

Two successive calcium-dependent transitions mediate membrane binding and oligomerization of daptomycin and the related antibiotic A54145

The final publication is available at Elsevier via <http://doi.org/10.1016/j.bbamem.2016.05.020> © 2016. This manuscript version is made available under the CC-BY-NC-ND 4.0 license <http://creativecommons.org/licenses/by-nc-nd/4.0/>

Robert Taylor^a, Khalida Butt^a, Bradley Scott^a, TianHua Zhang^a, Jawad K. Muraih^b, Evan Mintzer^c, Scott Taylor^{ad}, and Michael Palmer^{ad}

^aDepartment of Chemistry, University of Waterloo, Waterloo N2L 3G1, Ontario, Canada

^bDepartment of Chemistry, University of Al-Muthanna, Samawah, Al-Muthanna, Iraq

^cDepartment of Chemistry and Physics Lander College of Arts and Sciences, Touro College 1602 Ave. J, Brooklyn, NY 11230

^dCorresponding authors. Email: mpalmer@uwaterloo.ca, s5taylor@uwaterloo.ca

Abstract

Daptomycin and A54145 are homologous lipopeptide antibiotics that permeabilize the cell membranes of Gram-positive bacteria. Membrane permeabilization depends on the presence of both phosphatidylglycerol (PG) and calcium, and it involves the formation of oligomeric transmembrane pores that consist of approximately 6-8 subunits. We here show that each lipopeptide molecule binds two calcium ions in separable, successive steps. The first calcium ion causes the lipopeptide molecule to bind to the target membrane, and likely to form a loosely associated oligomer. Higher calcium concentrations induce binding of a second ion, which produces the more tightly associated and more deeply membrane-inserted final, functional form of the oligomer. Both calcium-dependent steps are accompanied by fluorescence signals that indicate transition of specific amino acid residues into less polar environments, suggestive of insertion into the target membrane. Our findings agree with the earlier observation that two of the four acidic amino acid residues in the daptomycin molecule are essential for antibacterial activity.

Introduction

Daptomycin and A54145 are two homologous acidic lipopeptide antibiotics (Figure 1) that permeabilize the cell membranes of Gram-positive bacteria in a calcium-dependent manner. The only molecule in the target membrane proven to be specifically required for antibacterial activity is phosphatidylglycerol, which is a major constituent of bacterial cell membranes [1]. The activity of both antibiotics coincides with the formation of membrane-associated oligomers [2]. Mixtures of daptomycin with CB-182,462, a semisynthetic A54145 derivative, form hybrid oligomers that are partially functional, indicating that daptomycin and A54145 are functionally closely related [2].

All known steps in the activity of daptomycin and A54145 can be recapitulated in a simple liposome model that contains only phosphatidylcholine (PC) and phosphatidylglycerol (PG). In this model, it can be shown that calcium is required for membrane binding [3, 4], and that PG and calcium are required for oligomerization [5] and pore formation [6]. While the requirement of calcium for activity is thus well documented, the interaction is not clearly understood in molecular detail, and the stoichiometric ratio of calcium to daptomycin is not clear. Circular dichroism (CD) experiments showed that daptomycin may undergo two calcium-dependent structural transitions. A first, minor transition can be observed when calcium is added to daptomycin in solution; the second, major transition occurs in the presence of calcium and membranes containing PG [4]. NMR studies of daptomycin in solution yielded a stoichiometry of one calcium ion per lipopeptide molecule [7].

Using isothermal calorimetry and fluorescence spectroscopy, we here show that daptomycin and A54145, when mixed with PG-containing membranes, successively bind two calcium ions per lipopeptide molecule. A singly bound state can be detected in which the lipopeptide molecules are associated with the membrane, but in which their peptide moieties do not tightly interact with one another, although aggregation of the acyl tails is evident from experiments reported in a separate study. Binding of the second calcium ion converts these loosely connected oligomers to a more tightly associated and more deeply membrane-inserted state, presumably mediated by conformational changes that ultimately also lead to transmembrane pore formation.

Materials and Methods

Liposome preparation. Large unilamellar vesicles (LUV) for use in both ITC and fluorescence experiments were prepared by polycarbonate membrane extrusion [8] from equal proportions of phosphatidylglycerol (PG) and phosphatidylcholine (PC) in HEPES-buffered saline as described

previously [5]. In fluorescence experiments, dimyristoyl lipids (DMPG, DMPC) were used, whereas dioleoyl lipids (DOPG, DOPC) were used for ITC experiments. All lipids were obtained from Avanti Polar Lipids, Alabaster, AL.

Isothermal calorimetry (ITC). ITC titrations were carried out on a MicroCal ITC 200 instrument (GE Healthcare). LUV (DOPG/DOPC) were loaded into the ITC cell at final concentrations of 3.55 to 4.55 mM total lipid, together with 150 to 490 μM of daptomycin; the concentration of the latter was adjusted by spectrophotometry. The single kynurenine residue of daptomycin absorbs at 360 nm; the molar extinction coefficient was taken to be 4,500 [9]. The temperature was kept at 25°C.

Calcium chloride was loaded into the syringe at 5 or 10 mM and injected in increments of 1 or 2 μL . Heat peaks were baseline-corrected and integrated using the Origin software supplied with the instrument. The integrated heats were fitted using models that assumed a single type of binding sites, or the sequential binding to two types of sites, with either fixed or variable calcium/daptomycin stoichiometry, as detailed in the Results section.

