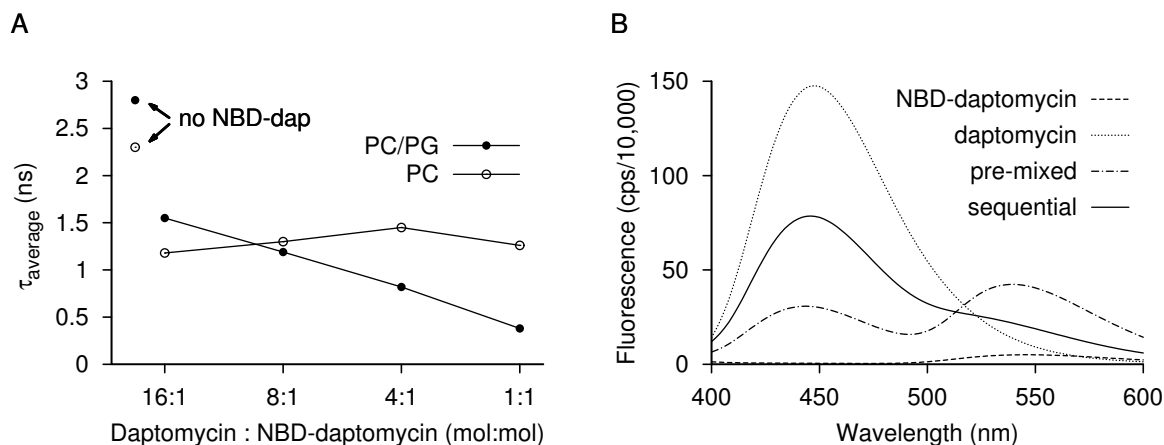


Figure 2.5 FRET experiments on the oligomerization of daptomycin. **A:** Kynurenine fluorescence lifetimes in mixtures of daptomycin and NBD-daptomycin, on PC/PG and PC membranes with saturating concentrations of calcium. The concentration of NBD-daptomycin was $1.36 \mu\text{M}$, where present; the concentration of unlabeled daptomycin is implied by the indicated molar ratios. The two unconnected data points were obtained with unlabeled daptomycin only. **B:** Fluorescence emission spectra of daptomycin ($4.8 \mu\text{M}$), and of NBD-daptomycin ($0.91 \mu\text{M}$), either alone or together, on DMPC/DMPG membranes. In the premixed sample, daptomycin and NBD-daptomycin were mixed before addition of membranes and calcium. In the sequential sample, daptomycin and calcium were added to the membranes and incubated for 30 minutes before application of NBD-daptomycin.

and PC/PG membranes, the kynurenine lifetime is significantly longer than with those samples that contain the equivalent amount of donor but with NBD-daptomycin present. This difference reflects FRET between molecules that are not part of the same oligomers, and thus is not an indication of oligomerization.

In steady-state fluorescence spectra (Figure 2.5B), the kynurenine peak was much lower with mixtures of daptomycin and NBD-daptomycin than with daptomycin only, indicating extensive FRET. This FRET should occur in part within oligomers and in part between oligomers. When daptomycin and NBD-daptomycin were applied to PC/PG membranes sequentially, with time allowed for the oligomerization of NBD-daptomycin before addition of the daptomycin, the drop in donor intensity was reduced by half. The most likely interpretation is that in this sample the labeled and the unlabeled molecules had formed separate oligomers, thus removing the component of FRET that occurred within hybrid oligomers, and leaving only the compo-

ment between separate oligomers. The spectra of such samples did not change substantially over two hours, indicating that most oligomers remained intact over this length of time.

2.3.4 Detection of oligomerization by NBD self-quenching; oligomerization on bacterial membranes

Like many other fluorophores, NBD undergoes self-quenching at high concentration, which likely involves FRET from monomeric NBD molecules to non-fluorescent aggregated pairs [21]. In oligomers that consist of NBD-daptomycin only, the local concentration of NBD groups, and thus the likelihood of aggregation will be higher than in mixed oligomers with unlabeled daptomycin. Therefore, the fluorescence signal of a certain given amount of NBD-daptomycin—after direct excitation at 465 nm, eliminating any FRET from kynurenine—should increase in the presence of unlabeled daptomycin. On the other hand, without oligomerization, the extent of self-quenching should be unaffected by the presence of unlabeled daptomycin, and therefore the NBD-fluorescence should remain unchanged. Therefore, self-quenching of NBD-daptomycin should also be a viable method for detecting daptomycin oligomerization.

These expectations are borne out by observations on PC/PG membranes and PC membranes, respectively. Addition of unlabeled daptomycin relieves self-quenching of NBD-daptomycin on PG/PC membranes (Figure 2.6A), while no such effect occurs with PC alone (Figure 2.6B). These observations confirm that oligomerization occurs only in the presence of PG.

The composition of *Bacillus cereus* and *Bacillus subtilis* cell membranes has been modeled with liposomes consisting of cardiolipin (TOCL, 17%), phosphatidylglycerol (DOPG, 40%) and phosphatidylethanolamine (POPE, 43%) [39, 86]. On such liposome membranes, relief of NBD self-quenching by addition of unlabeled daptomycin is again observed (Figure 2.6C). The same applies to membrane vesicles prepared from *Bacillus subtilis* bacterial cells (Figure 2.6D). Therefore, daptomycin oligomers form on bacterial cell membranes.

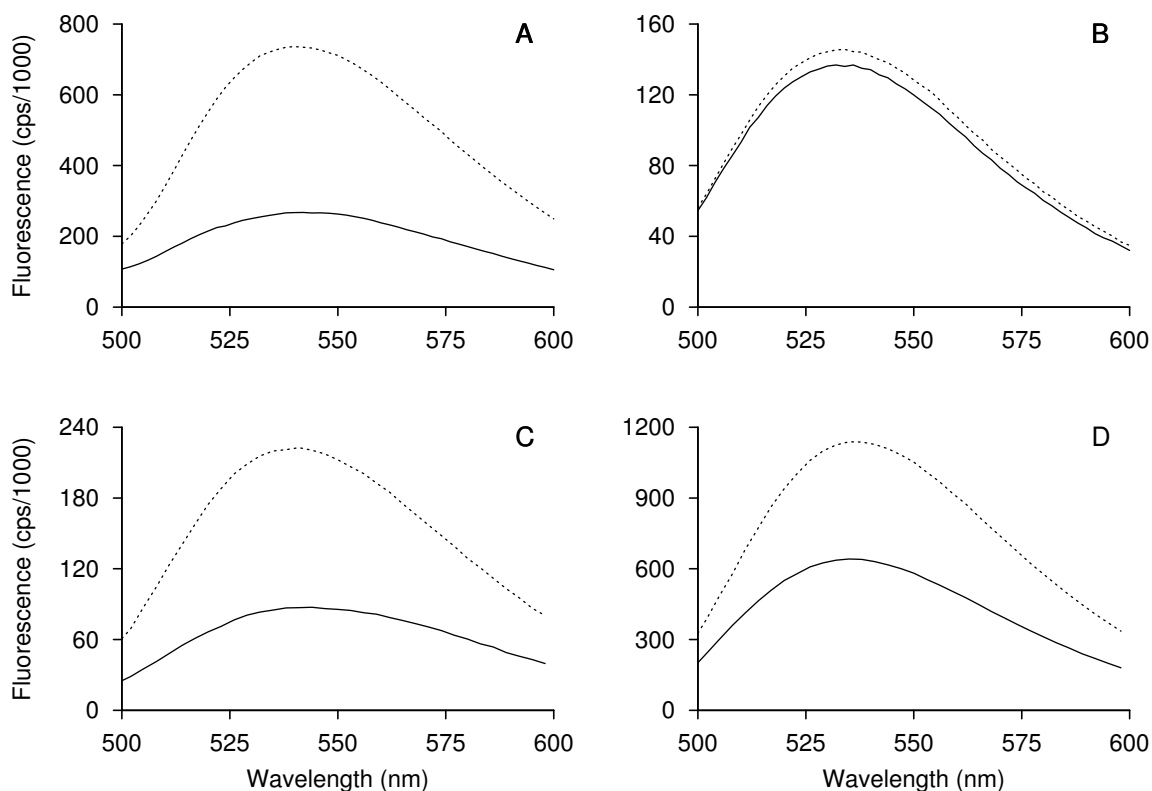


Figure 2.6 Fluorescence of NBD-daptomycin ($0.91 \mu\text{M}$) on (A) DMPC/DMPG liposomes, (B) PC liposomes, (C) POPE/DOPG/TOCL liposomes and (D) *Bacillus subtilis* membrane vesicles, without (solid lines) or with (dashed lines) a fourfold excess with unlabeled daptomycin. Since the excitation wavelength (465 nm) was outside the absorbance spectrum of kynurenine, differences in intensity are not due to FRET from kynurenine but to NBD self-quenching within oligomers.

2.3.5 Daptomycin does not form oligomers in solution at antimicrobially active concentrations

It has been previously reported that daptomycin forms oligomers in solution in the presence of calcium. This was observed by both NMR [9, 68] and ultracentrifugation [61].

In both of the previous studies [9, 68], the concentration of daptomycin was in the millimolar range. Fluorescence allows the observation of oligomerization at much lower concentrations, similar to those required for antimicrobial activity. Because kynurenine has a very low fluorescence intensity in aqueous solution, we here used daptomycin labeled with Alexa Fluor

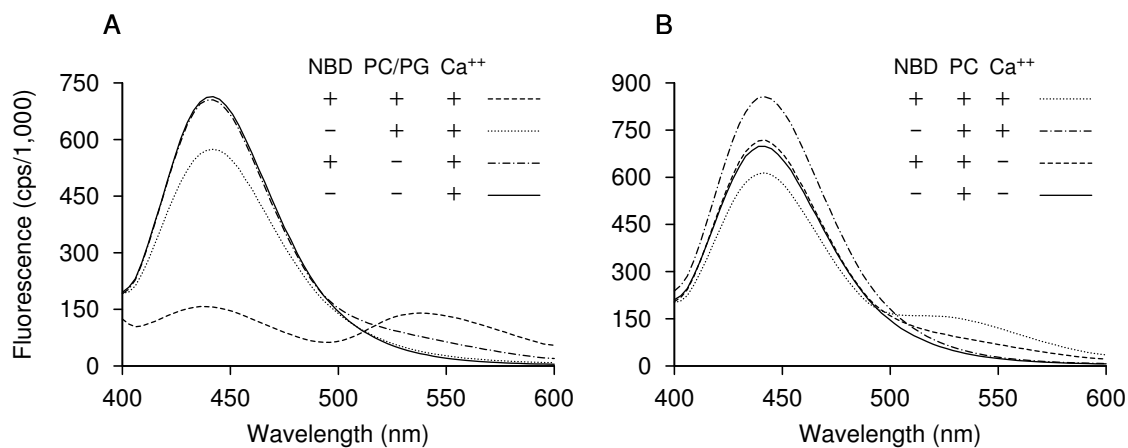


Figure 2.7 Requirements for daptomycin membrane binding and oligomerization. A: Alexa Fluor 350-daptomycin ($0.175 \mu\text{M}$; A) was incubated with Ca^{2+} (3 mM), with or without NBD-daptomycin ($0.88 \mu\text{M}$; N) and with or without PC/PG membranes ($125 \mu\text{M}$; M). The two labeled species readily form mixed oligomers on PC/PG membranes, as evident from the suppression of Alexa Fluor fluorescence through FRET. Without membranes, no FRET is observed, indicating absence of oligomerization. B: Alexa Fluor 350-daptomycin (A) was incubated with PC membranes (M), with or without NBD-daptomycin (N) and with or without Ca^{2+} (200 mM ; all other concentrations as in panel A). In the presence of both PC membranes and calcium, Alexa Fluor 350-daptomycin undergoes an increase in fluorescence intensity, which is likely due in part to kynurenine. In contrast, with the two labeled species, the emission at 450 nm is diminished through FRET. Since daptomycin does not oligomerize on PC membranes, this FRET is due to membrane binding alone. The absence of FRET in the sample with NBD-daptomycin but without calcium indicates absence of membrane binding.

350, which has a high intensity in both polar and apolar environments. The spectral positions of excitation and emission of Alexa Fluor 350 are such that this labeled derivative can again be combined with NBD-daptomycin in FRET experiments [110].

