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Letter

Binding Studies Reveal Phospholipid Specificity and Its Role in the Calcium-Dependent Mechanism of Action of Daptomycin

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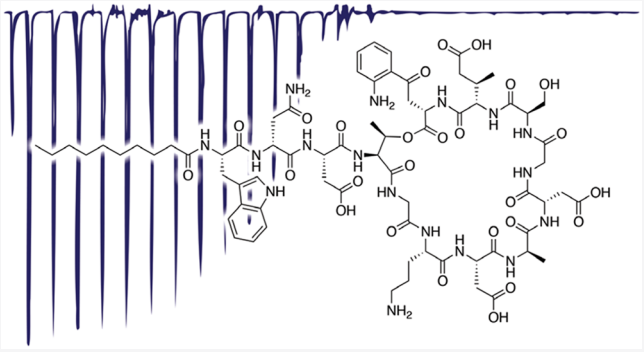
ABSTRACT: Multidrug-resistant bacteria pose a serious global health threat as antibiotics are increasingly losing their clinical efficacy. A molecular level understanding of the mechanism of action of antimicrobials plays a key role in developing new agents to combat the threat of antimicrobial resistance. Daptomycin, the only clinically used calcium-dependent lipopeptide antibiotic, selectively disrupts Gram-positive bacterial membranes to illicit its bactericidal effect. In this study, we use isothermal titration calorimetry to further characterize the structural features of the target bacterial phospholipids that drive daptomycin binding. Our studies reveal that daptomycin shows a clear preference for the phosphoglycerol headgroup. Furthermore, unlike other calcium-dependent lipopeptide antibiotics, calcium binding by daptomycin is strongly dependent on the presence of phosphatidylglycerol. These investigations provide new insights into daptomycin's phospholipid specificity and calcium binding behavior.



KEYWORDS: *daptomycin, calcium dependent lipopeptide antibiotics, mechanism of action, isothermal titration calorimetry, phospholipid specificity*

Daptomycin (Figure 1) is the prototypic calcium-dependent lipopeptide antibiotic (CDA).[1] First approved in 2003 for the treatment of complicated skin and skin-structure infections,[2] daptomycin was subsequently approved in 2006 for the treatment of right-sided endocarditis and bacteremia.[3] Daptomycin is generally prescribed as a last resort agent in treating infections due to Gram-positive pathogens including methicillin-resistant and vancomycin-resistant *Staphylococcus aureus* (MRSA & VRSA) and vancomycin-resistant *Enterococci* (VRE). The emergence of daptomycin-resistant phenotypes is consistently linked with alterations in the composition of the bacterial membrane and fortification of the Gram-positive cell-wall.[4] Specifically, daptomycin resistance is often linked to changes related to the bacterial phospholipid phosphatidylglycerol (PG).[5−10] For instance, daptomycin resistance in *S. aureus* is classically associated with mutations in mprF (multiple peptide resistance factor), which encodes a bifunctional transmembrane enzyme that performs lysylation of PG,[5,6] effectively masking PG on the membrane. Another daptomycin resistance mechanism reported for *S. aureus* involves the secretion of PG-rich membrane domains. This phenomenon, described as phospholipid shedding,[7] is hypothesized to antagonize the activity of daptomycin by diverting the antibiotic into the extracellular space. Using a related strategy, daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic from the division septum by redistributing cardiolipin-rich domains across its cell membrane.[8] Furthermore, daptomycin resistant mutants have been reported to entirely omit PG and cardiolipin from their phospholipid bilayers, due to loss-of-function mutations in their phospholipid synthases.[9,10]

The role of PG as a target for daptomycin is further supported by its capacity to antagonize daptomycin's antimicrobial activity *in vitro*.[11] A number of mechanistic studies have also provided insights into the interactions of daptomycin with PG[12−17] as well as cardiolipin[18,19] and more recently, peptidoglycan precursors.[20] Multiple investigations using a variety of techniques have shown that daptomycin oligomerizes and induces significant changes to vesicles containing PG or PG/cardiolipin mixtures in a calcium dependent manner.[12−15] Recently, a fluorescence microscopy study showed that daptomycin colocalized with PG lipids in giant unilamellar vesicles and subsequently induced formation

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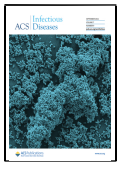