Synthesis of acrylodan-labeled A54145 and daptomycin. Acrylodan is a polarity-sensitive fluorescent dye whose emission undergoes a large blue shift and a pronounced increase in quantum yield upon transition from aqueous solutions to apolar environments such as lipid bilayers [10]. It reacts most readily with thiol groups but also with free amino groups [11]. Acrylodan (Setareh Biotech, Eugene/OR) was attached to the unique free side chain amino groups of lysine-8 in A54145 Factor D and of ornithine-6 in daptomycin (both generously provided by Jared Silverman, Cubist Inc.). In the case of A54145, acrylodan was reacted directly with the amino group (cf. Figure 1). 5 mg of A54145 was dissolved in 250 μL of sodium phosphate (50 mM, pH 8.0) and supplemented with acrylodan (1 mg, dissolved in 250 μL of DMF), and the reaction was incubated for 5 days at room temperature.

With daptomycin, the amino group of ornithine-6 was first converted to a sulfhydryl group using Traut's reagent [12], to which acrylodan was then attached. To a solution of daptomycin (20 mg, 0.0123 mmol) in potassium phosphate buffer (50 mM, pH 8.0, 0.5 mL) was added Traut's reagent (3.0 mg, 0.0281 mmol, 1.8 equiv). The mixture was stirred for 1 h. A solution of acrylodan (5.5 mg, 0.0246 mmol, 2 equiv) in DMF (1 mL) was added and the mixture stirred for 24 h.

The products were purified by HPLC on a reversed-phase column (Higgins Analytical, C18 10 μm) using a mobile phase of 0.1% aqueous trifluoroacetic acid with acetonitrile gradients from 40% to 50% (for the A54145 adduct) or from 50% to 80% (for the daptomycin adduct). Product purities and identities were confirmed by analytical HPLC and mass spectrometry. Concentrations were determined using molar extinction coefficients at 365 nm of 16,400 $\text{M}^{-1} \text{cm}^{-1}$ for the A54145 adduct and of 20,900

$M^{-1} cm^{-1}$ for the daptomycin adduct (the higher coefficient in the latter case accounts for the kynurenine residue contained in daptomycin). The labeled derivatives, purified to homogeneity, retained approximately 50% (A54145) or 25% (daptomycin) of the antibacterial activities of their respective parent compounds (data not shown).

Fluorescence measurements. Fluorescence emission spectra as well as anisotropy measurements were acquired using a PTI QuantaMaster 4 instrument. The samples contained Hepes-buffered (pH 7.4) NaCl (150 mM) with native and labeled daptomycin and A54145, alone or in combination at final total concentrations of 3 μM , as well as calcium at various concentrations, as indicated in the Results section. Excitation wavelengths were 280 nm for tryptophan in native A54145, 360 nm for acrylodan, and 365 nm for kynurenine in native daptomycin. All fluorescence measurements were carried out at 37 $^{\circ}C$.

Results

Stoichiometry of calcium binding by ITC. The calcium-dependent membrane binding of daptomycin can be observed by ITC [2, 13]. We here used this method to determine the stoichiometric ratio of calcium to lipopeptide. Calcium (5 or 10 mM) was titrated into a sample that contained between 150 and 510 μM of daptomycin and an excess of DOPC/DOPG liposomes.

Figure 2 shows a single, representative ITC experiment fitted to various kinetic models. Panel A shows the raw trace and the baseline generated by the Origin software. The data points in panels B and C are the peak areas integrated relative to this baseline. An initial downward slope is apparent in the integrated data points but not in the height of the raw peaks; this discrepancy is most likely due to a decrease in the reaction rate as daptomycin becomes saturated with calcium.

When a single class of binding sites is assumed that binds all calcium ions with the same affinity, a stoichiometry of 2 fits the data better than 1 or 3, although none of the models fits particularly well (panel B). If we allow the stoichiometry to vary freely, a model that assumes two sequential binding sites with distinct affinities produces a better fit, and a more plausible stoichiometry (2.06 calcium ions per daptomycin molecule), than a model that assumes a variable number of sites with uniform affinity (2.40 calcium ions per daptomycin molecule). In six independent experiments, the sequential two-site model produced a stoichiometry of 1.81 ± 0.29 . The most plausible integral value for the stoichiometry therefore is 2. The enthalpies and dissociation constants also obtained using this model are reported in Table 1.

Calcium-dependent changes in the fluorescence emission of native daptomycin and acrylodan-

daptomycin. It has previously been reported that both native *daptomycin* [3, 4] and *daptomycin* that was labeled at its ornithine-6 residue with nitrobenzoxadiazole (NBD) [5, 14] undergo fluorescence increases when binding to phospholipid membranes in the presence of calcium. For this study, we replaced NBD with acrylodan, another fluorescent label that exhibits greater sensitivity to changes in environmental polarity [10]. (While acrylodan overlaps both the excitation and the emission spectra of the kynurenine residue natively contained in *daptomycin*, both its extinction coefficient and its quantum yield are significantly higher. Accordingly, acrylodan dominates the emission spectrum of the labeled compound.)