Figure 2.7A shows the interaction of Alexa Fluor 350-daptomycin and NBD-daptomycin in solution and on PC/PG membranes, in the presence of calcium. On the membranes, Alexa fluorescence is strongly reduced by FRET to NBD, which is consistent with oligomerization. In contrast, no FRET occurs when the membranes are missing. This indicates that no oligomerization is happening; calcium-mediated oligomerization in solution therefore is restricted to much higher concentrations and not relevant to the antimicrobial effect of daptomycin. As

an aside, it is evident that without NBD-daptomycin, the fluorescence of Alexa Fluor 350-daptomycin on membranes is still somewhat lower than without membranes; this suggests that Alexa Fluor 350 is also subject to some degree of self-quenching upon oligomerization.

Figure 2.7B shows the interaction of Alexa Fluor 350-daptomycin and NBD-daptomycin on PC membranes, with and without calcium. With calcium, there is FRET, albeit less than on PC/PG membranes. Since no oligomerization occurs on PC, this FRET is due to membrane binding of both Alexa Fluor 350-daptomycin and NBD-daptomycin; membrane binding brings them much closer to each other than they are in solution. No FRET occurs with PC but without calcium, indicating that calcium is required for membrane binding.

2.3.6 Effect of EDTA on membrane-bound daptomycin oligomers

Calcium induces changes of daptomycin fluorescence and presumably of conformation on both neutral and negatively charged membranes; judging by both fluorescence and ability to oligomerize, these conformations are different. Calcium also commonly forms bridges between negative charges on proteins and phospholipid membranes; examples are annexins and coagulation factors that bind to phosphatidylserine in a calcium-dependent manner. Taken together, these observations suggest that there may be different types of calcium binding sites in the daptomycin/PC/PG system, some or all of which may involve PG. If so, the kinetic rates of Ca^{2+} dissociating from these sites would likely be different, which in turn might be reflected in the time course of fluorescence changes triggered by calcium depletion.

Figure 2.8 shows the time course of the kynurenine fluorescence of daptomycin on PC/PG membranes, following the addition of EDTA at time zero. The decrease in fluorescence is evidently not single-exponential. It can be fit with three exponential components, which comprise two short lifetimes (48 and 140 seconds, respectively) and one that is much longer (3351 seconds). This suggests the existence of at least two different classes of calcium binding sites. On PC membranes, the fluorescence dropped to a low, stable level within a few seconds of EDTA addition (data not shown). Together, these results suggest that the negative charge of

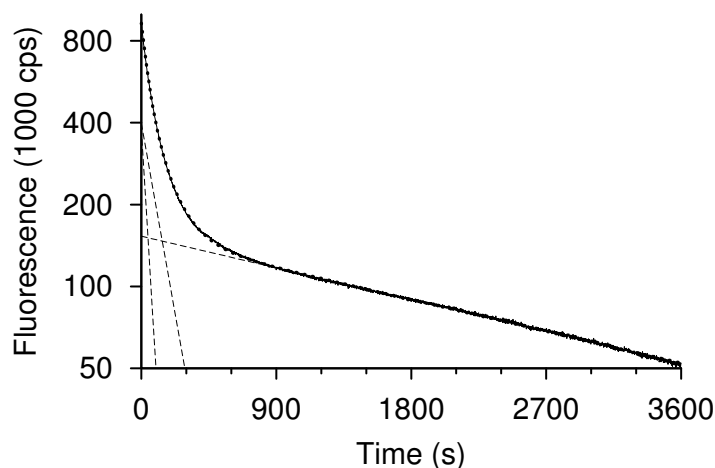


Figure 2.8 Time course of kynurenine fluorescence of daptomycin incubated with 6 mM Ca^{2+} and PC/PG membranes, after addition of 10 mM EDTA at 0 seconds. The dotted line superimposed to the experimental data points is the sum of three fitted exponential components that are represented by straight dashed lines. The time constants of these components are 48, 140 and 3351 seconds, respectively. A baseline of 240,000 cps that also resulted from the fit was subtracted from the experimental curve before plotting.

PG is involved in the slowly dissociating calcium binding sites, and possibly also the faster dissociating ones observed on PC/PG membranes.

If the distinction between fast-dissociating and slow-dissociating Ca^{2+} binding sites is valid, it is conceivable that one but not the other is necessary for oligomerization. When EDTA was added to a sample of daptomycin and NBD-daptomycin, sequentially incubated with PC/PG membranes as in (Figure 2.5B), and Ca^{2+} was then replenished after 180 seconds, the kynurenine fluorescence rebounded but to a much reduced level, whereas the NBD fluorescence was increased. This suggests that withdrawal of Ca^{2+} from the fast-dissociating sites suffices to disrupt the initially segregated daptomycin and NBD-daptomycin oligomers; the released monomers then mingle and reassemble into hybrid ones that show an increased level of FRET. Ca^{2+} bound to the fast-dissociating sites therefore appears to be required for oligomer stability.

2.4 DISCUSSION

While it has been proposed previously that daptomycin forms oligomers on membranes, this study provides the first such experimental evidence. Oligomerization on membranes correlates with the presence of phosphatidylglycerol, which coincides with the involvement of phosphatidylglycerol in bacterial susceptibility to daptomycin [47] and suggests that the daptomycin oligomer is the functional membrane lesion.

Our findings can be summarized in the model of daptomycin activity shown in Figure 2.9. In this model, binding of calcium to daptomycin causes a conformational change that in turn triggers membrane binding, or, at sufficiently high concentration, oligomerization in solution [68]. Membrane-bound daptomycin then interacts with phosphatidylglycerol, resulting in a second conformational transition that leads to the formation of the oligomer. We propose that the oligomer forms the functional membrane lesion, possibly enclosing a central aperture as is the case with many pore-forming proteins and peptides, although differences in the rate of daptomycin-induced membrane depolarization [125] and the absence of non-specific membrane permeabilization [27] suggest that a classic pore may not be formed. Daptomycin oligomerization was inferred here for PC/PG membranes from FRET experiments. This is further corroborated by the highly cooperative change of kynurenine fluorescence as a function of Ca^{+2} concentration on PC/PG membranes, which is characterized by a Hill coefficient of 2.7 (Figure 2.4). Conversely, the Hill coefficient of 1 on PC membranes again corroborates the lack of oligomerization on these membranes.

The key aspects of this model, in particular the roles of calcium in membrane binding and of phosphatidylglycerol in oligomerization, are supported by evidence; however, several details remain hypothetical. The postulated changes in conformation are inferred from those in function. The initial change triggered by calcium binding may lead to an increased exposure of hydrophobic moieties, possibly the N-terminal acyl chain; such a change would promote both oligomerization in solution and binding to membranes. The second change, triggered by phosphatidylglycerol, is depicted as causing not only oligomerization but also deeper mem-

brane penetration. Both kynurenine [77] and NBD in NBD-daptomycin show a greater gain of fluorescence intensity on PC/PG membranes than on PC membranes, indicating a more hydrophobic environment on the former. However, on PC/PG membranes, daptomycin will not only interact with surrounding lipid molecules but also other daptomycin molecules within the oligomer that forms only on these membranes. It should also be noted that PC/PG membranes may be subject to lateral phase segregation, which is further promoted by calcium ions. If this occurs, membranes may become leaky, which may expose additional sites for hydrophobic interaction. For these reasons, the increase in environmental hydrophobicity cannot be unequivocally ascribed to deeper membrane penetration.

In Figure 2.9, the interaction of daptomycin with phosphatidylglycerol is assumed to be mediated by calcium. Daptomycin has only one intrinsic positive charge, which is the amino group in the side chain of the ornithine residue. With NBD-daptomycin, as well as with previously characterized ornithine-modified derivatives [59, 123], even this single charge is masked (see Figure 2.1); nevertheless, these derivatives retain antimicrobial activity, which leaves calcium as the only available positive charge available to interact with the negative charge of phosphatidylglycerol. Not many more conclusions can be drawn as to the structure of the oligomer, other than that it is fairly stable on a time scale of minutes to hours, as indicated by the experiment shown in Figure 2.5. The structure of the membrane-associated oligomer thus remains to be determined.

Regarding the experimental strategies used in this study, it should be noted that FRET between daptomycin (or Alexa Fluor 350-daptomycin) and NBD-daptomycin can be quite extensive even in the absence of oligomerization, as is evident from Figures 2.5 and 2.7. The extent of FRET will also vary with the ratio of total daptomycin to membrane lipids, and it will also be affected by uneven distribution of membrane-bound daptomycin, as has been demonstrated by fluorescence with bacterial cells [52]. In contrast, the relief of self-quenching of NBD-daptomycin, caused by the addition of unlabeled daptomycin, is much less sensitive to such influences, as long as the amount of NBD-daptomycin remains constant. Without oligo-

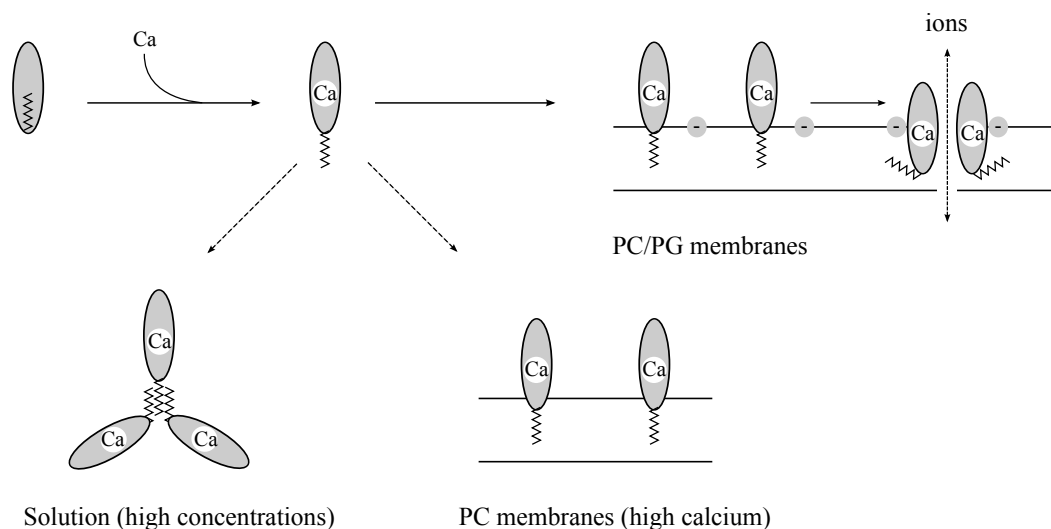


Figure 2.9 Model of daptomycin oligomerization on membranes. Calcium binds to daptomycin in solution and causes a conformational change, which at high concentration facilitates oligomerization in solution. It also facilitates binding to PC membranes, on which however no oligomerization occurs. On PC/PG membranes, as well as on bacterial membranes, the interaction of daptomycin/calcium with the negatively charged head-group of phosphatidylglycerol induces a second conformational change that induces oligomerization and deeper membrane insertion.

merization, the addition of unlabeled daptomycin should cause no change in quenching, since the unlabeled daptomycin would simply not interfere with the NBD-daptomycin in any way. In contrast, with oligomerization, the addition of unlabeled daptomycin must reduce quenching, since within the hybrid oligomers the NBD-daptomycin molecules will be less likely to closely interact with one another. Therefore, the more robust quenching assay was preferred to detect oligomerization on bacterial cell membranes.

In summary, we have shown that daptomycin oligomerizes on liposomal and bacterial membranes. The requirements for oligomerization resemble those established for bacterial cell membrane permeabilization, suggesting that the oligomer is the functional membrane lesion. The detailed structure of the oligomer remains to be established.

Chapter 3

Characterization of daptomycin oligomerization with excimer fluorescence

3.1 INTRODUCTION

Daptomycin is a cyclic lipopeptide that consists of 13 amino acid residues, including one kynurenine residue and several other non-standard amino acids, and a decanoyl fatty acyl residue attached to the exocyclic N-terminal tryptophan residue (see Figure 3.1). It is produced by the soil bacterium *Streptomyces roseosporus*. Daptomycin is used in the treatment of infections by Gram-positive bacteria such as enterococci and staphylococci, including strains that are resistant to penicillin, methicillin and vancomycin [133, 132]. Daptomycin binds and depolarizes the bacterial cytoplasmic membrane [125]. Its activity requires calcium and correlates with the target membrane's content of phosphatidylglycerol (PG); increased conversion of PG to lysyl-PG confers partial resistance to bacterial cells [52, 53].