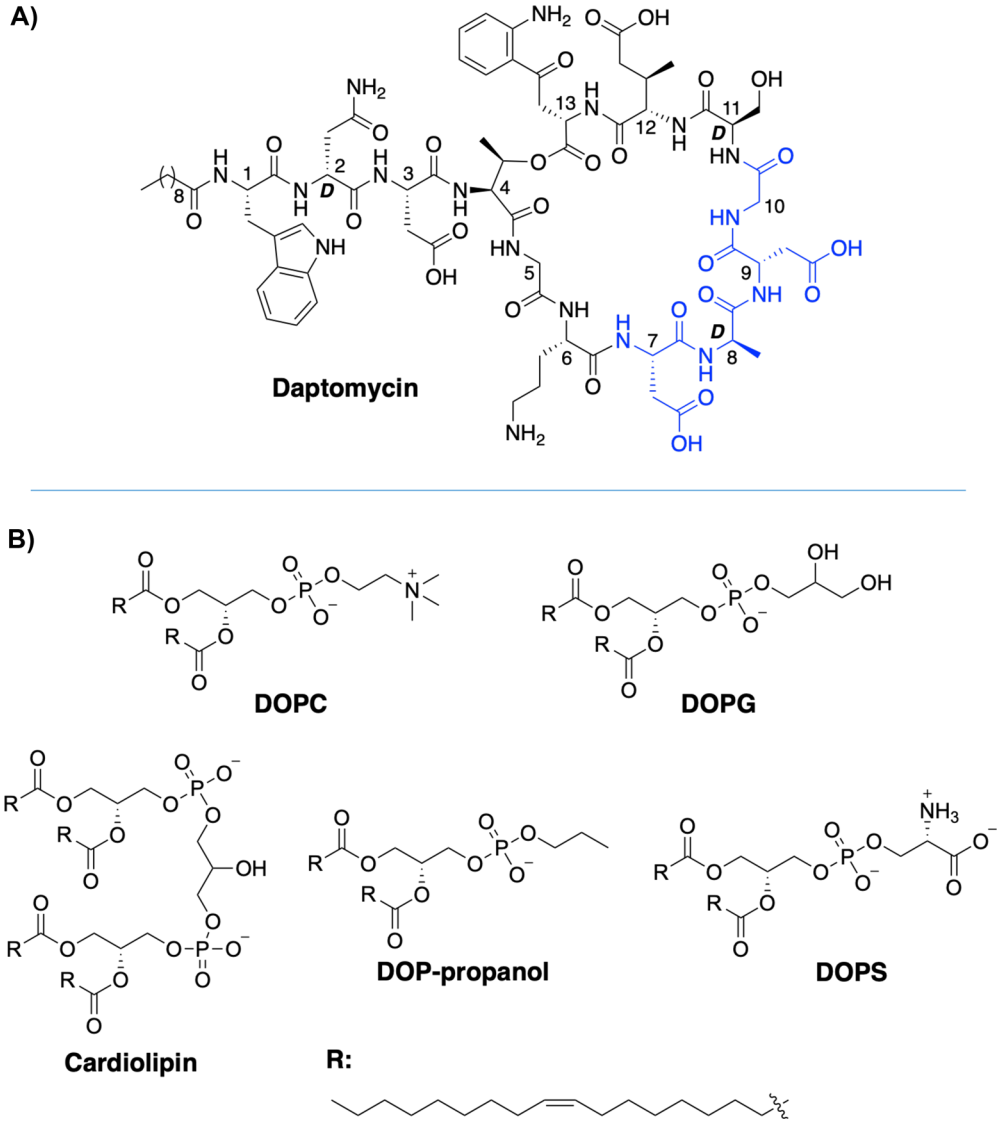

Figure 1. (A) Structure of daptomycin. Indicated in blue is the Aps-X-Asp-Gly calcium binding motif conserved among the CDAs. (B) Different phospholipids explored in the present study.

of daptomycin-PG clusters.[21] Palmer and co-workers have also used Isothermal Titration Calorimetry (ITC) to demonstrate the avidity with which daptomycin binds PG-containing large unilamellar vesicles (LUVs) in the presence of calcium ions.[18,22,23] Numerous biophysical studies have led to a generally accepted model wherein daptomycin is incorporated into a lipid assembly on the target membrane which results in changes in the molecular packing and overall fluidity and permeability of bilayers.[19,24,25] This membrane effect, in turn, impacts several essential membrane-associated processes such as the regulation of cell division and cell wall synthesis.[26,27,20]

The structure of daptomycin is characterized by a 10 amino acid macrolactone and an exocyclic linear tripeptide, which is *N*-terminally acylated with decanoic acid. Over the past decade, synthetic advances have provided access to structural analogues of daptomycin revealing important Structure–Activity Relationship (SAR) information.[28−31] The lipid tail of daptomycin is indispensable for antibacterial activity, and it is also known that elongation of the fatty acyl chain enhances daptomycin's activity.[1,32] With regard to the peptide core of daptomycin, a number of structural analogues prepared by Taylor and co-workers as well as our own group and others have provided important insights.[32−34] Changes to the highly conserved Asp-X-Asp-Gly calcium-binding motif (indicated in blue in Figure 1A) are poorly tolerated as the side chain carboxylates of the Asp^7 and Asp^9 are assumed to be essential for daptomycin's interactions with Ca^{2+}.[32] In addition, the noncanonical amino acids 3-methyl glutamic acid ($MeGlu^{12}$) and kynurenine (Kyn^{13}) are also important for full activity.[29] Notable, however, is a recent report describing the synthesis of "kynomycin" a daptomycin analogue containing an *N*-methylated Kyn^{13} residue described as having enhanced antibacterial activity both *in vitro* and *in vivo*.[35]

We here describe the use of ITC to investigate the structural features present in the target phospholipids that are responsible for recognition by daptomycin. Specifically, the binding of daptomycin to vesicles containing PG or other PG-related phospholipids including cardiolipin, dioleoylphosphatidylserine (DOPS), and dioleoylphosphatidyl propanol (DOP-propanol) was evaluated, providing insights into the role of the phospholipid headgroup (Figure 1B). The ITC binding experiments were performed using mixed LUVs

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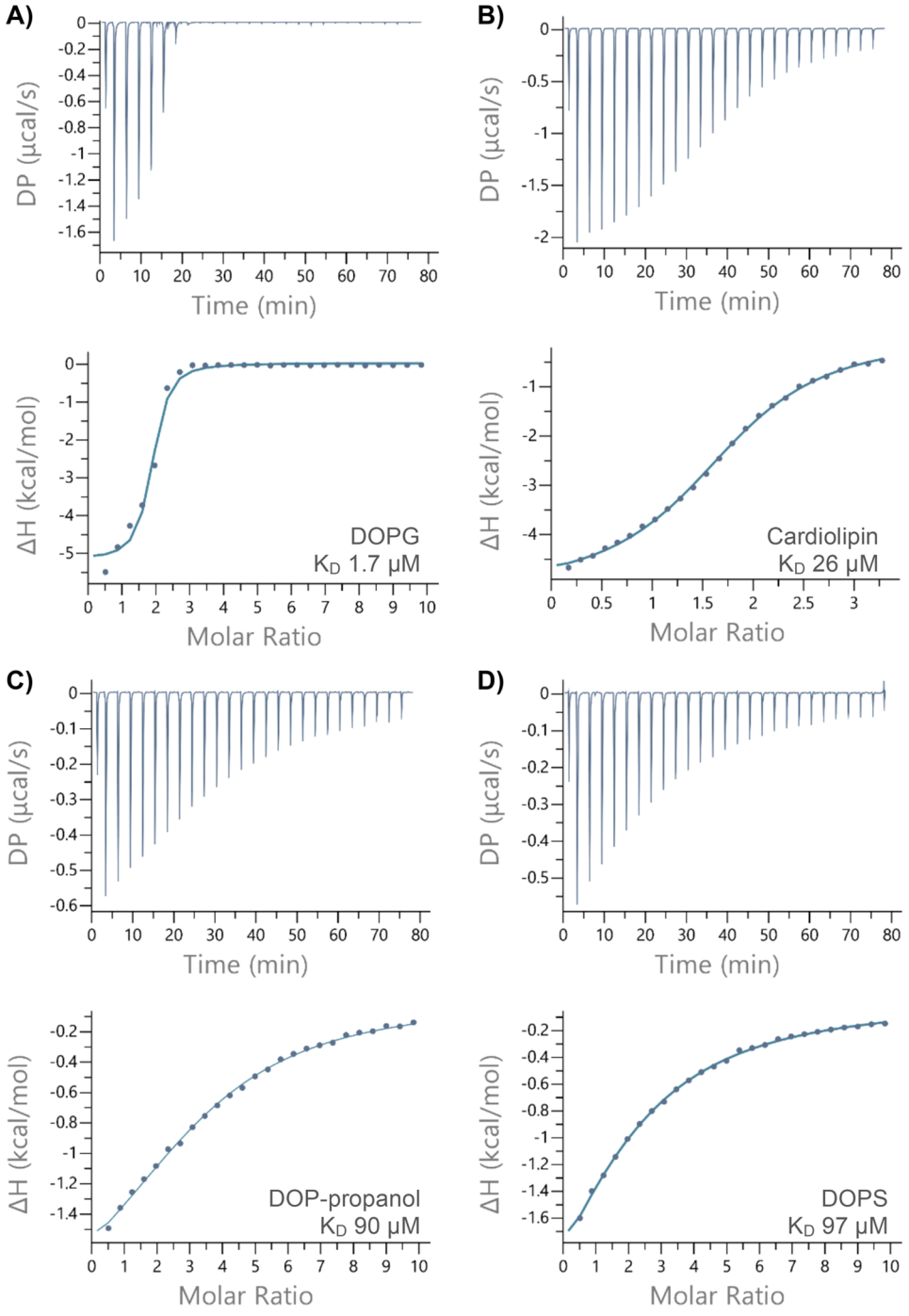