We separately incubated native *daptomycin* (Figure 3A) and acrylodan-*daptomycin* (Figure 3B) with PC/PG membranes and observed their changes in fluorescence as the concentration of calcium was raised stepwise from 0 to 10 mM. The intrinsic fluorescence of kynurenine-13 in native *daptomycin* shows little response up to 0.1 mM calcium but then rapidly increases, approaching saturation at 1 mM (Figure 3A). In contrast, the fluorescence of acrylodan attached to ornithine-6 rises up sooner and already peaks at 0.1 mM calcium but then decreases again at higher concentrations (Figure 3B). The different responses to increasing calcium concentrations are clearly evident in Figure 3D: acrylodan at ornithine-6 responds first and peaks when kynurenine just starts to react. Acrylodan emission then decreases progressively as kynurenine emission approaches its maximum.

The most straightforward explanation for the decrease in acrylodan fluorescence at higher calcium concentrations consists in concentration-dependent self-quenching, which also occurs with the label NBD attached to the same residue, and which is indicative of *daptomycin* oligomer formation [5]. In keeping with this assumption, the extent of quenching is reduced when acrylodan-*daptomycin* is mixed with an excess of unlabeled *daptomycin* before addition to the membranes (Figure 3C); within the hybrid oligomers formed from the mixtures, the acrylodan molecules will mostly be separated from one another. (The unlabeled species used in this experiment was the synthetic *daptomycin* variant E12W13, in which the kynurenine residue has been replaced with tryptophan [15], and which therefore does not contribute any fluorescence upon excitation at 360 nm.)

Concomitantly with calcium binding, the emission spectra of kynurenine and particularly of acrylodan also undergo significant blue shifts. The observed fluorescence signals of both kynurenine and acrylodan are compatible with their transition to a more hydrophobic environment, which likely corresponds to the apolar interior of the target membrane.

Calcium-dependent fluorescence changes in native and acrylodan-labeled A54145. The lipopeptide A54145 resembles *daptomycin* structurally and functionally, but unlike *daptomycin* does not contain

kynurenine, so that the intrinsic fluorescence of its tryptophan residue is unmasked [2]. Figure 4A shows that the tryptophan residue undergoes an appreciable blue shift, again suggestive of insertion into a hydrophobic environment, at calcium concentrations greater than 0.1 mM.

Figure 4B shows the response of A54145-acrylodan to incremental addition of calcium. As with acrylodan-daptomycin, calcium induces a pronounced blue-shift, and the emission intensity increases rapidly up to 0.1 mM calcium but drops back when calcium is raised further. This drop is again due to oligomer formation and self-quenching, since it can be suppressed by mixing the acrylodan derivative with an excess of unlabeled A54145 prior to incubation with membranes (Figure 4C).

Figure 4D compares the fluorescence signals of native and acrylodan-labeled A54145. We can observe that the initial increase in acrylodan fluorescence precedes the tryptophan emission shift, which in turn slightly precedes the drop in the acrylodan emission at higher calcium concentrations. The findings are again compatible with the binding of calcium in two successive steps. Like ornithine-6 of daptomycin, lysine-8 of A54145 inserts into the membrane when the first calcium ion binds, and it interacts with the same residue in adjacent subunits when the second calcium ion binds. Similarly, like kynurenine-13 of daptomycin, tryptophan-1 of A54145 inserts into the membrane concomitantly with binding of the second calcium ion.

Two successive steps of membrane interaction can also be detected by fluorescence anisotropy measurements (Figure 5). The anisotropy of tryptophan-1 in A54145 rises concomitantly with the emission blue shift (cf. Figure 4D), which is accounted for by the higher viscosity inside the membrane than outside. In contrast, the anisotropy of kynurenine-13 in daptomycin rises more rapidly, reaching a maximum already at 0.1 mM of calcium; that is, with this residue, the increase in anisotropy precedes the spectral shift that signals membrane insertion. The early increase in anisotropy may be due to a low mobility of this residue relative to the body of the peptide, which would restrict the motion of kynurenine as soon as any part of the peptide becomes membrane-attached. The anisotropy declines again as calcium is raised further. This may be caused by increasing homo-FRET concomitantly with the aggregation of daptomycin into larger oligomers. Overall, therefore, the anisotropy experiments are compatible with the interpretations proposed for the spectral changes.

Discussion

Figure 6 summarizes the experimental findings of this study. In this figure, the fluorophores contained in labeled and in unlabeled daptomycin and A54145 are combined into a single schematic structure. Binding of the first calcium ion enhances the fluorescence of the extrinsic labels attached to ornithine-6

in daptomycin and to lysine-8 in A54145. Binding of the second calcium ion elicits signals from the intrinsically fluorescent residues tryptophan-1 and kynurenine-13. Concomitantly, the fluorescence signals of the labeled residues 6 and 8 undergo self-quenching due to mutual interaction. All four fluorophores transition to a more hydrophobic environment upon calcium binding. Thus, residues 1, 6, 8, and 13 likely all participate in the membrane interaction of daptomycin/A54145, and residues 6 and 8 additionally participate in the mutual interaction of subunits within the membrane-bound oligomer. Whether or not this mutual interaction involves subunits located in opposite membrane leaflets (as suggested by the cartoon) remains to be determined.