The results presented in this chapter have been published in: "Characterization of daptomycin oligomerization with perylene excimer fluorescence: stoichiometric binding of phosphatidylglycerol triggers oligomer formation", by Muraih, J.K., Harris, J., Taylor, S.D., Palmer, M. (2012), *Biochim Biophys Acta* 1818:673-8.

Authors' contributions: The preparation of pyrene- and perylene-daptomycin, as described in Sections 3.2.1 and 3.2.2, was performed by J. Harris and S.D. Taylor. All other experiments were performed by the thesis author.

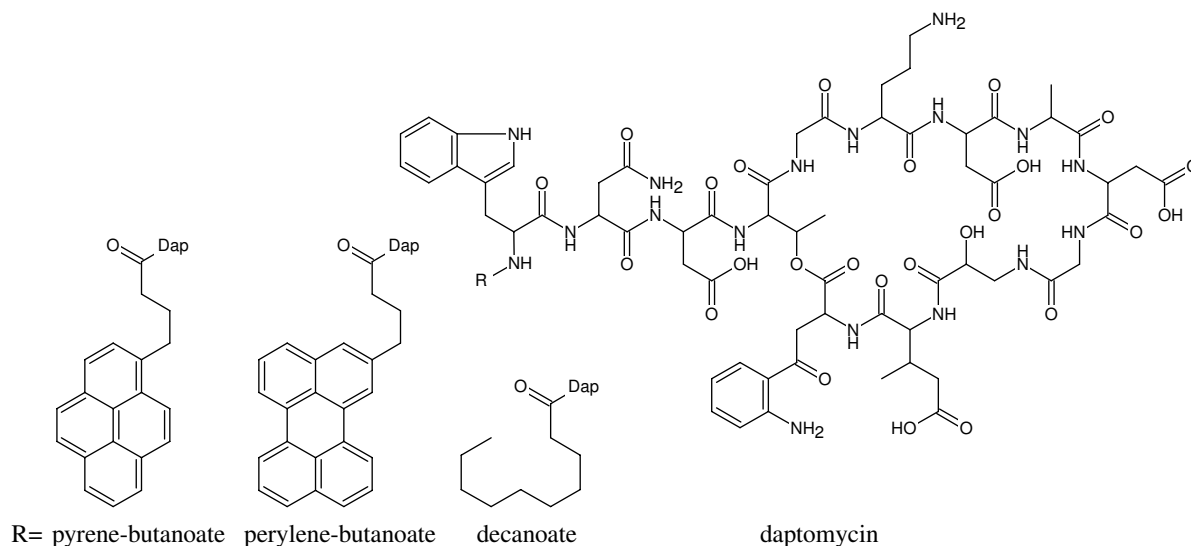


Figure 3.1 Structures of daptomycin, and of the substituents pyrene- and perylene-butanoic acid that were substituted for the decanoyl residue of the native compound in this study.

Using FRET between the kynurenine residue of unlabeled daptomycin and a fluorescently labeled derivative of daptomycin, we have shown previously that daptomycin forms oligomers on liposome membranes (see Chapter 2). Oligomerization occurs under conditions that resemble those required for its antibacterial activity, suggesting that the oligomer is involved in the bactericidal effect.

In this study, we have used excimer fluorescence in order to further characterize the membrane-associated daptomycin oligomer. An excimer, or excited dimer, is a complex that forms from one fluorophore molecule that is in the excited state and another one in the ground state [78]. Since this requires direct contact between two fluorophore molecules, the detection of excimer fluorescence imposes a more stringent constraint on the distance of the interaction than that implied by the observation of FRET.

The fluorophore most widely used to study excimer fluorescence is pyrene, whose excimer and monomer spectra are well separated. However, as will be shown below, pyrene incorporation into daptomycin results in FRET from pyrene to kynurenine, which prevents the observation of excimer fluorescence. To circumvent this difficulty, we therefore chose to incorporate

perylene, which emits at longer wavelengths [66] and therefore should not engage in FRET to kynurenine.

A prior study has shown that substitutions of the N-terminal decanoyl moiety of daptomycin are quite compatible with its antibacterial activity, as long as the introduced substituents retain an overall hydrophobic character [33]. Pyrene and perylene are very hydrophobic molecules, and accordingly they were introduced into daptomycin by replacing the decanoyl chain with pyrene-butanoic acid and perylene-butanoic acid, respectively. The derivatives retained antibacterial activity, albeit at a slightly reduced level in the case of perylene-daptomycin. Nevertheless, oligomer formation of perylene-daptomycin was clearly evident through excimer fluorescence, under conditions that mirrored those used in the previous FRET experiments. This result indicates that the fatty acyl residues of adjacent subunits of the membrane-associated daptomycin oligomer are within immediate reach of one another.

In a lipid bicelle system, the excimer fluorescence could be titrated with phosphatidylglycerol. Conversion of monomer fluorescence to excimer fluorescence approached saturation at an equimolar ratio of phosphatidylglycerol to daptomycin. Therefore, a single molecule of phosphatidylglycerol is sufficient to convert daptomycin to a conformation that is competent for oligomerization.

Our study provides significant additional insight into the process of daptomycin oligomerization. At the same time, it demonstrates that perylene excimer fluorescence is useful as a probe in the experimental study of biochemical systems.

3.2 METHODS AND MATERIALS

3.2.1 Synthesis of perylene-daptomycin

To a suspension of deacylated daptomycin bearing a Boc protecting group on the ornithine residue (120 mg, 0.077 mmol; generously provided by Jared Silverman of Cubist Inc.) in dry dimethylformamide (0.5 mL) was added 4-(3-perylenyl)butanoic acid succinimidyl ester

(0.035 g, 0.08 mmol). The mixture was stirred for 24 h and then concentrated by high vacuum rotary evaporation. The residue was dissolved in water and lyophilized. The lyophilized powder was dissolved in an ice-cold solution of trifluoroacetic acid (TFA) containing 2% thioanisole, and the resulting solution was stirred for 15 min at 0 °C (ice bath). The mixture was concentrated using a high vacuum rotary evaporator, keeping the heating bath below 40 °C. The residue was dissolved in water and lyophilized. The resulting crude powder was purified by semi-preparative reversed-phase HPLC employing a linear gradient starting with 95% water (0.1% TFA) to 5% acetonitrile to 100% acetonitrile over 50 minutes ($t_r = 34$ min). Fractions containing the desired product were pooled and concentrated using a high vacuum rotary evaporator keeping the heating bath below 40 °C. The resulting residue was lyophilized to give 0.047 g (34% yield) of perylene-daptomycin as a yellow powder. The molecular weight of the purified perylene-daptomycin determined by mass spectrometry was 1784.76 Da.

The analytical reversed-phase HPLC chromatogram of the purified material, using a linear gradient starting with 95% water (0.1% TFA) and 5% acetonitrile to 100% acetonitrile over 50 min. Relevant figures and more details are provided in the supporting data for reference [100].

3.2.2 Synthesis of pyrene-daptomycin

This compound was prepared using essentially the same procedure as that described above for perylene-daptomycin, substituting 0.0081 g (0.021 mmol) 1-pyrenebutanoic acid succinimidyl ester for the analogous perylene compound. The amount of Boc-protected deacylated daptomycin used was 0.030 g (0.019 mmol). The crude reaction product was purified by HPLC as described above for perylene-daptomycin ($t_r = 28$ min). Fractions containing the desired product were pooled and concentrated and the resulting residue lyophilized to give 17 mg (51% yield) of pyrene-daptomycin as a white powder. The molecular weight of the purified pyrene-daptomycin determined by mass spectrometry was 1734.75 Da. Analytical data are contained in the supporting data for reference [100].

3.2.3 Antibacterial activity test for pyrene- and perylene-daptomycin

The two labeled derivatives as well as native daptomycin were tested for antibacterial activity by broth dilution. LB broth was supplemented with calcium (5 mM), and the daptomycin derivative in question was added at concentrations of 10, 5, 3, 2, 1.5, and 1 $\mu\text{g}/\text{ml}$. Each tube was inoculated with *Bacillus subtilis* ATCC 1046 and incubated overnight at 37 °C. Growth inhibition was evaluated visually by turbidity. Growth controls without antibiotic were prepared for comparison.

3.2.4 Lipids and liposomes

The preparation of large unilamellar vesicles (LUV) was performed as described above in Section 2.2.3. Dihexanoyl-PC (DHPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL) and used directly.

3.2.5 Fluorescence measurements

Emission spectra were acquired on a QuantaMaster 4 spectrofluorimeter (Photon Technology International, London, ON). Pyrene fluorescence was excited at 340 nm and the emission recorded from 360 to 600 nm. Perylene fluorescence was excited at 430 nm and the emission recorded from 440 to 600 nm. Bandpasses for excitation and emission were between 2 and 5 nm, depending on the sample concentration. Emission spectra were corrected for wavelength-dependent instrument response using a quinine sulfate standard and a tabulated normalized quinine spectrum listed in reference [78]. Fluorescence lifetime measurements were performed on a FluoTime 100 Lifetime spectrometer (PicoQuant, Berlin, Germany), using diode laser light sources emitting at 370 nm and at 440 nm for pyrene and perylene, respectively. The emission was isolated using 460 ± 10 nm or 560 ± 10 nm bandpass filters (Melles-Griot, Brossard, QC). Experimental decays were numerically fitted with two or three exponentials, with resulting χ^2 values typically between 1.1 and 1.3. From these fitted exponential components, average

lifetimes were calculated according to the equation

$$\langle \tau \rangle = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad (3.1)$$

where α_i represents the amplitude at time zero and τ_i the lifetime of the i^{th} component.

All measurements were acquired at room temperature. The concentration of calcium was 200 mM with liposomes consisting of PC only. With all other samples, the calcium concentration was 5 mM, unless stated otherwise.

3.2.6 Deconvolution of perylene excimer and monomer spectra

Perylene fluorescence decays were measured at 560 ± 10 nm on DMPC/DMPG membranes, both with pure perylene-daptomycin and with a mixture of perylene-daptomycin and a fivefold excess of unlabeled daptomycin. The decays were fitted to a two-exponential model. The fractional emission intensities of the monomer and the excimer at 560 nm were obtained from these data according to

$$\langle \tau \rangle = \frac{\alpha_1 \tau_1}{\alpha_1 \tau_1 + \alpha_2 \tau_2} \quad (3.2)$$

and

$$F_{excimer,560} = 1 - F_{monomer,560} \quad (3.3)$$

From the spectrum of perylene-daptomycin only, P, and the spectrum of the mixture of perylene-daptomycin with unlabeled daptomycin, U, the pure excimer spectrum, E, was calculated as

$$E = P - aU \quad (3.4)$$

with

$$a = \frac{F_{P, monomer, 560} I_{P, 560}}{F_{U, monomer, 560} I_{U, 560}} \quad (3.5)$$

where $I_{P, 560}$ and $I_{U, 560}$ denote the intensity of P and U at 560 nm, respectively. The pure monomer spectrum M was obtained analogously. After normalizing E and M to the same quantity of molecules, the quantum yield of the excimer, relative to that of the monomer, was calculated from the ratio of the areas of the two normalized spectra.

3.2.7 Titration of perylene excimer fluorescence with phosphatidylglycerol

To a solution of 100 μM perylene-daptomycin, 1 mM Ca^{2+} and 20 mM DHPC in HEPES buffer (HBS), a mixture of DMPC and DMPG (molar ratio, 4:1) was added in a stepwise fashion. After each addition, the mixture was incubated for 2 min before acquisition of the perylene emission spectrum. Repeated scans showed that this short incubation sufficed to reach equilibrium. From each spectrum, the ratio of the emission intensities at 521 nm and 480 nm was calculated, and the increment was plotted as a function of the molar ratio of DMPG to perylene-daptomycin.

3.3 RESULTS

3.3.1 Antibacterial activity of pyrene- and perylene-daptomycin

The antibacterial activities of pyrene-daptomycin and perylene-daptomycin (structures shown in Figure 3.1) against *B. subtilis* strain ATCC 1046 were determined by broth dilution, using native daptomycin as a reference. The minimum inhibitory concentration of perylene-daptomycin was 3 $\mu\text{g}/\text{ml}$, compared to 1 $\mu\text{g}/\text{ml}$ for native daptomycin and pyrene-daptomycin. Therefore, the specific activity of perylene-daptomycin is approximately 3 times lower than that of native daptomycin and of pyrene-daptomycin.