Figure 2. Representative binding isotherms for the titrations of 10 mM DOPC LUVs containing 25 mol % of: (A) DOPG (titrated into 50 μM daptomycin); (B) cardiolipin (titrated into 150 μM daptomycin); (C) DOP-propanol and (D) DOPS (both titrated into 50 μM daptomycin). Buffer used for all binding experiments consisted of 20 mM HEPES, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.4.

Table 1. Thermodynamic Parameters for Daptomycin Binding Anionic Membranes by ITC

Phospholipid[a]	K_D (μM)	N (sites)	ΔH (kcal/mol)	$-T\Delta S$(kcal/mol)	ΔG (kcal/mol)
DOPG[b]	1.72 ± 0.08	1.72 ± 0.03	−5.26 ± 0.05	−2.60 ± 0.04	−7.86 ± 0.03
Cardiolipin[c]	26.23 ± 1.35	1.81 ± 0.04	−4.98 ± 0.11	−1.25 ± 0.11	−6.23 ± 0.01
DOP-propanol[b]	90.00 ± 1.90	ND	ND	ND	−5.52 ± 0.01
DOPS[b]	97.00 ± 19.71	ND	ND	ND	−5.49 ± 0.12

[a]LUVs (10 mM, 25 mol % phospholipid:75 mol % DOPC) were titrated into the ITC sample cell containing daptomycin in 5 mM $CaCl_2$, 20 mM HEPES, 150 mM NaCl, pH 7.4. The results shown are the average of three experiments with the standard deviation indicated. ND: For binding of daptomycin to DOP-propanol and DOPS, reliable values for N, ΔH, and -TΔS could not be determined due to the shape of the isotherms resulting under the experimental conditions used. [b]50 μM daptomycin. [c]150 μM daptomycin.

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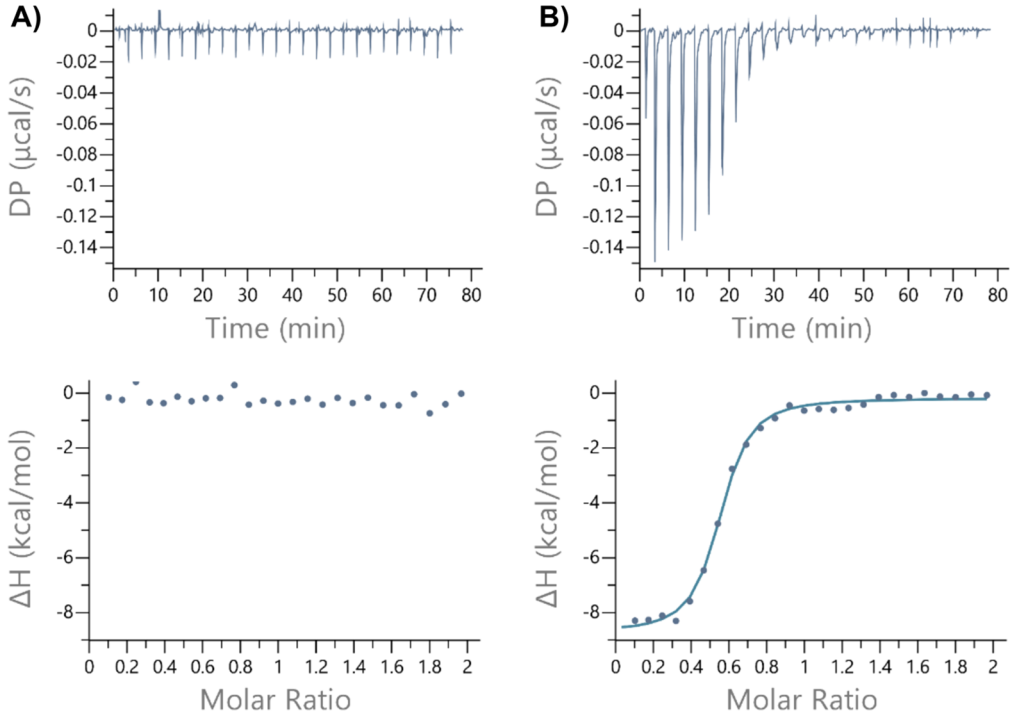

Figure 3. Titrations of LUVs containing 2 mol % lipid II and 20 mol % DOPG in DOPC (10 mM) into a solution of: (A) 20 μM daptomycin, 2 mM DOPG or (B) 20 μM nisin, 2 mM DOPG. In both cases, the buffer used consisted of 20 mM HEPES, 5 mM $CaCl_2$, 150 mM NaCl, pH 7.4.

comprising 25 mol % of the target phospholipid under investigation mixed with 75 mol % dioleoylphosphatidylcholine (DOPC) in buffer containing 5 mM $CaCl_2$. All lipids used contained the same 1,2-dioleoyl-*sn*-glycero-3-phospho motif allowing for comparison of the contributions to binding due specifically to the varying headgroups. The most reproducible results were obtained by titrating the vesicle preparations into the ITC sample-cell which contained a solution of daptomycin in the same buffer. Figure 2 provides representative binding isotherms obtained for each of the different phospholipids evaluated and the results of the titrations are summarized in Table 1 (all thermograms and experimental conditions are provided in the Supporting Information).

