# An acyl-linked dimer of daptomycin is strongly inhibited by the bacterial cell wall

Robert M. Taylor,[‡,§] Bradley Scott,[‡,‖] Scott Taylor,[*,‡,⊥] and Michael Palmer[*,‡,⊥]

*Department of Chemistry, University of Waterloo, 200 University Ave W, Waterloo ON N2L3G1, Canada*

E-mail: s5taylor@uwaterloo.ca; mpalmer@uwaterloo.ca

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[*] To whom correspondence should be addressed

[‡] Department of Chemistry, University of Waterloo, 200 University Ave W, Waterloo ON N2L3G1, Canada

[§] These authors contributed equally to this work

[‖] These authors contributed equally to this work.

[⊥] Corresponding author

**Abstract**

The lipopeptide antibiotic daptomycin is active against Gram-positive pathogens. It permeabilizes bacterial cell membranes, which involves the formation of membrane-associated oligomers. We here studied a dimer of daptomycin whose two subunits were linked through a bivalent aliphatic acyl chain. Unexpectedly, the dimer had very low activity on vegetative *Staphylococcus aureus* and *Bacillus subtilis* cells. However, activity resembled that of monomeric daptomycin on liposomes and on *B. subtilis* L-forms. These findings underscore the importance of the bacterial cell wall in daptomycin resistance.

## Keywords

lipopeptide antibiotics, antibiotic resistance, cell wall permeability, bacterial L-forms



Daptomycin is a calcium-dependent lipopeptide antibiotic that depolarizes the cell membranes of Gram-positive bacteria (*1*, *2*). In previous studies, we have shown that permeabilization involves the formation of daptomycin oligomers on target cell membranes (*3*, *4*). Oligomer formation has an entropy cost, and we reasoned that lowering this cost by joining two daptomycin molecules together might produce a derivative with greater antimicrobial activity. Within the daptomycin oligomer, the fatty acyl tails of adjoining subunits are in close proximity (*5*, *6*), suggesting that joining them covalently should not impose any unfavourable steric constraints on oligomer formation; we thus used a bivalent fatty acyl moiety to effect dimerization (see Figure 1).

The dimer was then tested for antibacterial activity against several strains of *Staphylococcus aureus* and *Bacillus subtilis*. Unexpectedly, the MIC was higher than that of the monomer with all tested strains by one to two orders of magnitude (see Tables 1 and 2).

