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Establishing the Structure-Activity Relationship of Daptomycin

Hoi Yee Chow,† Kathy Hiu Laam Po,‡ Kang Jin,† Guanlin Qiao,† Zhenquan Sun,† Wenjie Ma,† Xiyun Ye,† Ning Zhou,† Sheng Chen‡* and Xuechen Li†,*

†Department of Chemistry, State Key Lab of Synthetic Chemistry, The University of Hong Kong, Pok Fu Lam, Hong Kong

‡Department of Infectious Diseases and Public Health, Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, Hong Kong

KEYWORDS. Daptomycin, calcium-dependent antibiotics, Structure-activity relationship, cyclic depsipeptides, total chemical synthesis

ABSTRACT: Daptomycin is effective in treating infections caused by antibiotic-resistant Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and vancomycin-resistant *S. aureus* (VRSA). Due to its distinct mechanism of action towards multidrug-resistant bacteria, daptomycin provides an attractive structural motif to generate new daptomycin-based antibiotics to combat the problem of bacterial resistance. In this study, we used total synthesis method to produce daptomycin analogues with a variety in terms of types and sites of modifications. Five classes of daptomycin analogues were synthesized and the antimicrobial activities of the analogues were analyzed by several biological assays. From this study, we established a comprehensive structure-activity relationship of daptomycin which will lay foundation for the further development of daptomycin- based antibiotics.

The emergence of multidrug resistance in bacterial pathogens has severely undermined the effectiveness of public health management.[1] Despite many research efforts, there is currently no reliable solution to deal with the problem of the rapid development of resistance to clinically important antimicrobial agents and the resulting global dissemination of resistant organisms. Furthermore, the current drug development programs appear to be insufficient to provide therapeutic coverage in the foreseeable future, partly due to scientific challenges in discovering new class of antibiotics, and new compounds in the established class, as well as financial considerations of pharmaceutical companies.[1] Methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as one of the most important pathogens, both in hospital and community-acquired infections.[2]

Daptomycin is a lipodepsipeptide isolated from *Streptomyces roseoporus* obtained from a soil sample from Mount Ararat by the scientists at Eli Lilly. Daptomycin has potent bactericidal activities against the otherwise antibiotic-resistant Gram-positive pathogens, including MRSA, vancomycin-resistant *Enterococci* (VRE) and vancomycin-resistant *S. aureus* (VRSA).[3] It has been proposed that daptomycin, in the presence of calcium ions, undergoes conformational changes upon association with bacterial membrane.[4] Although the mechanism of action (MOA) of daptomycin is not fully elucidated, with the most recent study suggesting that daptomycin works by interfering the cell wall biosynthesis,[5] its distinct mechanism of action renders daptomycin useful in treating infection caused by multidrug-resistant bacteria. Daptomycin was approved by the FDA in 2003 and marketed in the United States under the trade name Cubicin by Cubist Pharmaceuticals Inc. (now Merck & Co.) for the treatment of skin and skin structure infections and right-side endocarditis caused by MRSA, as well as patients with prolonged MRSA bacteremia (>7 days) which are at high risk for metastatic complications and death. As daptomycin has been introduced to the market for over 15 years, there are increasing reported cases of infection caused by daptomycin-resistant Gram-positive clinical pathogens such as *S. aureus*, *Enterococci* and *Streptococci* in clinical settings in recent years.[6-9] As the first-in-class lipodepsipeptide antibiotic with a distinct mechanism of action, daptomycin has the potential to be derivatized to produce next-generation daptomycin-based antibiotics as seen in the successful development of several generations of β-lactam–based antibiotics.

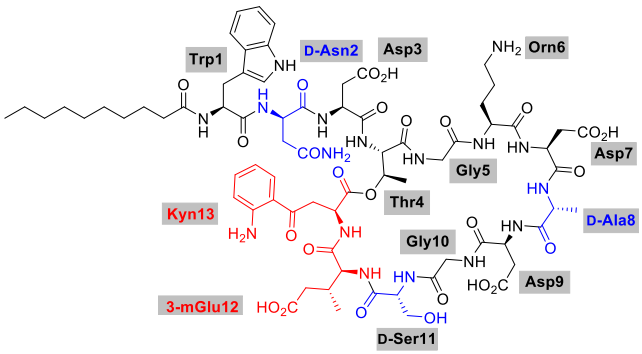


Figure 1. The structure of daptomycin. Red: nonproteinogenic amino acids, blue: D-amino acids.