In line with expectation,[18] the titration with DOPG-containing LUVs resulted in a strongly exothermic binding isotherm (Figure 2A). This binding is attributable to the interaction of daptomycin with DOPG, given that no such exothermic signal results from the titration of "blank" LUVs containing 100 mol % DOPC (see Supporting Information Figures S1 & S2). A dissociation constant (K_D) of 1.7 ± 0.08 μM was thus determined for daptomycin binding to DOPG-containing LUVs using a one-site binding model with the strength of the interaction (determined by the free energy of binding ΔG) being ruled by an enthalpic contribution (Table 1). Building upon these findings, we proceeded to assess the binding of daptomycin to the other phospholipids. We next investigated daptomycin's interaction with cardiolipin, a bisphosphatidyl-glycerol lipid variant common in bacterial membranes.[36] Previous studies have indicated that increased levels of cardiolipin in bacterial membranes may contribute to daptomycin resistance[4] and binding studies with LUVs containing mixtures of PG and cardiolipin indicate that daptomycin does interact with cardiolipin.[18] Given that cardiolipin contains a second 1,2-dioleoyl-*sn*-glycero-3-phosophate moiety in place of one of the hydroxyl groups found in PG, additional ionic interactions with daptomycin are possible. Notably, the additional phosphatidic acid moiety present in cardiolipin does not appear to increase the negative net charge of the LUV suspensions; the zeta potential of 25 mol % DOPG LUVs is of the same magnitude as the corresponding cardiolipin LUVs, as assessed by electrophoretic light scattering (see Supporting Information Table S1). As illustrated in Figure 2B, a clear interaction is observed when cardiolipin-containing LUVs are titrated into daptomycin with a corresponding K_D value of 26.23 ± 1.35 μM. This binding is weaker than that measured for DOPG and suggests that the presence of an additional negatively charged phosphatidic acid moiety does not compensate for the concomitant loss of an H-bond donor/acceptor.

These findings indicate that the calcium-dependent interaction of daptomycin with phospholipid headgroups is dependent on the availability of the hydrogen bonding interactions provided both glycerol hydroxyl groups. In the case of cardiolipin-containing mixed membranes, the lone 2′-hydroxyl group of cardiolipin may therefore not suffice for inducing daptomycin to adopt its fully active configuration. This rationale is also in line with the finding that high cardiolipin content in PG/PC bilayers prevents the characteristic daptomycin-mediated membrane disruption:[18] an observation which was also recently corroborated by atomic force microscopy based investigations.[24]

To further investigate the contribution of H-bonding and electrostatic interactions to daptomycin's affinity for phospholipids, we next assessed its binding to LUVs containing dioleoylphosphatidyl-propanol (DOP-propanol) and the naturally occurring anionic phospholipid dioleoylphosphatidylserine (DOPS).[36] While DOP-propanol contains the same aliphatic backbone as DOPG, it lacks both hydroxyl groups of the glycerol moiety. This results in a significant loss of binding affinity with a measured K_D value of 90.00 ± 1.90 μM

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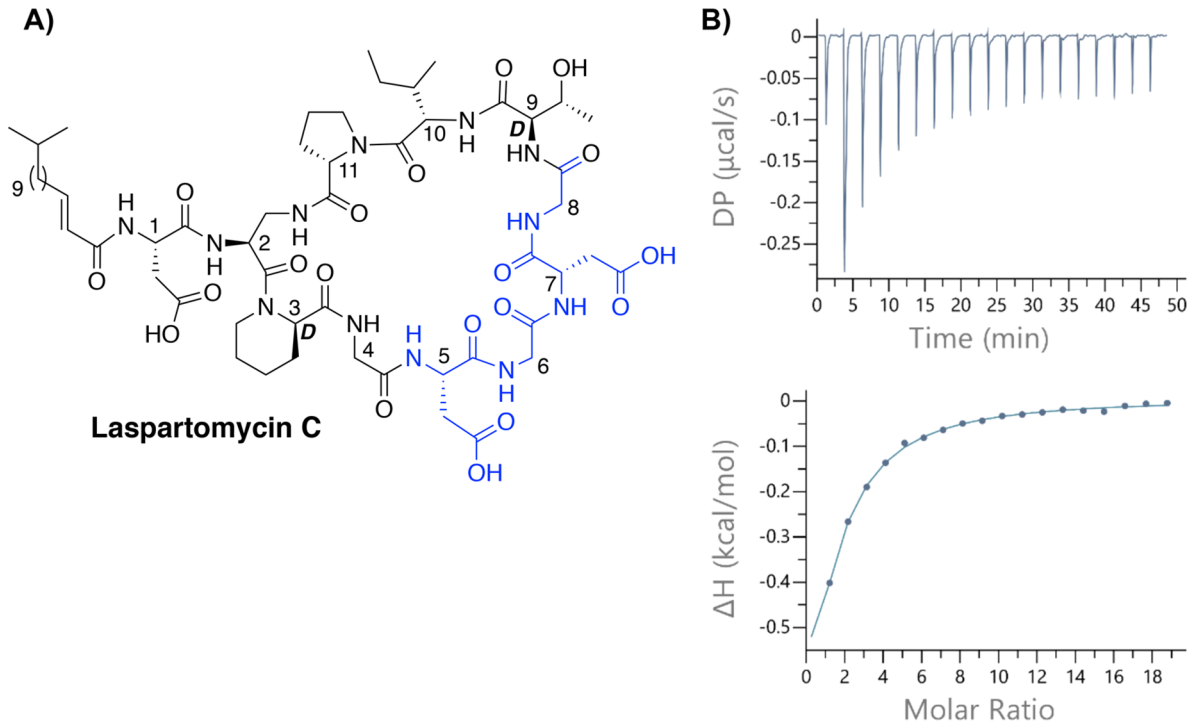

Figure 4. (A) Structure of laspartomycin C with calcium binding motif highlighted in blue; and (B) representative thermogram resulting from titration of 5 mM $CaCl_2$ into 50 μM laspartomycin C. Buffer used for all binding experiments consisted of 20 mM HEPES, pH 7.4.