3.3.2 Fluorescence of pyrene-daptomycin

Figure 3.2 shows the emission spectra of pyrene-daptomycin in solution and on liposomes consisting of phosphatidylcholine (PC) and phosphatidylglycerol (PG). Pyrene monomer flu-

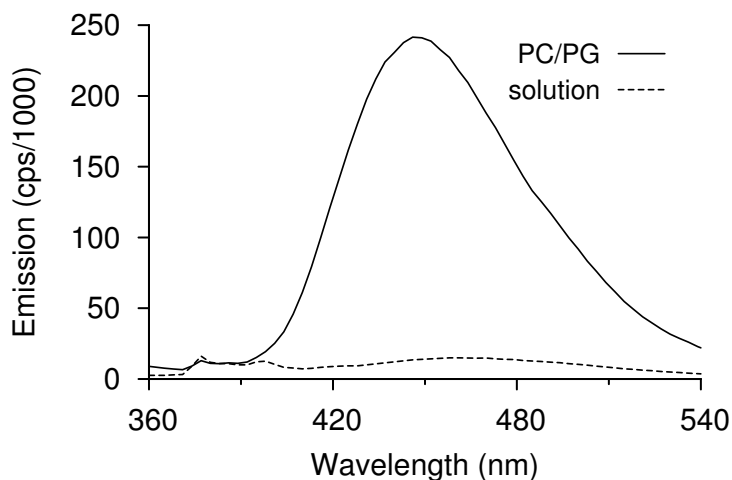


Figure 3.2 Fluorescence emission spectra of pyrene-daptomycin in solution and on DMPC/DMPG liposomes, in the presence of 6 mM calcium. The excitation wavelength was 340 nm.

orescence, which is apparent in the small peaks to both sides of 380 nm, is very low in both samples. There is a broad peak around 460 nm that greatly increases after incubation with the liposomes, on which daptomycin has been shown to form oligomers. While oligomer formation should be accompanied by excimer formation, the latter should be accompanied by a decrease of monomer emission; however, monomer emission is very low to begin with and does not decrease any further. Furthermore, the peak at 460 nm has an average excited state lifetime of approximately 3.4 ns as shown in Table 3.1, whereas the typical lifetimes of pyrene excimers are much longer. On the other hand, the observed lifetime is very similar to that found previously for the kynurenine residue of daptomycin [102].

The monomer emission spectrum of pyrene significantly overlaps the absorption spectrum of the kynurenine residue, which should cause fluorescence energy transfer from excited pyrene to kynurenine. The increase in the sensitized kynurenine emission upon membrane binding mirrors that of directly excited kynurenine fluorescence reported earlier [77]. In sum, because of spectral overlap between pyrene emission and kynurenine absorption, pyrene-daptomycin does not produce experimentally useful excimer fluorescence.

Table 3.1 Fluorescence lifetimes of pyrene-daptomycin and native daptomycin on DMPC/DMPG and DMPC liposome membranes. Both pyrene and kynurenine fluorescence decays were measured by using diode laser light source at 370 nm. Fluorescence emission was isolated using a 460 ± 10 nm bandpass filter. The decays were fitted to a two-exponential model, and average lifetimes were calculated according to equation 3.1.

	PC/PG	PC
Native daptomycin	2.941 ns	1.645 ns
Pyrene-daptomycin	3.387 ns	2.049 ns

3.3.3 Observation of perylene-daptomycin excimer fluorescence

The emission spectrum of perylene-daptomycin in solution resembles that of monomeric, free perylene in hexane [66], with three distinct peaks between 450 and 520 nm (Figure 3.3A). A similar spectrum is obtained when perylene-daptomycin is incubated with DMPC membranes (Figure 3.3B). In contrast, on membranes composed of DMPC and DMPG, as well as on bacterial cells, the shape of the spectrum changes significantly (Figure 3.3C, D). The two leftmost peaks are greatly decreased, and a new, broad peak emerges that overlaps the monomer fluorescence.

While the new peak is centered around 525 nm and therefore is blue-shifted relative to the excimer of free perylene [66], its shape nevertheless is suggestive of excimer fluorescence. This interpretation is corroborated by time-resolved fluorescence measurements. Figure 3.4 shows fluorescence decays of perylene-daptomycin on PC liposomes (A) and on PC/PG liposomes (B), measured at 560 nm, where monomer intensity is very low. Both decays can be fitted quite well with a two-exponential model (see fit parameter values in the figure), and the lifetime parameters obtained from these fits are similar to those of perylene monomers and excimers reported earlier [66]. Note that the pre-exponentials are different between the two membranes, which reflects a higher prevalence of excimers on the PC/PG membranes. Also, the fitted fluorescence lifetime of the putative excimer component is shorter in PC than in PC/PG membranes.

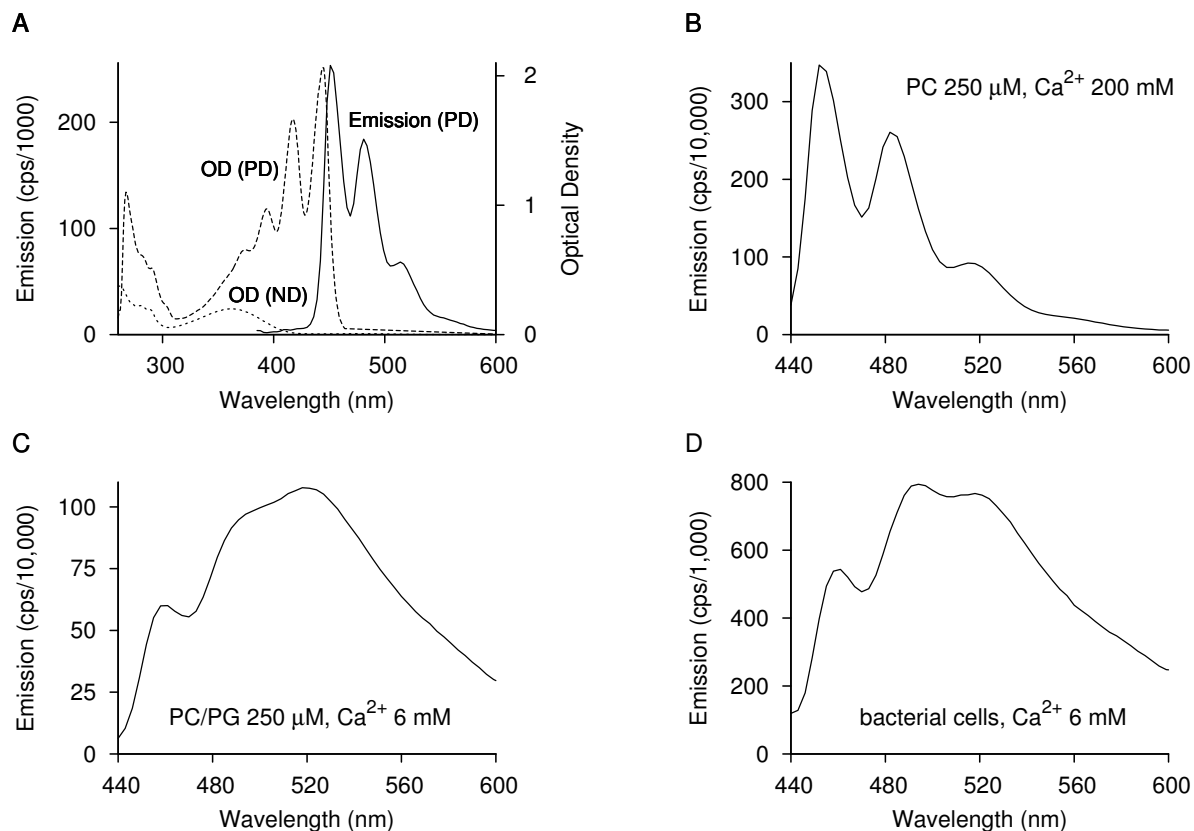


Figure 3.3 Fluorescence emission spectra of perylene-daptomycin in solution (A), on DMPC liposomes (B), on DMPC/DMPG liposomes (C), and on bacterial cells (D). The emission spectra were measured with 2.5 μM perylene-daptomycin. In (A), the absorption spectra (OD) of perylene-daptomycin (PD) and of native daptomycin (ND) at 50 μM in solution are also shown. The absorption spectrum of native daptomycin on membranes is indistinguishable (not shown). In (D), the cells were incubated with perylene-daptomycin and calcium, then washed repeatedly by centrifugation to remove unbound daptomycin and resuspended before acquisition of the spectrum.

Excimer fluorescence only occurs when a monomer in the excited state binds directly to another monomer that is in the ground state. It should therefore be greatly facilitated by the formation of stable oligomers, since within these the perylene moieties should remain close to one another throughout. On the other hand, a mixture of perylene-daptomycin with an excess of unlabeled daptomycin should produce hybrid oligomers, within which the perylene-labeled molecules should become separated from one another by intervening unlabeled molecules. This should lead to an increase in monomer fluorescence and a decrease in excimer fluores-

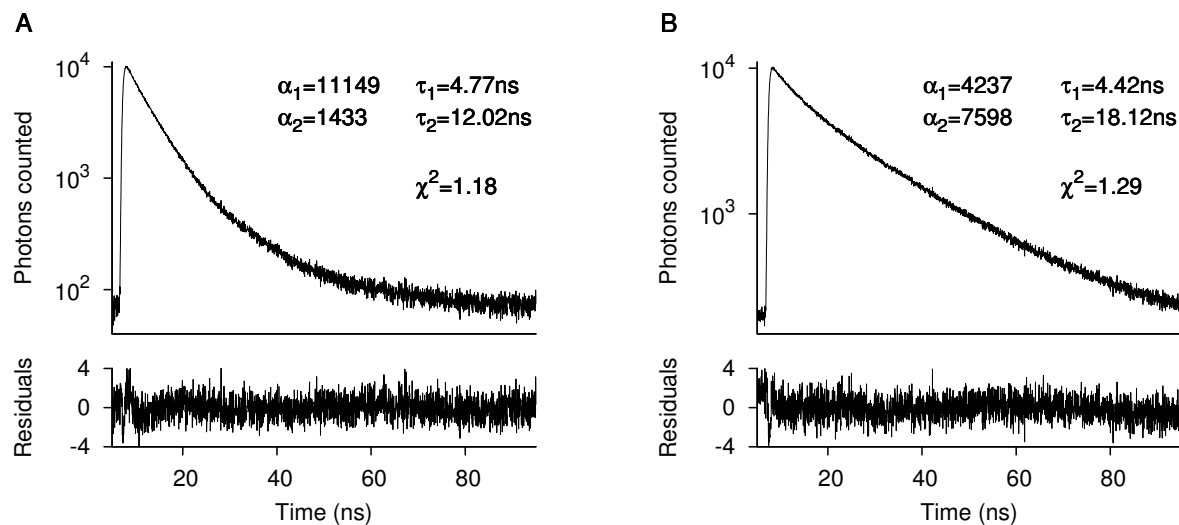


Figure 3.4 Time-resolved fluorescence measurements of perylene-daptomycin on DMPC (A) and DMPC/DMPG (B) membranes. Sample composition was as in Fig. 3B and C, respectively. Excitation was with a 440 nm diode laser. Emission was measured using a 560 ± 10 nm bandpass filter. The decays were fitted to a two-exponential model. Pre-exponentials (α) and lifetimes (τ) of the fitted components, as well as the residuals and χ^2 of the fits are given.

cence. Figure 3.5 shows that this is indeed the case. It also shows that, on PC membranes, the ratio of excimer fluorescence to monomer fluorescence is not affected by the addition of unlabeled daptomycin. This is in line with the assumption that stable oligomers do not form on these membranes, and that excimer fluorescence arises by diffusional collision of individual perylene-daptomycin molecules only.

Large unilamellar vesicles consisting of POPE, DOPG and TOCL are considered models of the cytoplasmic membranes of bacteria [39, 86]. With such liposomes, the spectra and fluorescence lifetimes closely resembled those observed with PC/PG liposomes. These results agree with previous observations using FRET between daptomycin molecules (see Chapter 2).