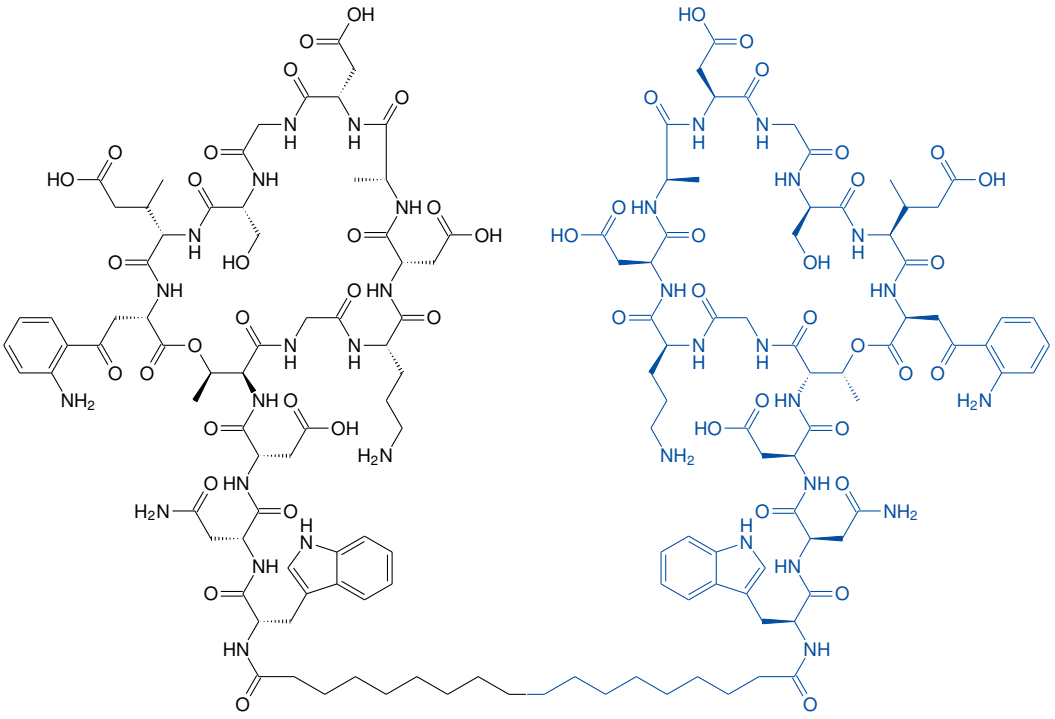

Figure 1: Structure of the semisynthetic daptomycin dimer characterized in this study. Each half of the molecule corresponds to a native daptomycin monomer. Details on the synthetic procedure and on the characterization are given in the Supplementary Materials.

Several explanations might conceivably account for the marked reduction in activity. Firstly, the steric constraints introduced by dimerization might disrupt the membrane-permeabilizing activity. To test this possibility, we tested the dimer in a liposome model that recapitulates both the oligomer formation (*7*) and the membrane permeabilization (*8*) which are also observed in bacterial cells (*1*, *3*–*5*, *9*). Membrane binding and oligomerization is accompanied by a large increase in the fluorescence intensity of daptomycin's intrinsic kynurenine residue (*10*, *11*). The fluorescence increase occurs in a virtually indistinguishable manner with the monomer and the dimer (Figure 2A). Similarly, when phosphatidylglycerol was omitted from the liposomes, both the monomer (*3*) and the dimer required much higher calcium concentrations for membrane binding. These findings suggest that the dimer exhibits normal membrane interaction.

Liposome permeabilization was tested with a coupled fluorescence assay (*8*). Permeabilization occurred more rapidly with the dimer than the monomer. Moreover, unlike the monomer, the dimer did not require addition of the proton ionophore CCCP to effect permeabilization,

Table 1: Minimal inhibitory concentrations (MIC; units: μg/mL) of monomeric and dimeric daptomycin on various strains of *Staphylococcus aureus*—either susceptible (MSSA) or resistant (MRSA) to methicillin. Test medium: Mueller-Hinton broth, supplemented with 1.25 mM $CaCl_2$. MRSA strains are distinct clinical isolates not contained in type culture collections.

| | MSSA | | MRSA | |
|---|---|---|---|---|
| | ATCC 6538 | ATCC 25293 | isolates 1-3 | isolates 4,5 |
| Daptomycin | 0.25 | 0.5 | 0.5 | 0.5 |
| Daptomycin dimer | 5 | 75 | 30 | 75 |

suggesting that it may form pores large enough to permit passage of protons bound to buffer ions. (Indeed, as in the example experiment shown in Figure 2B, CCCP caused a minor but reproducible decrease in the pyranine fluorescence signal. We suspect, but have not rigorously determined, that this is a fluorescence artifact which arises when both pyranine (*12*) and CCCP interact with liposomes membranes.) Overall, the liposome experiments indicated that the dimer retained its membrane-permeabilizing activity, while the apparent change in functional diameter suggests that some subtle structural difference exists between the membrane-associated forms of monomeric and dimeric daptomycin, respectively.