(Figure 2C). In the case of DOPS, the glycerol motif is replaced by a serine residue linked via its hydroxyl side chain to the 1,2-dioleoyl-*sn*-glycero-3-phosphate moiety. Thus, while DOPS lacks the two hydroxy groups of the glycerol moiety, it does contain a zwitterionic amino acid unit. These additional charged amino and carboxylate functionalities do not, however, compensate for the missing hydroxyl groups and, in fact, further reduce daptomycin's affinity for the DOPS containing LUVs with an associated K_D value of 97.00 ± 19.71 μM (Figure 2C).

Collectively, our analyses reveal the specific role of the glycerol moiety in the recognition of PG by daptomycin. These findings are well in line with the colocalization of daptomycin to PG-rich membrane domains in vesicles[21,19] and in bacterial cells.[20,26,23]

Bacterial membrane regions rich in PG and cardiolipin also typically contain the machinery for cell wall biogenesis.[26,27,37] While previous studies have suggested that daptomycin interferes with cell wall synthesis,[26,27] a recent report from Schneider and co-workers provides evidence for a direct interaction of daptomycin with the cell wall building block lipid II and its precursors undecaprenyl phosphate (C_{55}−P) and undecaprenyl pyrophosphate (C_{55}−PP).[20] Using a range of biochemical assays, the interaction of daptomycin with these cell wall precursors was shown to be dependent on the presence of Ca^{2+} ions and PG. Given our group's previous success in using ITC to assess lipid II binding by peptide antibiotics including nisin[38] and teixobactin,[39] we were curious to see if a similar approach could be used to characterize the interaction of daptomycin with lipid II. To do so, we prepared LUVs containing a range of lipid II (1−2 mol %) and DOPG (1−20 mol %) concentrations and titrated them into solutions containing daptomycin. However, despite investigating a variety of conditions, we were not able to detect any measurable differences relative to the titrations performed using DOPG-containing LUVs lacking lipid II. A possible explanation is that the heat produced by the interaction of daptomycin with PG effectively drowns out any heat signal due to lipid II binding (see Supporting Information Figure S3). In an attempt to isolate the lipid II effect from the heat associated with daptomycin's interaction with PG, mixed DOPG/DOPC LUVs containing 2 mol % lipid II were titrated into a premixed solution of daptomycin and DOPG, but this also failed to produce any measurable signal (Figure 3A). As a positive control we performed the same binding experiment with nisin in place of daptomycin. When DOPG/DOPC LUVs containing 2 mol % lipid II are titrated into a mixture of nisin and DOPG, binding is readily detected (Figure 3B). These results suggest that nisin binds lipid II more tightly than daptomycin, a finding in keeping with previously reported antagonization studies with both antibiotics: it is known that PG antagonizes the activity of daptomycin[11] while lipid II does not.[40] Conversely, for nisin, the addition of lipid II very effectively antagonizes its antibacterial activity.[40] We are careful to note that while our results do not provide evidence for lipid II binding by daptomycin, this may also reflect a limitation of the ITC based methods used to detect the interaction.

We also performed a series of ITC investigations to characterize the binding of Ca^{2+} by daptomycin in comparison to another well-characterized CDA, laspartomycin C (Figure 4A). To date, laspartomycin C is the only CDA for which a crystal structure has been solved that shows the lipopeptide in complex with both Ca^{2+} and its target phospholipid (C_{55} P).[10,11] This crystal structure clearly reveals how the Asp-X-Asp-Gly calcium-binding motif and the phospholipid both participate in the binding of two calcium ions. In line with previously reported Circular Dichroism (CD) studies,[11] titration of Ca^{2+} into laspartomycin C alone, generated a clear exothermic signal (Figure 4B). In contrast to this,

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titration of $CaCl_2$, into daptomycin alone, gave no indication of binding. The unexpected difference in the calcium binding behavior of these two CDAs, indicates that despite sharing the conserved Ca^{2+} binding motif, the structural differences between laspartomycin C and daptomycin significantly impact their capacities to bind Ca^{2+}. In a follow-up experiment we found that when a solution of $CaCl_2$ was titrated in a mixture of daptomycin and PG-containing vesicles a significant exothermic signal is produced (Figure 5). We further

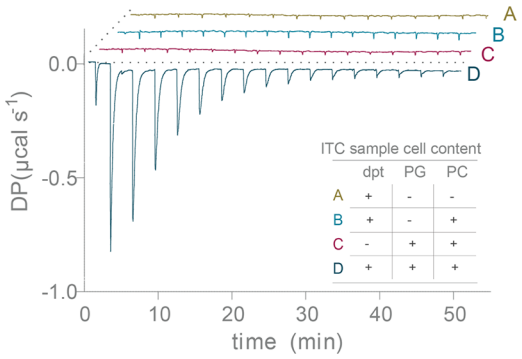

Figure 5. Calcium binding by daptomycin-PG. The stacked thermograms show the heat exchange upon the 16 first injections of 5 mM $CaCl_2$ into the ITC sample cell containing 50 μM daptomycin with or without LUVs of varying composition. Thermograms are representative of two experiments. All experiments and thermodynamic parameters are reported in the Supporting Information (Table S2 and Figures S5 and S6).

demonstrated that this heat is produced as a result of complex formation between all three components: daptomycin, PG, and Ca^{2+}, as no appreciable signal was detected when $CaCl_2$ was titrated into PG-vesicles, or neutral DOPC vesicles (Figure 5). These findings are in agreement with recent studies utilizing fluorescence microscopy[21] and CD methods[16,17] which also indicate that daptomycin's capacity to undergo the conformational changes needed to interact with target membranes is explicitly dependent on the presence of both Ca^{2+} and PG. As an extension of the binding studies illustrated in Figure 2 and summarized in Table 1, we also examined the impact of calcium ion concentration on the measured affinity of daptomycin for the different phospholipids evaluated in the present study. This revealed a clear effect wherein elevated Ca^{2+} concentration enhanced the measured binding of daptomycin to PG, cardiolipin, DOP-propanol, and DOPS, while reduced calcium ion concentrations had the inverse effect (see Supporting Information Table S3).