The figure also includes the key findings of another recent study, in which pyrene moieties were semisynthetically incorporated into the fatty acyl tail of A54145 [16]. As in the current study, two successive calcium-dependent transitions were observed, which occurred at calcium concentrations very similar to those reported here. In contrast to the present study, however, a mutual interaction of the labeled molecules, in the form of pyrene excimer fluorescence, was observed already after the first calcium-dependent transition. The excimer intensity increased further in the second transition, indicating that the interaction between labeled acyl residues became tighter and/or involved a larger number of subunits.

Collectively, the findings from both studies can be interpreted as follows: Binding of the first calcium ion causes monomeric lipopeptide molecules to bind to the membrane and to form a loosely associated structure, in which the acyl tails interact, but the peptide moieties – or at least, residues 6 and 8 – do not. This initial aggregate transitions to the final, tightly associated and functional oligomer concomitantly with binding of the second calcium ion. This mechanism is reminiscent of many structurally diverse oligomer-forming, membrane-permeabilizing protein toxins that also form oligomeric “pre-pores” prior to the functional, membrane-inserted pores [17-19].

The observations from fluorescence correlate well with the findings from ITC experiments. With all binding models tested, the whole-number stoichiometric ratio of calcium to daptomycin that best fit the experimental data was 2. The assumption of successive binding to sites with different affinities leads to a notably improved fit relative to the simpler model that assumes a single class of binding sites. If we accept that each lipopeptide molecule binds two calcium ions, the question arises which amino acid residues are involved in this interaction. Prime candidates are the four anionic residues, three of which occur in conserved positions (cf. Figure 1). Using substitutions with uncharged amino acid residues, it has previously been shown that both aspartate-7 and aspartate-9 in daptomycin are essential for antibacterial activity, while replacement of the other two residues reduced, but did not abrogate activity

[20, 21]. It is noteworthy also that anionic residues in positions 7 and 9 are conserved between daptomycin/A45145 and the amphomycin/friulimicin family [22], which is also calcium-dependent but exhibits a different mode of antibacterial action [23]. On the other hand, a recently synthesized daptomycin derivative, in which kynurenine-13 was replaced with tyrosine, showed signs of significantly diminished calcium affinity [15], suggesting that one calcium binding site may involve methyl-glutamate-12, which is located next to residue 13. In any case, the two calcium binding sites remain to be unambiguously determined by future experimental studies.

A possible objection to the fluorescence experiments presented here is that the labelled derivatives displayed reduced antibacterial activity, which might be due to their impeded interaction with calcium and/or membrane lipids. While this possibility cannot be excluded, it should be noted that the labeled derivatives detected the first calcium-dependent transition, whereas the two native compounds detected the second transition. If indeed the two labelled compounds had reduced calcium affinity, this would imply that the first transition should precede the second one by a larger calcium concentration interval than suggested by the results presented here. It seems unlikely, however, that the distinction between both transitions as such arises solely as an artifact of the fluorescent labeling. In this context, it is also worth noting that two successive transitions were also evident in a related study that used only a single labeled derivative [16]. Moreover, with native daptomycin, the anisotropy of kynurenine-13 rises before the residue inserts into the lipid bilayer (cf. Figures 3A and 5, respectively). This observation, which does not involve any extrinsic label, also suggests two successive stages of membrane interaction.

In summary, our study combines ITC and fluorescence methods to determine the stoichiometry of calcium binding to daptomycin and A54145, and to clarify the relationship between calcium binding, membrane binding, and oligomer formation. The exact binding sites of the two calcium ions that bind to each lipopeptide molecule remain to be elucidated in future experiments.

Table 1: Thermodynamic parameters for calcium binding

Stoichiometries, reaction heats (ΔH), and association constants (K) for calcium binding to daptomycin in the presence of an excess of DOPC/DOPG vesicles. Parameters are reported only for the successive two-site model (cf. Figure 2). Averages and standard deviations were obtained from 6 independent experiments.

Parameter		Average	Standard deviation
Stoichiometry (calcium/lipopeptide)		1.81	0.29
First step	ΔH	-4.36 kJ/mol	0.48 kJ/mol
	K	$5.65 \times 10^4 \text{ M}^{-1}$	$3.65 \times 10^4 \text{ M}^{-1}$
Second step	ΔH	-6.57 kJ/mol	1.14 kJ/mol
	K	$2.98 \times 10^4 \text{ M}^{-1}$	$1.61 \times 10^4 \text{ M}^{-1}$

Figure legends

Figure 1: Structures of daptomycin and of A54145 Factor D. “R” represents the fatty acyl tail that is decanoic acid in daptomycin but variable in A54145. Residues that confer intrinsic fluorescence (kynurenine-13 in daptomycin and tryptophan-1 in A54145) as well as those derivatized with acrylodan (ornithine-6 in daptomycin and lysine-8 in A54145) are highlighted in blue; acidic residues are highlighted in red.

Figure 2: A representative isothermal calorimetry trace of calcium binding to daptomycin (510 μM) and LUV composed of DOPC and DOPG (1:1; final concentration of DOPG 3.55 mM). Calcium (10 mM) was injected in increments of 1 μL into a cell volume of 200 μL . A: raw thermal trace (black). The ΔH values in B and C represent the peak areas integrated relative to the baseline (red). The lipid control contained the same amount of lipids but no daptomycin; the experiment was terminated after 70 minutes. B: The ΔH values were fitted with a model that assumes a single set of binding sites, with stoichiometric ratios (n) of 1, 2, or 3 calcium ions per daptomycin molecule. C: The ΔH values were fitted with a model that assumes a variable number (n) of calcium ions binding to a single type of sites or to two successive sites, respectively.