3.3.4 Quantitative evaluation of excimer fluorescence

By combining lifetime data with steady state spectra, we can obtain an approximation of the spectrum of the excimer, as well as an estimate of its quantum yield relative to that of the

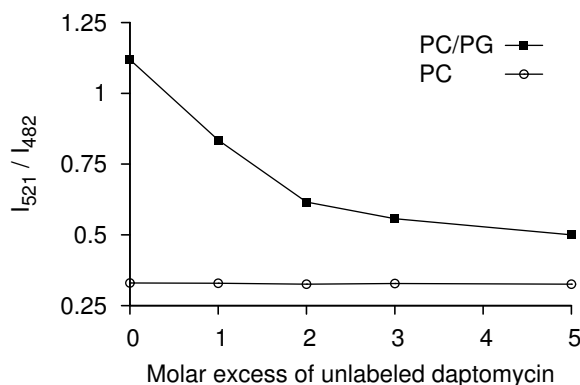


Figure 3.5 Effect of unlabeled daptomycin on the extent of excimer formation by perylene-daptomycin. Perylene-daptomycin ($2.5 \mu\text{M}$) was mixed with unlabeled daptomycin at the molar ratios indicated and incubated with DMPG/DMPC or DMPC liposomes with 6 and 200 mM of calcium, respectively. The change in fluorescence is represented the ratio of emission at 521 nm over that at 482 nm; a higher ratio indicates a greater extent of excimer formation.

monomer. The calculation, which is detailed in Section 3.2.6, yields the monomer and excimer spectra shown in Figure 3.6.

The quantum yield of the excimer, relative to that of the monomer, is 0.35. On PC/PG membranes, the observed excimer emission corresponds to less than 90% of all excitation events. If we neglect excimer emission due to collisional encounters of the few remaining monomers, we can infer that more than 90% of all daptomycin molecules are now located in oligomers. Considering that some excited perylene molecules will emit monomer fluorescence even when placed in the vicinity of other perylene molecules, the extent of oligomer formation may well be even higher.

At an oligomerization efficiency of 90%, the ratio of emission at 521 nm to that at 482 nm is 1.35 (Figure 3.3C). This ratio is 0.33 for PC liposomes (Figure 3.3B), on which perylene-daptomycin emits mostly as a monomer. On bacterial cells (Figure 3.3D), it is 1.16, which shows that most of the cell-bound perylene-daptomycin have become incorporated into oligomers. Therefore, on suitable membranes, oligomerization of daptomycin approaches or reaches completion.

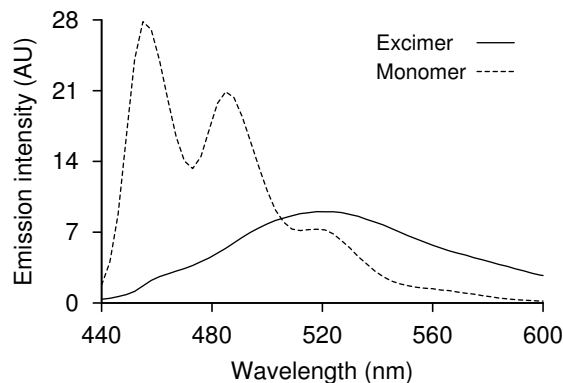


Figure 3.6 Deconvoluted excimer and monomer spectra of perylene-daptomycin on DMPG/DMPC membranes. The steps involved in the deconvolution are described in Section 3.2.6.

3.3.5 Titration of excimer fluorescence with phosphatidylglycerol

In previous studies (references [77, 86] and Chapter 2), phosphatidylglycerol was used in considerable molar excess over daptomycin, and therefore these experiments did not provide information on the stoichiometry of the interaction of daptomycin with PG. To examine this question, we used a mixture of DMPC and DMPG with an excess of dihexanoyl-PC. Mixtures of the latter with lipids of typical acyl chain length yield lipid bicelles, which are small, disk-like lipid aggregates that are suitable as membrane mimetics [49].

The advantage of bicelles over liposomes is twofold. Firstly, suspensions of bicelles are much less turbid. This allows measurements at higher concentrations of daptomycin and PG, which will help to drive the reaction between the two toward completion. Secondly, in a bicelle, both monolayers will be exposed to daptomycin. In contrast, with liposomes, only the outer monolayer may be accessible. Previous observation of membrane fusion induced by daptomycin in model liposomes [67] suggests that the membrane continuity may be disrupted, and both monolayers may be accessible to daptomycin in this system also. On the other hand, membrane fusion did not go to completion. In addition, no morphological discontinuities were observed in cell membranes of daptomycin-treated bacteria by electron microscopy [27], suggesting that the inner monolayer may not or only incompletely be accessible to daptomycin. The use of bicelles avoids this uncertainty.

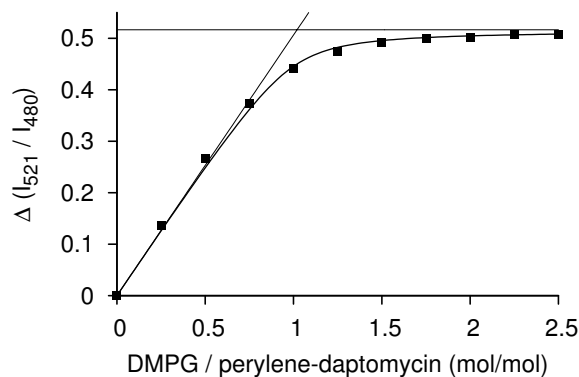


Figure 3.7 Titration of excimer formation with DMPG. Perylene-daptomycin was incubated with dihexanoyl-PC (20 mM) and calcium (1 mM). A suspension of DMPC/DMPG liposomes was added in a stepwise fashion, and the perylene fluorescence emission recorded after each addition. The increment in the ratio of the emissions at 521 nm and at 482 nm is plotted as a function of the molar ratio of PG to daptomycin. The curve represents a fit to the law of mass action, and the straight lines represent the tangents of the fit curve at zero and infinity, respectively.

Figure 3.7 shows the titration of 100 μM perylene-daptomycin, in 20 mM dihexanoyl-PC and 1 mM calcium, with a mixture of dimyristoyl-PC and dimyristoyl-PG. Excimer formation is monitored as the ratio of fluorescence intensities at wavelengths 521 nm and 482 nm. It can be seen that the extent of excimer formation, and thus oligomerization, approaches saturation after addition of a stoichiometric amount of phosphatidylglycerol.

3.4 DISCUSSION

In this study, we synthesized pyrene-daptomycin and perylene-daptomycin and used the latter to characterize the oligomerization of daptomycin on membranes. The perylene moiety was introduced into daptomycin by substituting it for the normal decanoyl chain. Considering the substantial bulk that is added to the molecule by this modification, the fact that the antimicrobial activity is reduced no more than threefold is quite remarkable, as is the observation that the introduction of pyrene caused no measurable decrease in activity at all. These observations are, however, in line with a previous study on a variety of acyl analogues, which revealed

only minor changes to the antibacterial activity, as long as the overall hydrophobic character of the acyl chain was preserved [33]. While the spectral overlap between pyrene and kynurenine prevented the observation of pyrene excimer fluorescence, perylene excimers could be observed and used to characterize the oligomerization of daptomycin. The perylene excimer fluorescence indicated the formation of daptomycin oligomers on both liposomes and bacterial cells. In liposomes, oligomerization correlates with the presence of phosphatidylglycerol. These findings are entirely consistent with those obtained using fluorescence energy transfer (see Chapter 2).

Beyond this confirmation, the use of perylene-daptomycin provides significant additional insights that could not be inferred from previous experiments. The first novel observation is the formation of daptomycin oligomers on live bacterial cells. Secondly, using perylene-daptomycin, the extent of oligomerization could be measured. This extent exceeds 90% on model membranes that contain 50% DMPG, and it is only slightly lower on bacterial membranes. This high extent of oligomerization supports the notion that it is the oligomer that causes the bactericidal effect.

Thirdly, the formation of perylene excimers shows that, in the oligomer, the N-terminal acyl chains interact directly with one another. Direct interaction of the daptomycin peptide moieties within the oligomer was previously inferred from the self-quenching of NBD-labels that had been attached to their unique ornithine residues (Chapter 2). Taken together, these two points of mutual interaction suggest that neighboring oligomer subunits are aligned in a parallel fashion, or form an acute angle with one another.

The fourth novel finding reported here is that daptomycin oligomerization is driven by a stoichiometric interaction with phosphatidylglycerol. This result suggests that PG interacts with daptomycin directly and specifically, rather than indirectly through influencing bulk properties of the membrane such as charge or lateral phase segregation. Enhanced conversion of PG to lysyl-PG by bacterial cells has been reported as a mechanism of partial resistance to daptomycin [52, 53]. Against this background, the observed stoichiometric interaction lends further

support to the hypothesis that PG-mediated oligomerization is essential for the bactericidal effect of daptomycin.

In summary, the current study provides significant new insights into the interaction of daptomycin with itself and with membrane lipids. At the same time, this study is the first one to use perylene excimer fluorescence for the characterization of peptide or protein oligomerization. The results provided here illustrate that perylene should be a valuable tool wherever the occurrence of intrinsic chromophores or fluorophores precludes the use of pyrene-labeled probes.

Chapter 4

The subunit stoichiometry of the daptomycin oligomer

4.1 INTRODUCTION

The lipopeptide daptomycin is an important reserve antibiotic for the treatment of infections by otherwise resistant strains of Gram-positive bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis* [16, 23, 83]. Its bactericidal effect involves membrane depolarization due to permeabilization for potassium ions [125].

In the preceding chapters of this thesis, daptomycin was shown to form oligomers on bacterial membranes and on liposome membranes containing phosphatidylglycerol. Oligomerization is dependent on calcium. The requirements of oligomerization for both calcium and phosphatidylglycerol mirror those of bactericidal action [10], which suggests that the oligomer

The results presented in this chapter have been published in: “Estimation of the subunit stoichiometry of the membrane-associated daptomycin oligomer by FRET”, by Muraih, J.K. and Palmer, M. (2012), *Biochim Biophys Acta* 1818:1642-7.

Authors’ contributions: The experiment depicted in Figure 4.6 was performed by J. Pogliano. All other experiments were performed by the thesis author. The Python program that implements the numerical simulation shown in Figure 4.5 was written by M. Palmer.

forms a functional membrane lesion, as is the case with many other membrane-permeabilizing peptides and proteins.

At high concentrations and in the presence of calcium, daptomycin also undergoes oligomerization in solution. Such oligomers have been structurally characterized by NMR and by density gradient centrifugation, and they reportedly consist of 14–16 subunits [61]. On the other hand, the action of daptomycin has also been compared to that of cationic antimicrobial peptides, many of which do not form discrete oligomers but rather follow the carpet model of membrane destabilization [120]. In the current study, we therefore set out to determine whether the membrane-associated oligomer has a defined subunit stoichiometry.

Daptomycin contains several non-standard amino acid residues. One of these, kynurenine, exhibits an experimentally useful intrinsic fluorescence that responds with a significant gain in quantum yield to membrane interaction [77]. Another one, ornithine, carries the molecule's single free amino group. Derivatization of this ornithine residue can be used to prepare fluorescent derivatives such as NBD-daptomycin, which retains antibacterial activity and forms hybrid oligomers with unlabeled daptomycin (Chapter 2). In NBD-daptomycin, kynurenine fluorescence is entirely suppressed due to fluorescence resonance energy transfer (FRET) from kynurenine to NBD. Moreover, in mixed oligomers, FRET also occurs between unlabeled daptomycin and NBD-daptomycin. The experimental approach used here to determine the subunit stoichiometry of the membrane-bound oligomer consists of a quantitative analysis of the latter effect. The results suggest that the membrane-associated oligomer is smaller and structurally different from the oligomer observed in solution.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of liposomes and daptomycin derivatives

Liposomes consisting of an equimolar mixture of DMPC and DMPG were prepared as described in Section 2.2. NBD-daptomycin and perylene-daptomycin was prepared as described

as in Section 2.2 and 3.2.1, respectively. Phospholipids were obtained from Avanti Polar Lipids Inc., and unlabeled daptomycin was provided by Cubist Pharmaceuticals.

4.2.2 Fluorescence measurements

Samples contained sample buffer (HEPES 20 mM, 150 mM NaCl, pH 7.4), LUV (250 μ M total lipid), calcium chloride (6 mM), and either perylene-daptomycin or daptomycin and NBD-daptomycin in various proportions and total concentrations. Steady state fluorescence emission spectra were obtained on a PTI QuantaMaster 4 instrument. For daptomycin and NBD-daptomycin, the excitation wavelength was 365 nm, and emission was recorded between 400 and 600 nm. For perylene-daptomycin, the excitation wavelength was 430 nm, and emission was recorded from 440 nm to 600 nm. Excitation and emission slit widths were adjusted to keep the maximal emission recorded below 10^6 counts/s; this latter value was determined to be within the linear range of the instrument's response.