In conclusion, we here report a comprehensive ITC study that further clarifies the parameters required for phospholipid binding by daptomycin and the necessity of these partner phospholipids for calcium ion binding. Our results make clear the optimal nature of the PG phospholipid headgroup and the essentiality of both hydroxyl moieties for tight binding. Also, a recent report describing a role for the bacterial cell wall precursor lipid II as a target for daptomycin prompted us to study this binding interaction by ITC. While clear lipid II binding was evident in titrations with nisin, a well characterized lipid II binding lanthipeptide, the same approach failed to provide a detectable signal for daptomycin suggesting that ITC methods may not be suitable for characterizing the daptomycin-lipid II interaction. Investigations into the capacity for daptomycin to directly bind Ca^{2+} also highlight notable differences among structurally related CDAs: while laspartomycin C binds Ca^{2+} in the absence of any added phospholipid, for daptomycin, the interaction with Ca^{2+} is absolutely dependent on the presence of phospholipids with PG providing the greatest effect. Collectively, our investigations serve to add new insights to the ever-growing body of mechanistic characteristics ascribed to daptomycin.

METHODS

Formulation of Large Unilamellar Vesicles (LUVs). Phospholipid stock solutions (10−30 mM) were prepared in chloroform. Gram positive Lipid II stock solutions (0.3−1.0 mM) were prepared in chloroform/methanol 1:1. Appropriate volumes of the stock solutions were mixed, and the organic solvents were evaporated under a stream of nitrogen at 35−40 °C. The resulting dry lipid films were hydrated with a buffer of specified $CaCl_2$ content (20 mM HEPES, 150 mM NaCl, pH 7.4) and homogenized by 5 cycles of freezing (−196 °C) and thawing (35−40 °C) to produce vesicle suspensions with a final concentration of 10 mM total lipid. The suspensions were passed through 2 opposite directed Whatman polycarbonate membranes, with a final pore size of 0.2 μm (Sigma-Aldrich, Taufkirchen, Germany) 11 times at room temperature with an Avanti mini extruder (Avanti Polar Lipids Inc., Alabaster, Alabama USA), to yield homogeneous (polydispersity index <0.1) LUV suspensions of ∼140 nm hydrodynamic diameter, as assessed by dynamic light scattering (DLS) spectroscopy at 25 °C, on a Zetasizer Nano S (Malvern Panalytical Ltd., Malvern, UK) using acrylic low-volume cuvettes (VWR international, Leuven, Belgium). The ζ potentials of DOPG and cardiolipin containing DOPC LUVs formulated in 20 mM HEPES, pH 7.4 were measured using laser Doppler electrophoresis on the same instrument with a ζ dip cell (Malvern Panalytical Ltd., Malvern, UK). Samples were diluted 100-fold before all DLS measurements.

Isothermal Titration Calorimetry (ITC). All binding experiments were performed using a MicroCal PEAQ-ITC Automated microcalorimeter (Malvern Panalytical Ltd., Malvern, UK). In **method A**, the samples were equilibrated to 25 °C prior to measurement. The titrations were conducted at 25 °C under constant stirring at 1000 rpm. Each experiment consisted of an initial injection of 0.3 μL followed by 25 separate injections of 1.5 μL into the sample cell of 200 μL. The time between each injection was 180 s, and the measurements were performed with the reference power set at 5 μcal s^{-1} and the feedback mode set at "high." In **method B**, the samples were equilibrated to 25 °C prior to measurement. The titrations were conducted at 25 °C under constant stirring at 750 rpm. Each experiment consisted of an initial injection of 0.4 μL followed by 18 separate injections of 2.0 μL into the sample cell of 200 μL. The time between each injection was 150 s, and the measurements were performed with the reference power set at 10 μcal s^{-1} and the feedback mode set at "high."

ITC Procedure for Phospholipid Binding. LUV suspensions of 2.5 mM anionic lipid 7.5 mM DOPC or 10 mM DOPC in buffer containing 0, 1, 5, or 10 mM $CaCl_2$ were titrated into daptomycin solution in the same buffer. Daptomycin solutions were prepared from a 2 mM stock in the same buffer which was stored in −20 °C for no more than 5 days prior to use. Blank titrations included the titration of buffer into daptomycin and LUVs into buffer. The titrations were conducted according to method A described above.

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ITC Procedure for Calcium Binding Daptomycin with LUVs. A solution of 5 mM $CaCl_2$ in 20 mM HEPES, 150 mM NaCl, pH 7.4 was titrated into a 0.05 mM solution of daptomycin with or without a suspension of 1 mM LUV in the same buffer. Blank titrations included the titration of buffer into daptomycin (0.05 mM) or daptomycin (0.05 mM) with LUV suspension (1 mM) and $CaCl_2$ (5 mM) into buffer or LUV suspension. The titrations were conducted according to method A described above.

ITC Procedure for Calcium Binding in Solution. A solution of 5 mM $CaCl_2$ in 20 mM HEPES, pH 7.4 was titrated into a 0.05 mM solution of daptomycin or laspartomycin C in the same buffer. Blank titrations included the titration of buffer into the test compounds and $CaCl_2$ into buffer. The titrations were conducted according to method B described above.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00316.

ITC thermograms for all binding experiments performed and characterization of LUVs prepared and used (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Debono, M.; Abbott, B. J.; Molloy, R. M.; Fukuda, D. S.; Hunt, A. H.; Daupert, V. M.; Counter, F. T.; Ott, J. L.; Carrell, C. B.; Howard, L. C. Enzymatic and chemical modifications of lipopeptide antibiotic A21978C: the synthesis and evaluation of daptomycin (LY146032). *J. Antibiot.* **1988**, *41*, 1093−1105.

(2) Arbeit, R. D.; Maki, D.; Tally, F. P.; Campanaro, E.; Eisenstein, B. I. The safety and efficacy of daptomycin for the treatment of complicated skin and skin-structure infections. *Clin. Infect. Dis.* **2004**, *38*, 1673−1681.