Several investigators have suggested that the bactericidal effect of daptomycin may not be due to membrane permeabilization alone but also involve the direct inhibition of biosynthetic pathways (*13*–*16*). Such a mechanism would require a specific interaction between daptomycin and a bacterial macromolecule or, possibly, certain type of membrane lipid domain (*16*, *17*). If this specific interaction were disrupted in the dimer, this could account for its greatly reduced activity on bacterial cells but not liposome membranes. Alternatively, the discrepancy might be explained by a reduced permeation of the dimer across the bacterial cell wall (*18*). Increases in cell wall thickness (*19*) and in the extent of D-alanine modification of cell wall lipoteichoic acids (*20*, *21*) have previously been associated with daptomycin resistance in clinical isolates, and it seemed possible that impeded diffusion of the dimer across the murein layer might prevent it from reaching the target membrane.

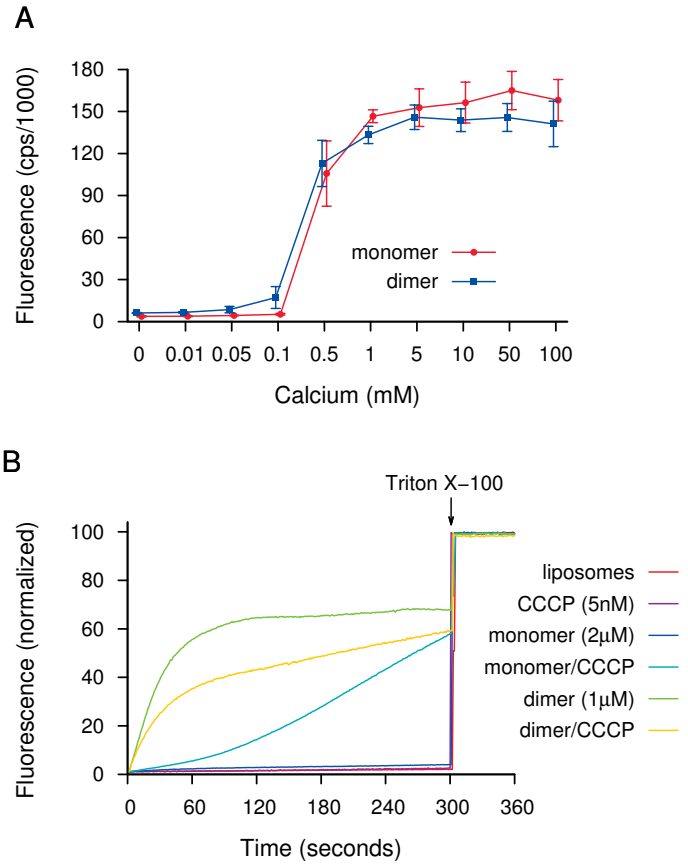


Figure 2: Interaction of daptomycin monomers and dimers with PC/PG liposomes. A: Increase in kynurenine fluorescence (excitation wavelength: 365 nm; emission wavelength: 445 nm) as a function of calcium concentration. Error bars represent standard deviations from two experiments. B: Membrane permeabilization. The fluorescence of pyranine entrapped in the liposomes rises with the internal pH as protons are exchanged across the membrane for sodium ions that permeate across pores formed by daptomycin. See Supplementary Materials for details.

To distinguish between these possibilities, we tested the activity of the dimer on bacterial L-forms, which are cell wall-less variants of bacterial cells that can survive and propagate when protected from osmotic lysis by inert osmolytes (e.g. 0.6M sucrose) in the growth medium. These were generated from the *Bacillus subtilis* strain PDC134, in which expression of the murE operon, which is required for murein synthesis, has been made dependent on xylose, so that xylose withdrawal triggers conversion to L-forms (*22*). On these L-forms, the MIC of the monomer at 5 mM $Ca^{++}$ decreased from 0.5 to 0.075 µg/mL; this increase in activity agrees with previous findings (*23*). With the dimer, however, activity increased dramatically, resulting in an MIC of

0.2 µg/mL which was only slightly (but reproducibly) above that of the monomer. Also note that the MIC for vegetative cells of PDC134 is similar to that observed with *B. subtilis* strain 1046, indicating that the mutations that characterize PDC134 (*22*) have no major effect on the susceptibility of vegetative cells.