Figure 3: Fluorescence response of native daptomycin (A,C) and of acrylodan-daptomycin (B,C) to increasing amounts of calcium. A: Native daptomycin (3 μM) was added to DMPC/DMPG LUV (250 μM total lipid). Calcium was added at the indicated concentrations, and the fluorescence of kynurenine-13 was excited at 365 nm. B: The same experiment was performed with acrylodan-daptomycin (3 μM). C: Acrylodan-daptomycin (0.5 μM) was mixed with a 5-fold molar excess of the synthetic daptomycin analogue E12W13 [14] prior to addition to membranes. Acrylodan fluorescence was excited at 360 nm. D: Maxima of acrylodan and kynurenine fluorescence emission at increasing calcium concentrations. The initial rise in acrylodan fluorescence subsides and partially reverses concomitantly with the rise in kynurenine fluorescence.

Figure 4: Fluorescence response of native A54145 (A,C,D) and acrylodan-labeled A54145 (B,C,D) to increasing concentrations of calcium. Experimental conditions as in Figure 3. A: The intrinsic fluorescence of tryptophan-1 was excited at 280 nm. B: The fluorescence of acrylodan was excited at 360 nm. C: Acrylodan fluorescence (excited at 360 nm) of a mixture of acrylodan-A54145 (0.5 μM final) and native A54145 (2.5 μM final) incubated with DMPC/DMPG vesicles and increasing calcium concentrations. D: Mean wavelength of tryptophan emission and intensity of acrylodan emission at 475 nm as functions of calcium concentration. The initial rise in acrylodan emission reverses concomitantly

with the blue shift of the tryptophan emission.

Figure 5: Fluorescence anisotropy of kynurenine-13 in daptomycin (3 μM) and of tryptophan-1 in A54145 (3 μM) in the presence of DMPG/DMPC membranes (250 μM total lipid) as a function of calcium concentration. Temperature: 37°C. Error bars represent standard deviations from 3 independent experiments.

Figure 6: Summary of experimental findings from the current study, as well as from a recent study using pyrene fluorescence [15]. The schematic of the lipopeptide molecule combines features from native daptomycin and A54145 as well as the labeled analogues. Circles represent fluorescent residues or labels, whereas semicircles represent calcium-binding sites (the exact locations of which are not certain). Pyrene, which was incorporated into the N-terminally attached fatty acyl tail, is represented by a polygon in dashed outline. The fluorescence signals of acrylodan attached to residue 6 (daptomycin) and residue 8 (A54145) are activated upon binding of the first calcium ion. Binding of the second calcium ion activates the fluorescence signals of tryptophan-1 and kynurenine-13, and at the same time reduces the label fluorescence of residues 6 and 8 through oligomerization and self-quenching. All four fluorophores respond in a manner that indicates increased membrane interaction upon calcium binding. Excimer formation of pyrene is detectable after binding of the first calcium but becomes more intense after binding of the second one.

Acknowledgments

This study was supported by operating grants by NSERC and CIHR to Scott Taylor and Michael Palmer. We thank Jared Silverman (formerly of Cubist) for supplying daptomycin and A54145, and him as well as Jean Duhamel for helpful discussions.