(3) Fowler, V. G.; Boucher, H. W.; Corey, G. R.; Abrutyn, E.; Karchmer, A. W.; Rupp, M. E.; Levine, D. P.; Chambers, H. F.; Tally, F. P.; Vigliani, G. A.; Cabell, C. H.; Link, A. S.; DeMeyer, I.; Filler, S. G.; Zervos, M.; Cook, P.; Parsonnet, J.; Bernstein, J. M.; Price, C. S.; Forrest, G. N.; Fätkenheuer, G.; Gareca, M.; Rehm, S. J.; Brodt, H. R.; Tice, A.; Cosgrove, S. E. Daptomycin versus standard therapy for bacteremia and endocarditis caused by Staphylococcus aureus. *N. Engl. J. Med.* **2006**, *355*, 653−665.

(4) Miller, W. R.; Bayer, A. S.; Arias, C. A. Mechanism of Action and Resistance to Daptomycin in Staphylococcus aureus and Enterococci. *Cold Spring Harbor Perspect. Med.* **2016**, *6*, No. a026997.

(5) Friedman, L.; Alder, J. D.; Silverman, J. A. Genetic changes that correlate with reduced susceptibility to daptomycin in Staphylococcus aureus. *Antimicrob. Agents Chemother.* **2006**, *50*, 2137−2145.

(6) Bayer, A. S.; Mishra, N. N.; Chen, L.; Kreiswirth, B. N.; Rubio, A.; Yang, S.-J. Frequency and distribution of single-nucleotide polymorphisms within mprF in Methicillin-Resistant Staphylococcus aureus clinical isolates and their role in cross-resistance to daptomycin and host defense antimicrobial peptides. *Antimicrob. Agents Chemother.* **2015**, *59*, 4930−4937.

(7) Pader, V.; Hakim, S.; Painter, K. L.; Wigneshweraraj, S.; Clarke, T. B.; Edwards, A. M. *Staphylococcus aureus* inactivates daptomycin by releasing membrane phospholipids. *Nat. Microbiol.* **2017**, *2*, 16194.

(8) Tran, T. T.; Panesso, D.; Mishra, N. N.; Mileykovskaya, E.; Guan, Z.; Munita, J. M.; Reyes, J.; Diaz, L.; Weinstock, G. M.; Murray, B. E.; Shamoo, Y.; Dowhan, W.; Bayer, A. S.; Arias, C. A. Daptomycin-resistant *Enterococcus faecalis* diverts the antibiotic molecule from the division septum and remodels cell membrane phospholipids. *mBio* **2013**, *4*, No. e00281.

(9) Tran, T. T.; Mishra, N. N.; Seepersaud, R.; Diaz, L.; Rios, R.; Dinh, A. Q.; Garcia-de-la-Maria, C.; Rybak, M. J.; Miro, J. M.; Shelburne, S. A.; Sullam, P. M.; Bayer, A. S.; Arias, C. A. Mutations in cdsA and pgsA correlate with daptomycin resistance in Streptococcus mitis and S. oralis. *Antimicrob. Agents Chemother.* **2019**, *63*, No. e01531.

(10) Goldner, N. K.; Bulow, C.; Cho, K.; Wallace, M.; Hsu, F.-F.; Patti, G. J.; Burnham, C.-A.; Schlesinger, P.; Dantas, G. Mechanism of high-level daptomycin resistance in *Corynebacterium striatum*. *mSphere* **2018**, *3*, No. e00371.

(11) Kleijn, L. H. J.; Vlieg, H. C.; Wood, T. M.; Sastre Toraño, J.; Janssen, B. J. C.; Martin, N. I. A High-resolution crystal structure that reveals molecular details of target recognition by the calcium-dependent lipopeptide antibiotic laspartomycin C. *Angew. Chem., Int. Ed.* **2017**, *56*, 16546−16549.

(12) Jung, D.; Rozek, A.; Okon, M.; Hancock, R. E. W. Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin. *Chem. Biol.* **2004**, *11*, 949−957.

(13) Jung, D.; Powers, J. P.; Straus, S. K.; Hancock, R. E. W. Lipid-specific binding of the calcium-dependent antibiotic daptomycin leads to changes in lipid polymorphism of model membranes. *Chem. Phys. Lipids* **2008**, *154*, 120−128.

(14) Muraih, J. K.; Pearson, A.; Silverman, J.; Palmer, M. Oligomerization of daptomycin on membranes. *Biochim. Biophys. Acta, Biomembr.* **2011**, *1808*, 1154−1160.

(15) Muraih, J. K.; Harris, J.; Taylor, S. D.; Palmer, M. Characterization of daptomycin oligomerization with perylene excimer fluorescence: Stoichiometric binding of phosphatidylglycerol triggers oligomer formation. *Biochim. Biophys. Acta, Biomembr.* **2012**, *1818*, 673−678.