Overall, removal of the cell wall increases the activity of both the monomer and the dimer, and it greatly reduces the difference in activity between them. These observations underscore the importance of the cell wall in bacterial resistance to daptomycin. It may be noted that increasing calcium concentrations to unphysiologically high concentrations increases the activity of daptomycin on vegetative *Bacillus subtilis* cells, and that again this increase is more marked with the dimer than the monomer (see Table 2). Since calcium should mask negative charges in both the cell wall and on daptomycin, this observation suggests that electrostatic repulsion plays an important role in impeding the permeation of daptomycin across the cell wall.

The small discrepancy that remains—the dimer at least matches the monomer's activity on liposomes, but it is somewhat less active on L-forms—may leave some scope for a possible contribution of mechanisms other than membrane permeabilization to the antibacterial effect of daptomycin, possibly the inhibition of some biosynthetic pathway (*16*, *24*, *25*) that may be disrupted or diminished in the dimer. However, the antibacterial activity of daptomycin varies with the length of the fatty acyl residue (*26*), and accordingly the replacement of daptomycin's native decanoyl residue by octadecanedioate to effect dimerization might also account for the observed difference in activity.

While a recent paper proposed that daptomycin inhibits cell bacterial murein synthesis by dislodging the enzymes MurG and PlsX (*16*), the observation made here and earlier (*23*) that daptomycin kills L-forms also reaffirms that daptomycin's bactericidal effect does not hinge on the disruption of cell wall synthesis, at least not with *Bacillus subtilis*. Permeabilization of bacterial cell membranes, while not confirmed by (*16*), has been observed repeatedly before (*1*, *2*, *9*) and accounts for the further observation that, unlike $\beta$-lactam antibiotics for example, daptomycin readily kills bacterial cells which are prevented from dividing by a bacteriostatic

antibiotic, and which are therefore protected from killing by inhibition of cell wall synthesis (*27*). It remains, of course, possible that daptomycin *both* permeabilizes membranes and inhibits cell wall biosynthesis, as does the lantibiotic nisin (*28*).

Table 2: Minimal inhibitory concentrations (MIC; units: μg/mL) of monomeric and dimeric daptomycin on L-forms and vegetative cells of *Bacillus subtilis*. Test medium: LB broth, supplemented with $CaCl_2$ as indicated. Assays were performed five times, with each assay run in triplicate.

| | *B. subtilis* strain | | | | |
|---|---|---|---|---|---|
| | 1046 | | | PDC134 | |
| L-forms (+/-) | - | - | - | - | + |
| $CaCl_2$ (mM) | 1.8 | 5 | 100 | 5 | 5 |
| Daptomycin | 1.0 | 0.75 | 0.5 | 0.5 | 0.075 |
| Daptomycin dimer | >100 | >100 | 7.5 | 25 | 0.2 |

In summary, our study illustrates that the permeability of the Gram-positive cell wall must be taken into account when designing lipopeptide derivatives with the goal of improving antibacterial activity. Furthermore, it also sheds some light on the assumed role of aggregation in the antibacterial activity of daptomycin. Based on experiments with model membranes and with daptomycin solutions of relatively high concentrations, it has been proposed earlier that daptomycin must approach its target membrane as a non-covalent aggregate of approximately 14 subunits in order to effect permeabilization (*29*). Considering that the cell wall is quite impervious for an adduct of even just two subunits, it appears unlikely that it would be more permeable toward a considerably larger aggregate. The present study thus supports our previous contention (*3*) that daptomycin diffuses towards and binds its target membrane as a monomer.

# Experimental procedures

## Synthesis of dimeric daptomycin

Briefly, the side chain amino group of the daptomycin's ornithine residue was protected with BOC, the decanoyl residue was enzymatically removed, and the exposed N-terminal amino

groups of two monomers were linked by acylation with activated octadecanedioate. For details on the procedures used for preparation and characterization, please refer to the Supplementary Materials.