References

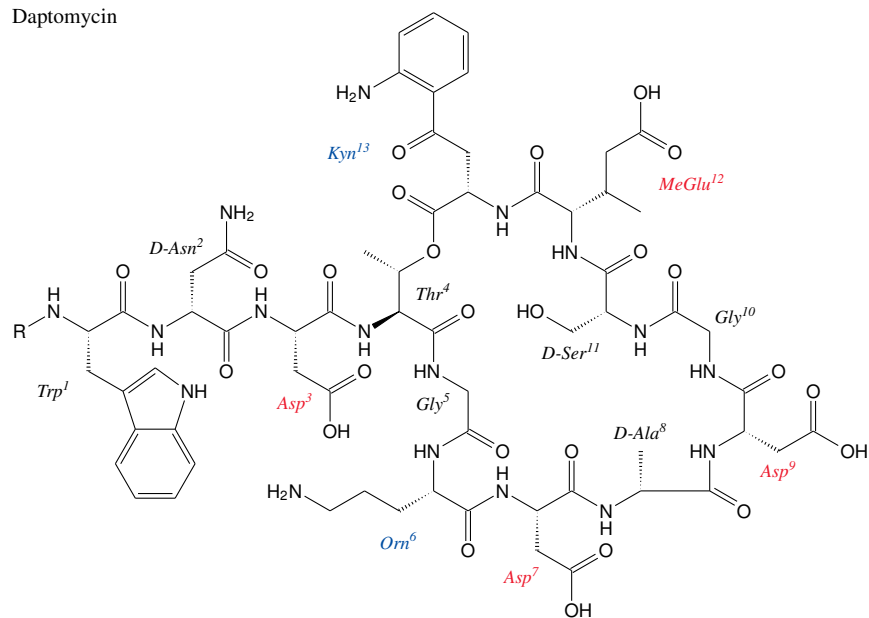
- [1] Baltz, R.H. Daptomycin: mechanisms of action and resistance, and biosynthetic engineering, *Curr Opin Chem Biol* 13 (2009) 144-151.
- [2] Zhang, T., Muraih, J.K., Mintzer, E., Tishbi, N., Desert, C., Silverman, J., Taylor, S. and Palmer, M. Mutual inhibition through hybrid oligomer formation of daptomycin and the semisynthetic lipopeptide antibiotic CB-182,462, *Biochim Biophys Acta* 1828 (2013) 302-308.
- [3] Lakey, J.H. and Ptak, M. Fluorescence indicates a calcium-dependent interaction between the lipopeptide antibiotic LY146032 and phospholipid membranes, *Biochemistry* 27 (1988) 4639-4645.
- [4] Jung, D., Rozek, A., Okon, M. and Hancock, R.E.W. Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin, *Chem Biol* 11 (2004) 949-957.
- [5] Muraih, J.K., Pearson, A., Silverman, J. and Palmer, M. Oligomerization of daptomycin on membranes, *Biochim Biophys Acta* 1808 (2011) 1154-1160.
- [6] Zhang, T., Muraih, J., MacCormick, B., Silverman, J. and Palmer, M. Daptomycin forms cation- and size-selective pores in model membranes, *Biochim Biophys Acta* 1838 (2014) 2425-2430.
- [7] Ball, L., Goult, C.M., Donarski, J.A., Micklefield, J. and Ramesh, V. NMR structure determination and calcium binding effects of lipopeptide antibiotic daptomycin, *Org Biomol Chem* 2 (2004) 1872-1878.
- [8] Mayer, L.D., Hope, M.J. and Cullis, P.R. Vesicles of variable sizes produced by a rapid extrusion procedure, *Biochim Biophys Acta* 858 (1986) 161-168.
- [9] Tsentalovich, Y.P., Snytnikova, O.A., Sherin, P.S. and Forbes, M.D.E. Photochemistry of kynurenine, a tryptophan metabolite: properties of the triplet state, *J Phys Chem A* 109 (2005) 3565-3568.
- [10] Prendergast, F.G., Meyer, M., Carlson, G.L., Iida, S. and Potter, J.D. Synthesis, spectral properties, and use of 6-acryloyl-2-dimethylaminonaphthalene (Acrylodan). A thiol-selective, polarity-sensitive fluorescent probe, *J Biol Chem* 258 (1983) 7541-7544.
- [11] Mims, M.P., Sturgis, C.B., Sparrow, J.T. and Morrisett, J.D. Acrylodan can label amino as well as sulfhydryl groups: results with low-density lipoprotein, lipoprotein[a], and lipid-free proteins, *Biochemistry* 32 (1993) 9215-9220.
- [12] Traut, R.R., Bollen, A., Sun, T.T., Hershey, J.W., Sundberg, J. and Pierce, L.R. Methyl 4-

mercaptobutyrimidate as a cleavable cross-linking reagent and its application to the Escherichia coli 30S ribosome, *Biochemistry* *12* (1973) 3266-3273.

- [13] Zhang, T., Muraih, J.K., Tishbi, N., Herskowitz, J., Victor, R.L., Silverman, J., Uwumarenogie, S., Taylor, S.D., Palmer, M. and Mintzer, E. Cardiolipin prevents membrane translocation and permeabilization by daptomycin, *J Biol Chem* *289* (2014) 11584-11591.
- [14] Muraih, J.K. and Palmer, M. Estimation of the subunit stoichiometry of the membrane-associated daptomycin oligomer by FRET, *Biochim Biophys Acta* *1818* (2012) 1642-1647.
- [15] Lohani, C., Taylor, R., Palmer, M. and Taylor, S. Solid-Phase Total Synthesis of Daptomycin and Analogs, *Organic Letters* *17* (2015) 748-751.
- [16] Zhang, T., Taylor, S., Palmer, M. and Duhamel, J. Membrane binding and oligomer formation of the calcium-dependent lipopeptide antibiotic A54145: a quantitative study with pyrene excimer fluorescence, *Biophys J* (*submitted*) (2016) .
- [17] Bravo, A., Gill, S.S. and Soberón, M. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control, *Toxicon* *49* (2007) 423-435.
- [18] Shepard, L., Shatursky, O., Johnson, A. and Tweten, R. The mechanism of pore assembly for a cholesterol-dependent cytolysin: Formation of a large pre-pore complex precedes the insertion of the transmembrane beta-hairpins, *Biochemistry* *39* (2000) 10284-10293.
- [19] Walker, B., Braha, O., Cheley, S. and Bayley, H. An intermediate in the assembly of a pore-forming protein trapped with a genetically-engineered switch, *Chem Biol* *2* (1995) 99-105.
- [20] Grünwald, J., Sieber, S.A., Mahlert, C., Linne, U. and Marahiel, M.A. Synthesis and derivatization of daptomycin: a chemoenzymatic route to acidic lipopeptide antibiotics, *J Am Chem Soc* *126* (2004) 17025-17031.
- [21] Lohani, C.R., Taylor, R., Palmer, M. and Taylor, S.D. Solid-phase synthesis and in vitro biological activity of a Thr4 → Ser4 analog of daptomycin, *Bioorg Med Chem Lett* *25* (2015) 5490-5494.
- [22] Schneider, T., Gries, K., Josten, M., Wiedemann, I., Pelzer, S., Labischinski, H. and Sahl, H. The lipopeptide antibiotic Friulimicin B inhibits cell wall biosynthesis through complex formation with bactoprenol phosphate, *Antimicrob Agents Chemother* *53* (2009) 1610-1618.
- [23] Strieker, M. and Marahiel, M.A. The structural diversity of acidic lipopeptide antibiotics, *Chembiochem* *10* (2009) 607-616.