(16) Lee, M. T.; Hung, W. C.; Hsieh, M. H.; Chen, H.; Chang, Y. Y.; Huang, H. W. Molecular state of the membrane-active antibiotic daptomycin. *Biophys. J.* **2017**, *113*, 82−90.
(17) Lee, M. T.; Yang, P. Y.; Charron, N. E.; Hsieh, M. H.; Chang, Y. Y.; Huang, H. W. Comparison of the effects of daptomycin on bacterial and model membranes. *Biochemistry* **2018**, *57*, 5629−5639.
(18) Zhang, T.; Muraih, J. K.; Tishbi, N.; Herskowitz, J.; Victor, R. L.; Silverman, J.; Uwumarenogie, S.; Taylor, S. D.; Palmer, M.; Mintzer, E. Cardiolipin prevents membrane translocation and permeabilization by daptomycin. *J. Biol. Chem.* **2014**, *289*, 11584−11591.
(19) Juhaniewicz-Dębińska, J.; Dziubak, D.; Sęk, S. Physicochemical characterization of daptomycin interaction with negatively charged lipid membranes. *Langmuir* **2020**, *36*, 5324−5335.
(20) Grein, F.; Müller, A.; Scherer, K. M.; Liu, X.; Ludwig, K. C.; Klöckner, A.; Strach, M.; Sahl, H. G.; Kubitscheck, U.; Schneider, T. Ca^{2+}-Daptomycin targets cell wall biosynthesis by forming a tripartite complex with undecaprenyl-coupled intermediates and membrane lipids. *Nat. Commun.* **2020**, *11*, 1455.
(21) Kreutzberger, M. A.; Pokorny, A.; Almeida, P. F. Daptomycin-phosphatidylglycerol domains in lipid membranes. *Langmuir* **2017**, *33*, 13669−13679.
(22) Zhang, T.; Muraih, J. K.; Mintzer, E.; Tishbi, N.; Desert, C.; Silverman, J.; Taylor, S.; Palmer, M. Mutual inhibition through hybrid oligomer formation of daptomycin and the semisynthetic lipopeptide antibiotic CB-182,462. *Biochim. Biophys. Acta, Biomembr.* **2013**, *1828*, 302−308.
(23) Taylor, R.; Butt, K.; Scott, B.; Zhang, T.; Muraih, J. K.; Mintzer, E.; Taylor, S.; Palmer, M. Two successive calcium-dependent transitions mediate membrane binding and oligomerization of daptomycin and the related antibiotic A54145. *Biochim. Biophys. Acta, Biomembr.* **2016**, *1858*, 1999−2005.
(24) Zuttion, F.; Colom, A.; Matile, S.; Farago, D.; Pompeo, F.; Kokavecz, J.; Galinier, A.; Sturgis, J.; Casuso, I. High-speed atomic force microscopy highlights new molecular mechanism of daptomycin action. *Nat. Commun.* **2020**, *11*, 6312.
(25) Mescola, A.; Ragazzini, G.; Alessandrini, A. Daptomycin strongly affects the phase behavior of model lipid bilayers. *J. Phys. Chem. B* **2020**, *124*, 8562−8571.
(26) Pogliano, J.; Pogliano, N.; Silverman, J. A. Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division poteins. *J. Bacteriol.* **2012**, *194*, 4494−4504.
(27) Müller, A.; Wenzel, M.; Strahl, H.; Grein, F.; Saaki, T. N. V.; Kohl, B.; Siersma, T.; Bandow, J. E.; Sahl, H. G.; Schneider, T.; Hamoen, L. W. Daptomycin inhibits cell envelope synthesis by interfering with fluid membrane microdomains. *Proc. Natl. Acad. Sci. U. S. A.* **2016**, *113*, E7077−E7086.
(28) Lam, H. Y.; Zhang, Y.; Liu, H.; Xu, J.; Wong, C. T. T.; Xu, C.; Li, X. Total synthesis of daptomycin by cyclization via a chemoselective serine ligation. *J. Am. Chem. Soc.* **2013**, *135*, 6272−6279.
(29) Lohani, C. R.; Taylor, R.; Palmer, M.; Taylor, S. D. Solid-phase total synthesis of daptomycin and analogs. *Org. Lett.* **2015**, *17*, 748−751.
(30) Wood, T. M.; Martin, N. I. The calcium-dependent lipopeptide antibiotics: Structure, mechanism, & medicinal chemistry. *MedChemComm* **2019**, *10*, 634−646.
(31) Karas, J. A.; Carter, G. P.; Howden, B. P.; Turner, A. M.; Paulin, O. K. A.; Swarbrick, J. D.; Baker, M. A.; Li, J.; Velkov, T. Structure-activity relationships of daptomycin lipopeptides. *J. Med. Chem.* **2020**, *63*, 13266−13290.
(32) Chow, H. Y.; Po, K. H. L.; Jin, K.; Qiao, G.; Sun, Z.; Ma, W.; Ye, X.; Zhou, N.; Chen, S.; Li, X. Establishing the structure-activity relationship of daptomycin. *ACS Med. Chem. Lett.* **2020**, *11*, 1442−1449.
(33) 't Hart, P.; Kleijn, L. H. J.; de Bruin, G.; Oppedijk, S. F.; Kemmink, J.; Martin, N. I. A combined solid- and solution-phase approach provides convenient access to analogues of the calcium-dependent lipopeptide antibiotics. *Org. Biomol. Chem.* **2014**, *12*, 913−918.
(34) Barnawi, G.; Noden, M.; Taylor, R.; Lohani, C.; Beriashvili, D.; Palmer, M.; Taylor, S. D. An entirely Fmoc solid phase approach to the synthesis of daptomycin analogs. *Pept. Sci.* **2019**, *111*, No. e23094.
(35) Chow, H. Y.; Po, K. H. L.; Gao, P.; Blasco, P.; Wang, X.; Li, C.; Ye, L.; Jin, K.; Chen, K.; Chan, E. W. C.; You, X.; Yi Tsun Kao, R.; Chen, S.; Li, X. Methylation of daptomycin leading to the discovery of kynomycin, a cyclic lipodepsipeptide active against resistant pathogens. *J. Med. Chem.* **2020**, *63*, 3161−3171.
(36) Sohlenkamp, C.; Geiger, O. Bacterial membrane lipids: Diversity in structures and pathways. *FEMS Microbiology Reviews* **2016**, *40*, 133−159.
(37) Müller, A.; Klöckner, A.; Schneider, T. Targeting a cell wall biosynthesis hot spot. *Nat. Prod. Rep.* **2017**, *34*, 909−932.
(38) 't Hart, P.; Oppedijk, S. F.; Breukink, E.; Martin, N. I. New insights into nisin's antibacterial mechanism revealed by binding studies with synthetic lipid II analogues. *Biochemistry* **2016**, *55*, 232−237.
(39) Chiorean, S.; Antwi, I.; Carney, D. W.; Kotsogianni, I.; Giltrap, A. M.; Alexander, F. M.; Cochrane, S. A.; Payne, R. J.; Martin, N. I.; Henninot, A.; Vederas, J. C. Dissecting the binding interactions of teixobactin with the bacterial cell-wall precursor lipid II. *ChemBioChem* **2020**, *21*, 789−792.
(40) Kleijn, L. H. J.; Oppedijk, S. F.; 't Hart, P.; van Harten, R. M.; Martin-Visscher, L. A.; Kemmink, J.; Breukink, E.; Martin, N. I. Total synthesis of laspartomycin C and characterization of its antibacterial mechanism of action. *J. Med. Chem.* **2016**, *59*, 3569−3574.

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