4.2.3 Determination of the corrected relative kynurenine fluorescence of daptomycin in mixed oligomer samples

The purpose of the experiments performed here is the quantitation of FRET between kynurenine in unlabeled daptomycin and NBD in NBD-daptomycin that occurs within hybrid oligomers of the two species. To this end, it is necessary to measure the kynurenine fluorescence of unlabeled daptomycin molecules within oligomers that have formed from mixtures of daptomycin with NBD-daptomycin, relative to the fluorescence of the same number of daptomycin molecules within oligomers formed without NBD-daptomycin, and corrected for FRET that will occur between pure daptomycin oligomers and NBD-daptomycin-containing oligomers.

Mixtures of daptomycin and NBD-daptomycin, in various proportions as indicated in the Results section, were prepared and added at a combined final concentration of 10 μ M to liposomes and calcium and incubated for 180 s to induce oligomerization before measuring the kynurenine emission.

To correct for FRET between (as opposed to within) oligomers, 4 μM unlabeled daptomycin was then added to the same sample and was again allowed to oligomerize for 180 s, and the kynurenine fluorescence was read again. The rationale for this calibration procedure is explained in the Results section. The relative kynurenine fluorescence was calculated using the following equation:

$$F_r = \frac{d_2}{d_1} \left(\frac{I_1 - I_0}{I_2 \frac{V_2}{V_1} - I_1} \right) \quad (4.1)$$

In equation 4.1, F_r is the relative kynurenine fluorescence, and d_1 and d_2 are the molar amounts of donor (unlabeled daptomycin) added in the first and second steps, respectively. I_0 , I_1 and I_2 are the emission intensities at 445 nm of a liposome blank, and of the oligomer sample after the first and second addition of daptomycin, respectively. V_1 and V_2 are the sample volumes before and after that addition, respectively; the term $\frac{V_2}{V_1}$ corrects for the dilution caused by the second addition of daptomycin, which amounted to 4 % of the sample volume.

4.2.4 Numerical simulation of hetero-oligomer formation

The simulation shown in Figure 4.5 was implemented as a Python program. The code of the program, with explanations, is contained in the supplementary file to reference [101].

4.3 RESULTS

4.3.1 Rationale of the overall experimental approach

The experimental approach used in this study is based on fluorescence resonance energy transfer (FRET) between the kynurenine in unlabeled daptomycin and NBD-daptomycin in hybrid oligomers. FRET involves the kynurenine residue in unlabeled daptomycin as the donor, and the NBD moiety of NBD-daptomycin as the acceptor. We made the following assumptions:

1. All daptomycin oligomers have the same number of subunits, n .

2. Conversion of monomeric daptomycin to oligomers is quantitative on the time scale of the experiment.
3. When native daptomycin and NBD-daptomycin are mixed before application to membranes, they will form oligomers randomly, without any positive or negative mutual discrimination.
4. In hybrid oligomers, the kynurenine fluorescence of all unlabeled daptomycin molecules is completely quenched by FRET. Therefore, any remaining kynurenine fluorescence originates from oligomers that consist of unlabeled daptomycin only.
5. Oligomers are stable on the time scale of the experiment.

Let d be the fraction of native daptomycin in a mixture with NBD-daptomycin (whose fraction is $1 - d$). After this mixture has undergone oligomerization according to conditions 1 and 3, the fraction of oligomers that contain only native daptomycin subunits but no NBD-daptomycin will be d^n . Then, the fraction D of all native daptomycin molecules from the original mixture that were incorporated into these donor-only oligomers will be given by

$$D = \frac{d^n}{d} = d^{n-1} \quad (4.2)$$

According to assumption 4, only the oligomers represented by D will emit observable kynurenine fluorescence. Therefore, the corrected relative kynurenine fluorescence F_r of a given amount of daptomycin that was mixed with NBD-daptomycin before oligomerization, as defined in equation 4.1, equals D . Substituting F_r for D in equation 4.2 and solving, n yields

$$n = 1 + \log_d(F_r) = 1 + \frac{\ln(F_r)}{\ln(d)} \quad (4.3)$$

Therefore, we can obtain the subunit stoichiometry of the oligomer by comparing the kynurenine fluorescence of mixed oligomers to that of pure native daptomycin oligomers.

In the following, we will first present some experiments that test the assumptions underlying the experimental approach, followed by the experiments to measure the oligomer subunit stoichiometry.

4.3.2 Kinetics and linear range of the fluorescence signal

Interaction of daptomycin with membranes causes a steep increase in the fluorescence emission of its single kynurenine residue [77]. Figure 4.1A shows the time course of kynurenine fluorescence after addition of daptomycin to PC/PG liposomes and calcium. The increase in the fluorescence signal is rapid and largely completed after 300 s. While the fluorescence increase suggests that the kynurenine residue has entered a more hydrophobic environment, such as the apolar interior of a lipid membrane, it may under certain conditions also be observed in the absence of oligomerization (see Section 2.3.3) and therefore does not prove that oligomerization has indeed occurred.

Oligomerization itself can be monitored with perylene-daptomycin by way of concomitant formation of perylene excimers (see Chapter 3). The fluorescence of perylene-daptomycin at 560 nm is predominantly due to excimers. Figure 4.1B shows that, as with the kynurenine fluorescence, the perylene excimer fluorescence develops and stabilizes very rapidly after the addition of perylene-daptomycin to liposomes and calcium. Therefore, oligomerization proceeds rapidly and reaches completion on the time scale of the experiment.

Figure 4.1C shows that the kynurenine emission signal is linear across the range of concentrations and under the measurement conditions of the experiment. Thus, the kynurenine emission can be used to measure the amount of fluorescence-emitting oligomers.

4.3.3 Kynurenine fluorescence of daptomycin in the presence of NBD-daptomycin

When daptomycin and NBD-daptomycin are mixed and then added to membranes, the kynurenine fluorescence of the unlabeled species is strongly suppressed (Figure 4.2A). The strong reduction of the kynurenine emission in this sample suggests that most unlabeled daptomycin

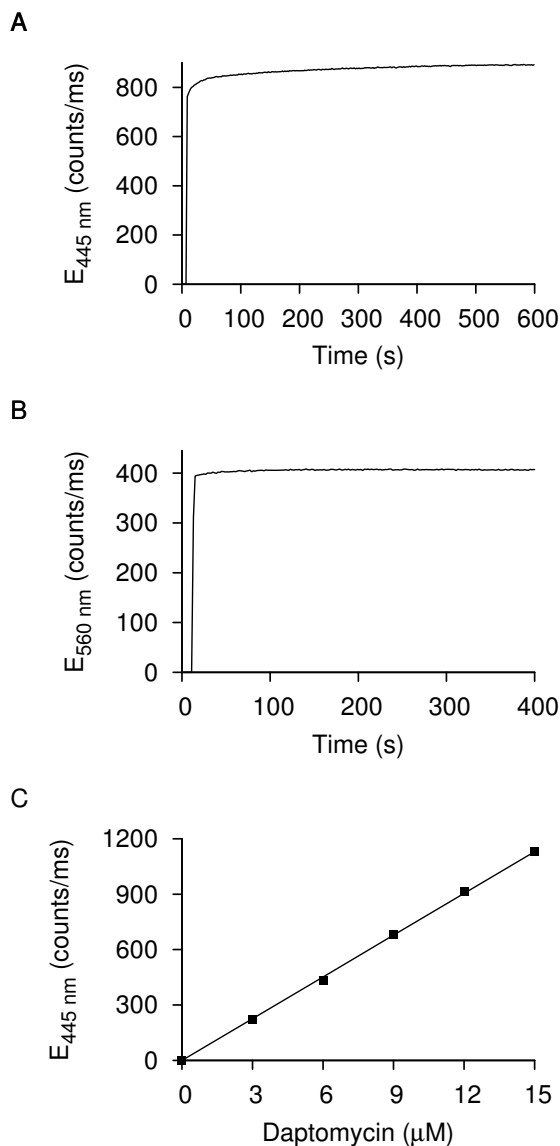


Figure 4.1 Time course and linear range of daptomycin fluorescence responses to interaction with liposomes. A: Time course of kynurenine fluorescence of 10 μM native daptomycin upon addition of PC/PG liposomes (250 μM total lipid) and calcium (6 mM) at time = 0. The small initial lag is caused by the shutter of the instrument. B: Time course of perylene fluorescence ($\lambda_{\text{ex}} = 430 \text{ nm}$, $\lambda_{\text{em}} = 560 \text{ nm}$) of 2.5 μM perylene-daptomycin to liposomes and calcium as before. The emission at 560 nm is mostly caused by excimers (see Chapter 3), indicating that oligomer formation is instantaneous. C: Linear range of kynurenine fluorescence upon addition of native daptomycin to liposomes and calcium as before and incubation for 300 s.

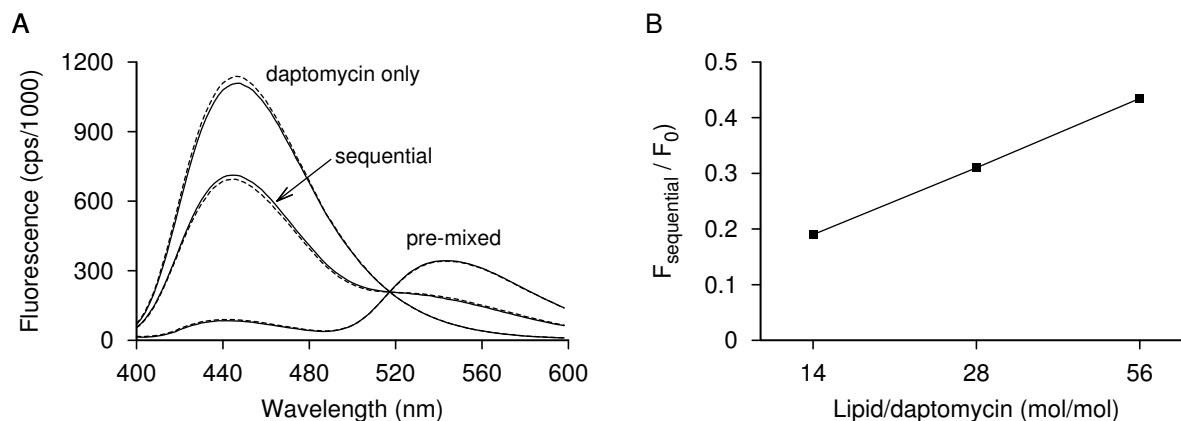


Figure 4.2 FRET within and between daptomycin oligomers. A: Fluorescence emission spectra of 2.4 μM daptomycin only, and of daptomycin (2.4 μM) plus NBD-daptomycin (0.9 μM), pre-mixed before addition to liposomes and calcium or added sequentially to the latter, with incubation for 3 min between the additions of the two species. Solid lines are spectra taken 5 min after sample preparation, and dashed lines are spectra acquired after an additional incubation for 10 min after the first measurement. The absence of an intensity decrease in the sequentially prepared sample indicates that the donor and acceptor oligomers remain distinct and stable on the time scale of the experiment. B: Kynurenine fluorescence intensity of samples containing native daptomycin and NBD-daptomycin at 2:1 molar ratio, and sequentially incubated with liposomes and calcium, at 250 μM total lipid and the indicated ratio of lipid to total daptomycin, relative to samples containing the same amount of native daptomycin but no NBD-daptomycin.

has indeed been incorporated into hybrid oligomers (assumption 3), and that its kynurenine fluorescence is effectively quenched within these hybrids (assumption 4).

Figure 4.2A also shows the fluorescence of a sample that was prepared sequentially, such that unlabeled daptomycin was applied to the membranes first, and NBD-daptomycin was applied 3 minutes later. Under these conditions, daptomycin and NBD-daptomycin should form oligomers separately. Here, the kynurenine signal is higher. It remains virtually unchanged after 10 min, indicating that the two oligomer species have remained separate and have not rearranged into hybrid oligomers; thus, individual oligomers are stable on this time scale (assumption 5).

On the other hand, the kynurenine fluorescence of the sequentially prepared sample in Figure 4.2A is below that of a sample containing no NBD-daptomycin at all. This indicates that,

in the sequentially prepared sample, the kynurenine fluorescence of the daptomycin-only oligomers is partially quenched by FRET to NBD-daptomycin oligomers in the vicinity. Raising the ratio of lipid to daptomycin reduces but does not eliminate FRET between oligomers (Figure 4.2B).