Figure 1

Daptomycin



A54145

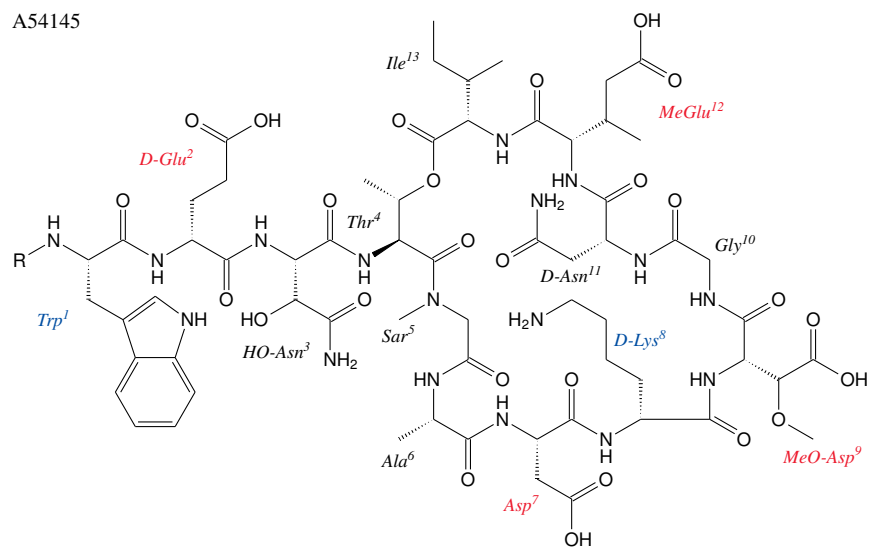


Figure 2

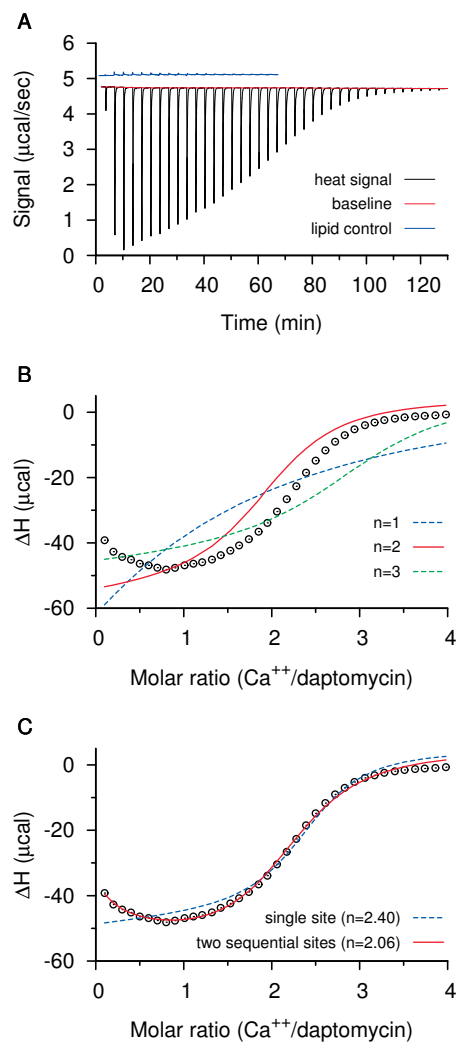


Figure 3