## Antimicrobial susceptibility testing of *S. aureus* and vegetative *B. subtilis* cells

Antimicrobial susceptibility tests were performed according to (*30*) with minor modifications. Serial dilutions of monomeric and dimeric daptomycin were prepared using 96-well microtitre plates in Luria-Bertani broth (*Bacillus subtilis*) or Mueller-Hinton broth (*Staphylococcus aureus*) that was supplemented with the concentrations of $CaCl_2$ indicated in Tables 1 and 2. Inoculation was performed by first diluting overnight cultures to a turbidity of McFarland standard 0.5, and then adding this diluted culture to the antibiotic samples at a volume ratio of 1:1.

Growth was assessed visually after incubation at 30°C (*B. subtilis*) or 37°C (*S. aureus*) overnight. Each antibiotic concentration was tested three times and in triplicate; growth and sterility controls were run in parallel.

## Generation and and antimicrobial susceptibility testing of *Bacillus subtilis* PDC 134 L-forms

The Bacillus subtilis strain PDC 134 has been engineered to make expression of several enzymes in murein synthesis dependent on xylose, so that L-forms can be obtained simply by withdrawing xylose (*22*). Xylose-free LB medium supplemented with MSM ($MgCl_2$, 20 mM; maleic acid, 20 mM; sucrose, 500 mM) at pH 7 was inoculated with a liquid culture of PDC 134 grown beforehand in the presence of xylose (0.5% w/v) and chloramphenicol (20 mg/ml). The culture was incubated at 30 °C overnight. To suppress growth of residual vegetative forms, ampicillin was added to this culture at 75 µg/mL. Conversion of the rod-shaped vegetative cells to spherical L-forms was confirmed by microscopy. Ampicillin was *not* present in the daptomycin MIC tests.

Antimicrobial activity tests were performed as above, except that LB-MSM medium was used and incubation at 30 °C was performed until the growth controls were unequivocally positive, which took 3-4 days.

## Kynurenine fluorescence

Liposomes (large unilamellar vesicles) were prepared either from an equimolar mixture of dimyristoyl-phosphatidylcholine (DMPC) and dimyristoyl-phosphatidylglycerol (DMPG) or from DMPC alone through polycarbonate membrane extrusion as described earlier (*3*). To a suspension of these liposomes (250 μM total lipid) in HEPES (20 mM, pH 7.4)/NaCl (150 mM), monomeric or dimeric daptomycin (3 μM and 1.5 μM final concentration, respectively) were added. $CaCl_2$ was present at the final concentrations indicated (0-50 mM). Samples were incubated at 37 °C for 3 minutes. Fluorescence spectra were acquired on a PTI QuantaMaster 4 instrument (excitation wavelength: 365 nm; emission wavelength: 400-600 nm).

## Liposome permeabilization

DMPC/DMPG liposomes loaded with pyranine and with an internal pH of 6.0 were prepared as described in (*8*), except that pyranine was used at only 1 mM rather than 5 mM, as this was found to significantly improve the stability of liposomes over time. (Another recent study also reported leakiness of liposomes loaded with higher concentrations of pyranine [4 mM] (*31*). On a similar note, we found that the liposomes became leaky at less than 0.5 mM calcium.) Moreover, while (*8*) used a series of different salts, only sodium chloride (100 mM) was used here.

Monomeric and dimeric daptomycin as well as the proton carrier carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP, Sigma) were added alone or in combination as indicated. At time 0, liposomes were added to a final concentration of 250 μM total lipid, and fluorescence (excitation wavelength: 460 nm; emission wavelength: 510 nm) was monitored for 300 seconds. Triton X-100 was then added to a final concentration of 0.1% to induce complete membrane disrup-

tion. The fluorescence intensity observed after Triton addition was used to normalize the entire curve.

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## Supporting Information Available

Experimental procedures and characterization data for dimeric daptomycin.

This material is available free of charge via the Internet at http://pubs.acs.org/.

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