For our intended use of equation 4.3, we need to determine the FRET that occurs within hybrid oligomers, as opposed to between them. According to assumptions 2 and 5, we can correct for FRET between oligomers experimentally by adding a fixed quantity of unlabeled daptomycin to the same sample after the hybrid oligomers have been formed. The principle of this correction method is illustrated in Figure 4.3A; its details are given in Section 4.2.3.

The experiments described so far support our assumptions 2 and 5. Assumptions 3 and 4 are supported to a degree: Hybrid oligomer formation and strong quenching by FRET are evident, but perfect randomness of oligomerization and quantitative FRET are hard to prove, as is the uniformity of the oligomer subunit stoichiometry (assumption 1). For now, these latter assumptions may be taken as working hypotheses; we will revisit them in the Discussion.

4.3.4 Kynurenine fluorescence in hybrid oligomers formed on membranes

In the experiments shown in Figure 4.4, NBD-daptomycin was combined with unlabeled daptomycin at various molar ratios; the total of both species was kept constant at 10 μM . The mixtures were added to PC/PG membranes (250 μM total lipid) and calcium (6 mM). The kynurenine fluorescence of the ensuing oligomers was measured and corrected for FRET between oligomers as described above, and the oligomer subunit stoichiometry, n , was calculated using equation 4.3. The calculated individual values for n vary between 5.7 and 7.5.

In sum, the results shown in Figure 4.4 support the notion that daptomycin forms oligomers with approximately six to seven subunits on membranes. This interpretation must be qualified in several ways, as is discussed below.

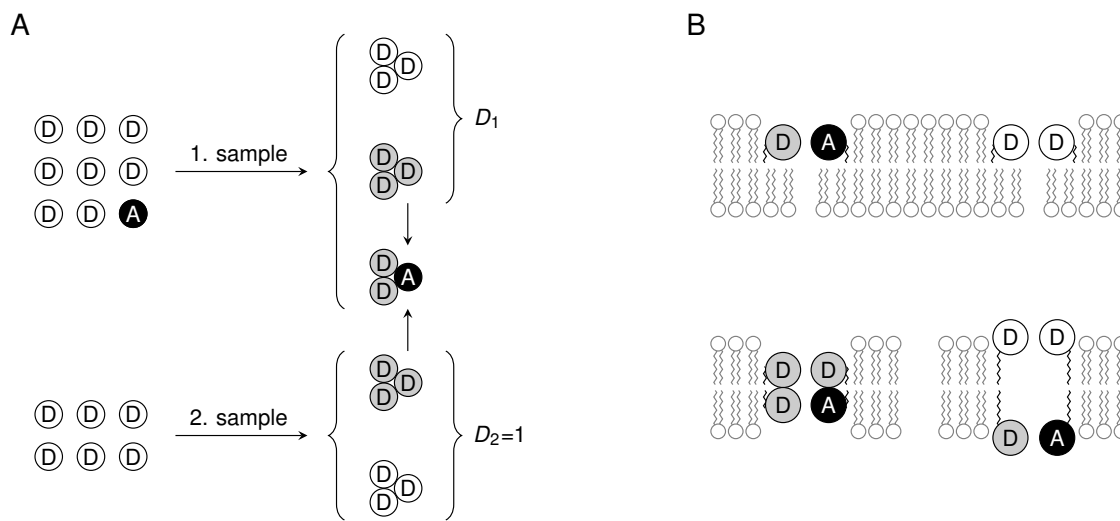


Figure 4.3 Illustration of the experimental approach used to measure oligomer subunit stoichiometry (A), and of hypothetical oligomer structures (B). A: When a mixture of donor (D; native daptomycin) and acceptor molecules (A; NBD-daptomycin) is applied to membranes, they randomly combine into oligomers. The fraction D_1 of donor monomers that become incorporated into pure donor oligomers is related to the number of oligomer subunits according to equation 4.2. In order to determine D_1 from the donor fluorescence, the latter must be corrected for FRET between donor-only oligomers and acceptor-containing oligomers in the vicinity. To this end, a second sample containing native daptomycin only is added subsequently. These molecules will all be converted to donor-only oligomers; that is, $D_2 = 1$. At the same time, they will be quenched by FRET to acceptor-containing oligomers to the same extent as the oligomers represented by D_1 . Their fluorescence intensity can therefore be used to calibrate the determination of D_1 from the donor fluorescence measured after the first step. B: Hypothetical daptomycin oligomer structures. It is presently unknown whether daptomycin becomes embedded only into the outer monolayer (top) or into both monolayers (bottom). In the latter case, it is conceivable that the distance between two subunits embedded in different monolayers exceeds the Förster radius for FRET between kynurenine and NBD (2.7 nm). In this case, the actual subunit stoichiometry may be up to twice the value obtained by the FRET experiments presented in this study.

4.4 DISCUSSION

This study aimed at determining the subunit stoichiometry of the membrane-associated daptomycin oligomer. The approach taken here is similar to that used previously for measuring the number of lipid II molecules that are incorporated into the transmembrane pores formed by the

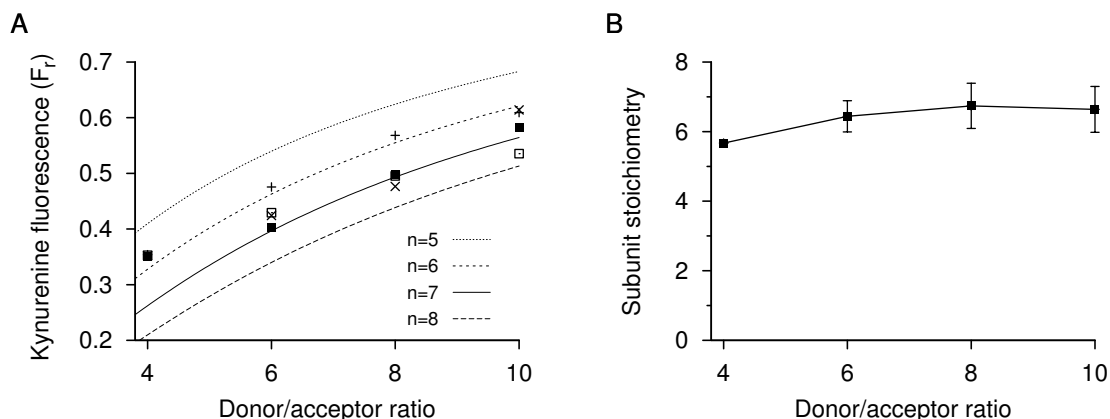


Figure 4.4 Determination of oligomer subunit stoichiometry by FRET. A: Kynurenine fluorescence intensities of 4 individual experiments, each performed at 4 different donor/acceptor ratios. The fluorescence intensities were corrected according to equation 4.1. The curves represent theoretical values calculated according to equation 4.2. B: Subunit stoichiometries calculated using equation 4.3 from the values shown in A (averages \pm standard deviation).

lantibiotic¹ nisin [56]. In both cases, random mixing of a fluorescently labeled derivative with the native species was assumed, and the oligomer subunit stoichiometry was extracted from the probability of mutual interaction of two fluorophore molecules. Both approaches also assume the interaction between two fluorophore molecules to occur with equal and complete efficiency, regardless of their respective positions and distance within the oligomer. In Ref. [56], the interaction in question was the formation of pyrene excimers, whereas FRET was employed in the current study. Heteromer analysis using FRET has also previously been used to characterize the dimeric nature of membrane-associated gramicidin [138].

Both excimer formation and FRET are sensitive to distance. In the case of FRET, the distance dependency is given by the Förster radius, R_0 , which for unlabeled daptomycin and NBD-daptomycin was previously estimated to 2.7 nm (Chapter 2). The thickness of a lipid bilayer is on the order of 4 nm. While the fluorescence changes of kynurenine in native daptomycin [77] and of NBD in NBD-daptomycin suggest that both fluorophores insert into the membrane, it is possible that they both remain close to the membrane surface. It is currently

¹The so-called “lantibiotics” are antibiotics that contain one or more lanthionine residues. Lanthionine contains two alanine residues linked by a thioether bridge.

unknown whether a single daptomycin oligomer interacts with one or both membrane leaflets. If indeed it interacts with both, subunits located in opposite leaflets may be beyond the reach of mutual FRET (see Figure 4.3B). The apparent oligomer stoichiometry obtained with the present method would then reflect the number of subunits in only one of the two monolayers, and the total number of subunits might in fact be twice as high. To resolve this question, it would be desirable to use probes that provide a greater effective R_0 ; however, several other fluorescent derivatives that we tested showed a significant reduction in their specific activity, which suggests that their oligomerization may be impaired, too, and therefore that they may not be able to randomly co-oligomerize with native daptomycin.

The extraction of the subunit stoichiometry from FRET within hybrid oligomers requires correction for FRET occurring between donor-only and acceptor-containing oligomers. In principle, it should be possible to separate oligomers from one another by dilution, that is, by making the ratio of daptomycin to lipid very small. However, this approach is not feasible in practice, because the fluorescence intensity of kynurenine is low; its molar extinction coefficient is 4000, and its quantum yield is only 0.04 (see Chapter 2). Furthermore, at high lipid concentration and in the presence of calcium, the PG-containing liposomes become very turbid, which interferes with the accurate measurement of fluorescence intensities. It was therefore necessary to correct for, rather than physically eliminate, FRET between oligomers.

Our fluorescence correction procedure assumes that all daptomycin oligomers are distributed evenly in the membrane, or alternatively that the native daptomycin applied in the second step will be distributed in the membrane in the same manner as the mixture of the native and NBD-daptomycin that was applied in the first step. In this context, it is noteworthy that the extent of FRET between oligomers (cf. Figure 4.2B) is greater than predicted for randomly distributed donors and effectors by the pertinent formula given by Wolber and Hudson [144] with the parameters of our system ($R_0 = 2.7$ nm, surface area of 0.7 nm² per lipid molecule).

It is known that acidic phospholipids within mixed membranes tend to cluster in the presence of calcium, and this tendency is further promoted by proteins and peptides that bind to

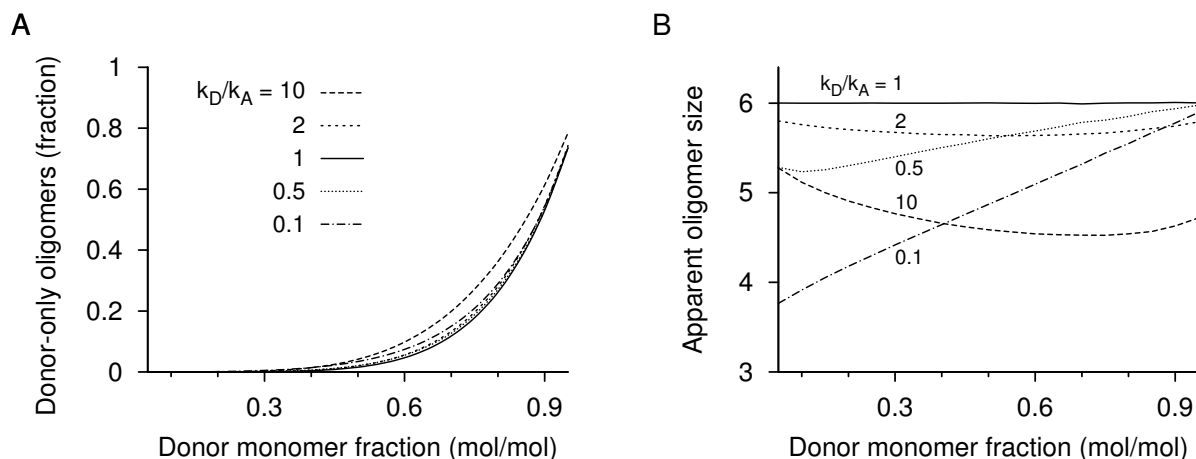


Figure 4.5 Numerical simulation of oligomerization with varying bimolecular rate constants for the incorporation of donor and acceptor monomers into growing oligomers. The oligomer is assumed to contain 6 subunits. The bimolecular rate constant k_A , which controls the addition of an acceptor monomer to an incomplete oligomer, was held at 1 in each case, whereas the rate constant k_D , which controls the addition of a donor monomer to an incomplete oligomer, was varied between 0.1 and 10. The program used to perform the calculation is mentioned in the supplementary file of the Ref. [101]. A: For each combination of rate constants, the fraction d of donors that are incorporated into donor-only oligomers is plotted as a function of the fraction of donor monomers d in the initial mixture (compare equation 4.2). B: The apparent subunit stoichiometries that would be inferred from the data shown in A according to equations 4.2 and 4.3 are plotted as functions of d .

them multivalently [142]. With bacterial cells, daptomycin clusters have indeed been observed on bacterial membranes. Importantly, however, in those experiments, two samples of daptomycin that carried two different labels and were added to the same cells at different times were found enriched and superimposed in the very same locations on the membrane (J. Pogliano, personal communication; see Figure 4.6). We assume that the same happens on our liposome membranes.