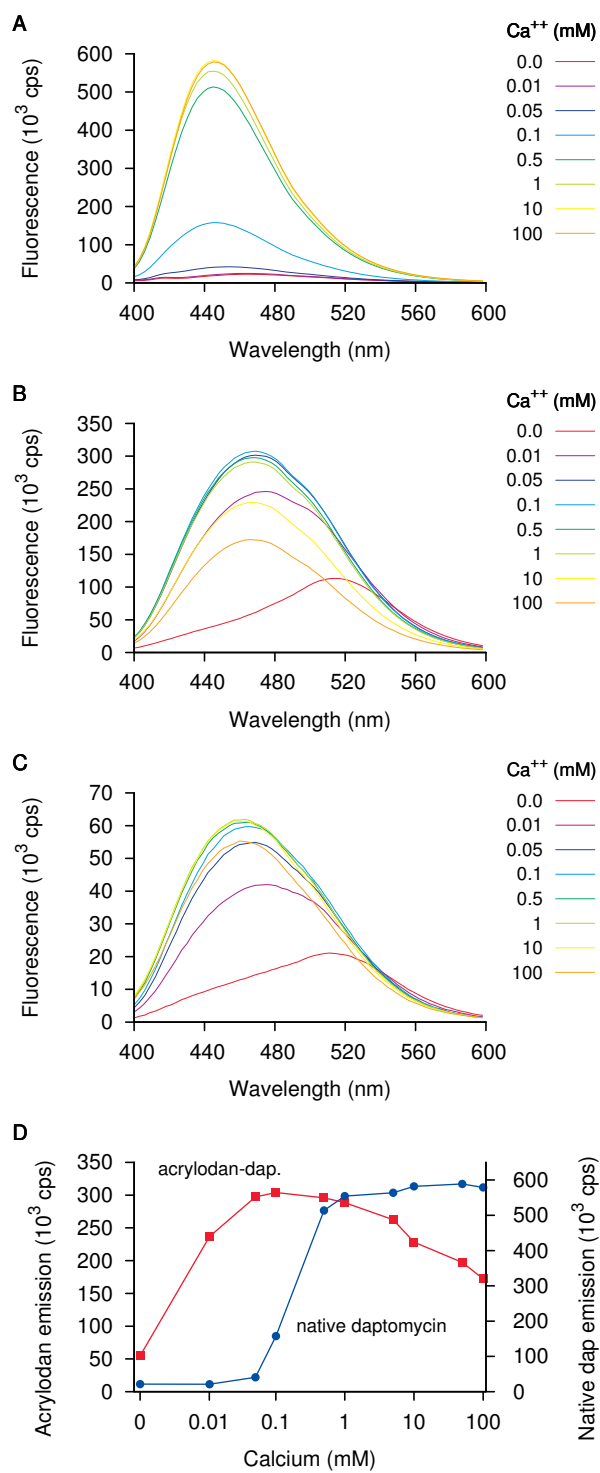


Figure 4

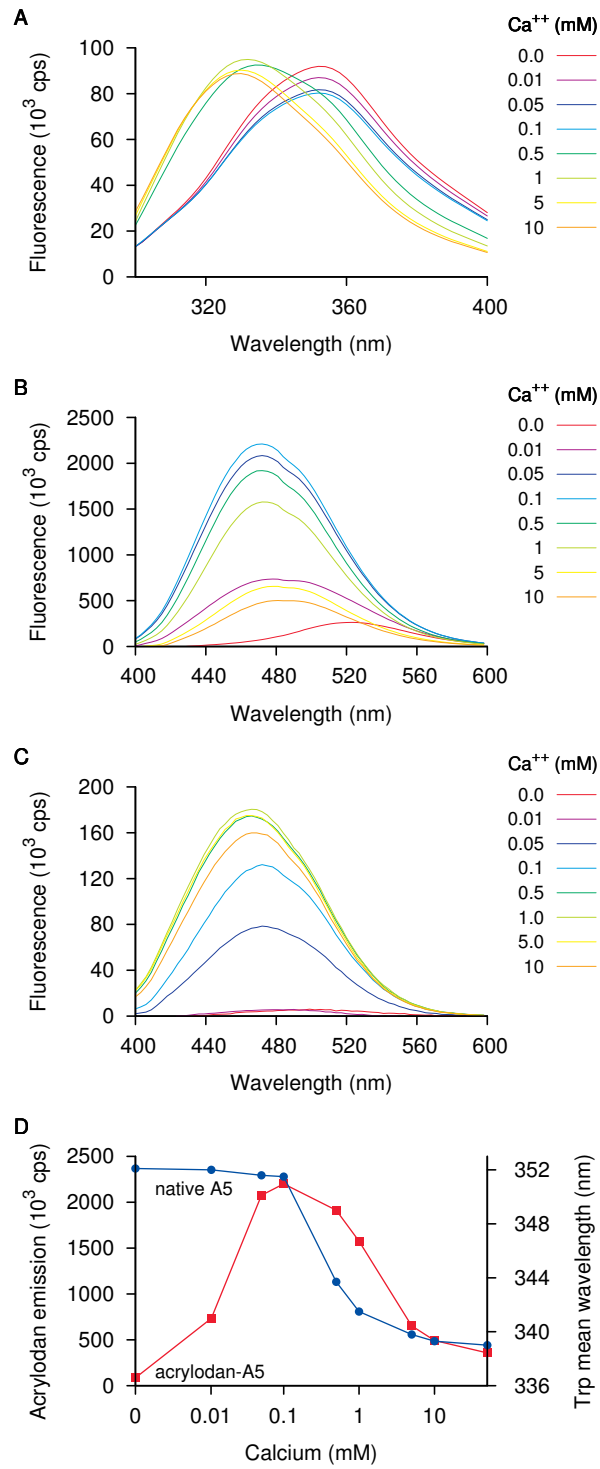


Figure 5

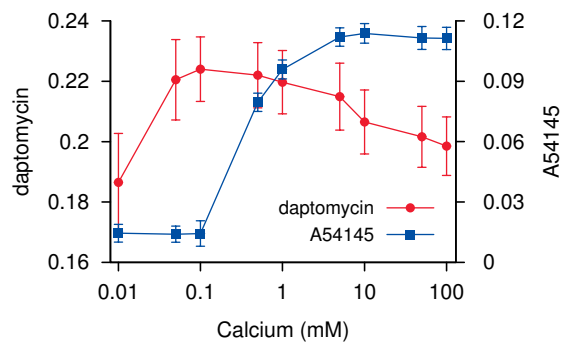


Figure 6