In the previously obtained NMR structure of daptomycin in water [9], the diameter of the peptide moiety, in a plane perpendicular to the extended fatty acyl chain, ranges from 0.8 to 1.2 nm. Therefore, even if we confine the consideration to subunits within the same monolayer, it is conceivable that the cumulative diameter of the oligomer subunits may exceed the range within which FRET can be assumed to be quantitative, as was done in the present study. In

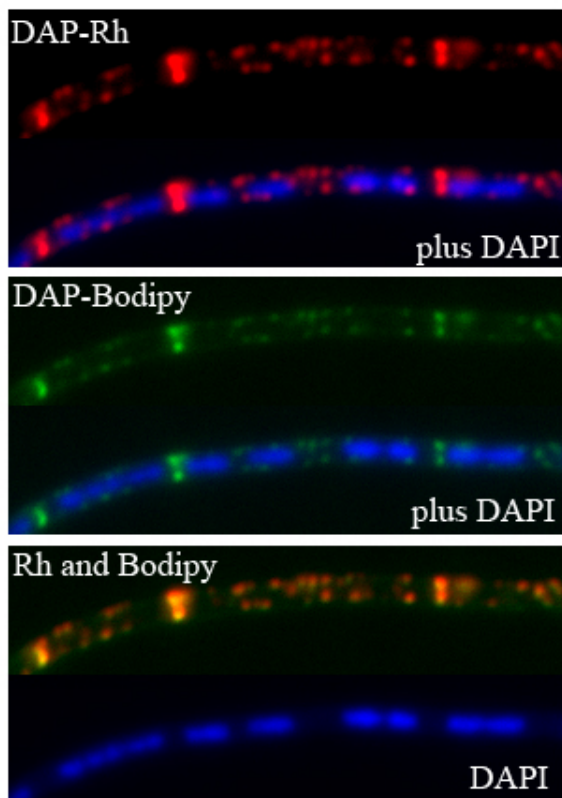


Figure 4.6 Dual labelling of bacterial cells with two colors of two labeled derivatives of Daptomycin. In sequential additions, Dap-Bodipy (16 $\mu\text{g/ml}$) was incubated with cells for five minutes and then Dap-Rh (16 $\mu\text{g/ml}$) was added for five more minutes. Excess labelled daptomycin was removed by washing the cells three times in LB. Cells were stained with DAPI (blue) to visualize the nucleoids. The same field of cells is shown in each panel. Experiment performed by J. Pogliano.

this context, however, it is interesting to note that, upon prolonged exposure of daptomycin oligomers that were formed on membranes to 10 mM of the detergent CHAPS, an apparent oligomer subunit stoichiometry of up to 15 was observed. This finding suggests that within the membrane—or, as discussed above, within a membrane monolayer—it is not the R_0 that limits the extent of FRET but rather the actual number of subunits present. (CHAPS was also found to induce rapid destabilization of oligomers, as evident from scrambling of donor and acceptor subunits, and therefore CHAPS-solubilized samples cannot be considered a valid model of the membrane-associated state of daptomycin.)

Another caveat concerning the method employed in this study concerns the relative rates of oligomer incorporation of the donor and the acceptor; these rates cannot currently be measured accurately. Figure 4.5 shows simulated data to illustrate what happens if the donor oligomerizes either more rapidly or more slowly than the acceptor. While native daptomycin and NBD-daptomycin have the same specific activity, it still seems possible that their rates of oligomer incorporation may be slightly different, which may then cause the estimated number of subunits to deviate from the true value.

Our analysis is also based on the assumption of uniform oligomer subunit stoichiometry. While our data do not provide direct evidence for or against this assumption, it seems to fit with the previously reported selective and specific nature of the permeability lesion caused by daptomycin [125, 2]. Less selective and possibly dose-dependent leakage of larger marker molecules might be expected in the case of inhomogeneous subunit stoichiometries of oligomers and associated membrane defects.

In sum, the results of our study indicate that the membrane-associated daptomycin oligomer contains approximately six to seven subunits. Whether this number applies to the entire oligomer or rather to a part of it that is embedded in one of the two membrane leaflets remains to be determined. In either case, the findings support the notion that daptomycin acts through the formation of oligomers of discrete size, rather than through detergent-like or carpet-mode wholesale membrane disruption. This conclusion is in line with the previously observed absence of morphological disruption of bacterial cell membranes exposed to daptomycin [27], as well as with the selective inhibition of membrane potential-dependent, but not of phosphotransfer-dependent membrane transport [4].

Chapter 5

Summary and future research work

5.1 SUMMARY

In this thesis, I have investigated the mode of action of the lipopeptide antibiotic daptomycin.

Chapter 2 focused on studying the oligomerization of daptomycin on liposomal and bacterial membranes as part of the mechanism of its antibacterial activity against Gram-positive bacteria. The previous studies only assumed that oligomerization of daptomycin molecules on membranes may be required for the bactericidal activity of daptomycin, but did not provide any experimental evidence. In our study, this evidence was obtained by using FRET, which occurred between kynurenine in native daptomycin as the donor and NBD in NBD-daptomycin as the acceptor. Oligomerization was also evident from the self-quenching of NBD-daptomycin fluorescence on membranes. The oligomers were stable on a time scale of several hours. They were detected on liposomal membranes containing phosphatidylglycerol (PG), combined with either phosphatidylcholine (PC) or phosphatidylethanolamine (PE) plus cardiolipin (CL), as

well as on membrane vesicles prepared from *Bacillus subtilis* (ATCC 6633) cells. No oligomerization was observed on membranes lacking PG. Therefore, we showed that oligomerization has the same requirements for Ca^{2+} and for PG that also applies to antibacterial activity. This strongly suggests that oligomerization is important for antibacterial action.

It was previously observed by using NMR that daptomycin can oligomerize in solution, and it was proposed that this step was integral to the mode of action of daptomycin [9]. However, those studies were performed at relatively high daptomycin concentrations, in the millimolar range. In contrast, our studies were conducted with fluorescence, which is more sensitive than NMR and permits daptomycin molecules to be observed under conditions and concentrations similar to MIC, both in solution and on membranes. Using a daptomycin derivative fluorescently labeled with Alexa Fluor 350, we showed that oligomerization did not occur in solution at daptomycin concentrations similar to MIC. Therefore, daptomycin most likely interacts with the membrane as a monomer and does not need to undergo aggregation or oligomerization in solution in order to acquire antibacterial activity.

The work described in Chapter 3 served to further characterize the membrane-associated daptomycin oligomer by using excimer fluorescence. An excimer, or excited dimer, is a complex that forms from one perylene fluorophore molecule that is in the excited state and another one in the ground state [78]. For this purpose, daptomycin was labeled with perylene instead of pyrene, which is more commonly used for excimer studies. This was necessary because the excitation energy of pyrene was almost quantitatively transferred to kynurenine by FRET, preventing the observation pyrene excimers. In contrast, perylene absorbs and emits at longer wavelengths than pyrene [66], and therefore was unaffected by FRET.

Under conditions resembling those used in the previous FRET experiments, oligomer formation of perylene-daptomycin was evident through excimer fluorescence on both liposomes and bacterial cells. Since excimer formation involves direct contact of the two labels in question, it is highly sensitive to distance. The observation of excimer fluorescence thus imposes a stricter constraint on the distance of the interaction than that implied by the observation of

FRET. Since perylene had been introduced into the fatty acyl residue position of daptomycin, the observation of excimer fluorescence indicates that the fatty acyl residues of adjacent subunits of the oligomer are interacting directly with one another.

Perylene-daptomycin excimer fluorescence was also used to directly demonstrate the efficient formation of daptomycin oligomers on intact bacterial cells. Perylene-daptomycin retained its antibacterial activity at a slightly reduced level. This gives further support to the notion that the oligomeric form of daptomycin is responsible for the antibacterial effect.

It is also noteworthy that our study is the first one to use perylene excimer fluorescence for the characterization of peptide or protein oligomerization. It therefore illustrates that perylene excimer fluorescence is useful in the experimental study of biochemical systems.

In a lipid bicelle system, we found that excimer fluorescence could be titrated with phosphatidylglycerol. The result showed that daptomycin oligomerization is driven by a stoichiometric interaction with phosphatidylglycerol (PG). Therefore, a single molecule of phosphatidylglycerol is adequate to convert daptomycin to a conformation that is proficient for oligomerization. This result confirms that PG interacts with daptomycin directly and specifically. This is consistent with but goes beyond the previous conclusion from FRET experiments (Chapter 2).

In Chapter 4, we tried to determine the subunit stoichiometry of the membrane-associated daptomycin oligomer. From the extent of energy transfer observed in such hybrid oligomers of labeled and unlabeled daptomycin, we calculated that the oligomer contains approximately 6 to 7 subunits. This calculation is based on the assumptions that all oligomers have the same number of subunits, and that donor and acceptor co-oligomerize in a perfectly random manner. We cannot exclude the possibility that the oligomer spans both membrane monolayers in a manner that reduces or prevents FRET between subunits embedded in opposite monolayers; in this case, the actual number of subunits might be up to twice as high as apparent from our experiments. While the number of subunits is therefore not yet known precisely, our findings do indicate a relatively small size of the daptomycin oligomer; this agrees with a relatively selec-

tive membrane permeabilization and with the absence of membrane discontinuities observable by electron microscopy shown in earlier studies.

In conclusion, the work in my thesis has shown that daptomycin forms small, stable oligomers on bacterial membranes. Oligomerization depends on a stoichiometric interaction with phosphatidylglycerol and very likely is required for the antibacterial effect of daptomycin. These findings significantly enhance our understanding of daptomycin's mode of action.

5.2 FURTHER RESEARCH WORK

The findings in my thesis raise additional questions that need to be addressed by future research.

5.2.1 Causative role of oligomerization in bactericidal action

While this thesis has provided solid evidence for a close correlation of oligomer formation on membranes and bactericidal action, a causative role has not been unequivocally proven. A possible approach to this question might be the use of mixtures of wild type daptomycin and a functionally impaired variant; a study cited earlier [104] has provided several such molecules. If oligomerization is *not* important, the mixtures should display strictly additive activity. On the other hand, if the phenotype of one species were to dominate the other, this would indicate that the mutual interaction of several molecules is critical for the activity of daptomycin.

5.2.2 Contribution of the negatively charged phospholipids other than PG to daptomycin binding, oligomerization, and bactericidal action

Binding and oligomerization of daptomycin on membranes require Ca^{2+} and correlate with the presence of phosphatidylglycerol (see Chapter 2). However, it has not yet been determined how other negatively charged phospholipids may affect the interaction of daptomycin with membranes.

Apart from PG, the most abundant negatively charged phospholipid in bacterial membranes is cardiolipin. We performed some initial experiments on cardiolipin, which showed that it facilitates calcium-dependent binding of daptomycin molecules to the membrane. However, there is no obvious evidence regarding formation of oligomers on membranes composed from PC and cardiolipin in ratio 4:1. The effect of cardiolipin on the antibacterial effect of daptomycin remains to be determined.

5.2.3 Functional characterization of the membrane defect

In addition to the structure, the functional properties of the membrane defect also remain incompletely understood. Exposure of *Staphylococcus aureus* to daptomycin has been reported to cause release of calcein from the cells, while the DNA-binding dye ToPro3 remains excluded [27]. The selectivity of the membrane defect for molecules differing in size and charge deserves to be more completely characterized.

5.2.4 Analysis of oligomer structure on membranes by NMR

The relatively small size of the membrane-associated daptomycin oligomer suggests that it should be feasible to determine its structure by NMR, and Dr. Dieckmann's group has begun such experiments. NMR in solution is, however, hampered by sample aggregation, which is due to the simultaneous requirement for Ca^{2+} and PG. Solid state NMR may be another possibility, and some initial experiments have been run by Dr. Ladizhansky at the University of Guelph. It is not yet possible to decide whether these experiments will succeed.

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