Daptomycin is a 13-amino acid cyclic lipodepsipeptide belonging to the nonribosomal peptide family (Figure 1). The establishment of the structure-activity relationship (SAR) of daptomycin will be of great value to search for daptomycin-based next-generation antibiotics for additional clinical applications and bacterial resistance preparedness. However, the structural complexity of daptomycin, due to the presence of two non-

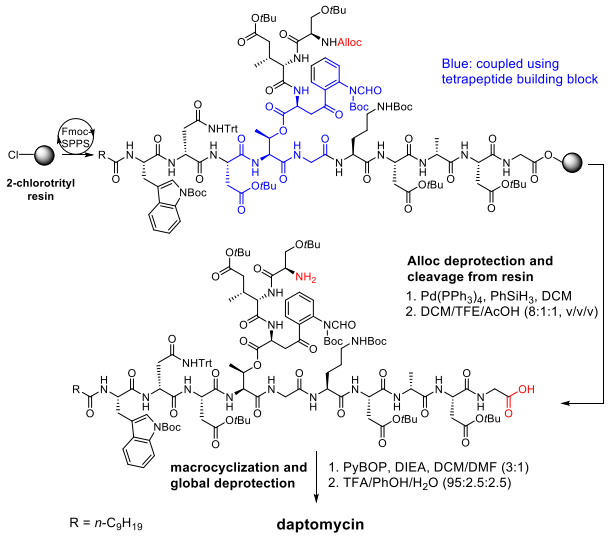
proteinogenic amino acids (Kyn and 3-mGlu) as well as the lactone bond in the cyclic structure, renders it a challenging target for total synthesis. The first total synthesis was reported by our group in 2013,[10] 10 years after its approval by the FDA. Before establishment of the total synthesis, only a limited number of daptomycin analogues with narrow choices of modification sites have been produced via genetic engineering of the non-ribosomal peptide synthetases in the daptomycin biosynthetic pathway or via semi-synthesis. For biosynthetic method, the produced analogues include modifications at position 8 (D-Ala), position 11 (D-Ser), position 12 (3-mGlu) and position 13 (Kyn) with a limited choice of amino acids that could be substituted at these sites. Notably, these studies have revealed that 3-mGlu is important for the antibacterial activity, since the replacement of 3-mGlu with Glu in daptomycin resulted in an 8-fold increase in minimal inhibitory concentration (MIC).[11] For semi-synthesis method, the modification site is limited to the rather easily chemically accessible exocyclic domain[12-15] and the reactive amino group on the side chain of Orn.[16,17]

Marahiel's group produced some analogues by enzyme-assisted lactone closure on unprotected acyclic peptides which were prepared by solid-phase peptide synthesis (SPPS).[18,19] This method could potentially allow modifications at more sites. However, the lack of absolute selectivity of the ring closure site resulted in a mixture of regioisomers, which were difficult to be separated and thus complicated the purification. The unexamined substrate scope of the enzyme and the inaccessibility of the enzyme to most synthetic labs make this method less preferred for analogue preparation. The lack of a comprehensive SAR studies of daptomycin was mainly due to the limitation in having an efficient method for producing analogues needed for SAR studies.

Chemical synthesis of daptomycin would facilitate generation of analogues with flexibility and precision. Besides the our total synthesis of daptomycin,[10] other syntheses have been reported.[20-23] Taylor and co-workers synthesized a number of daptomycin analogues, all of which contained Glu and Trp at position 12 and 13, respectively, in place of the crucial 3-mGlu and Kyn in daptomycin. The importance of 3-mGlu on the daptomycin antibacterial activity was illustrated by early study,[11] as well as our study[24] by synthesizing daptomycin analogues with 3*R*-mGlu being replaced by 3*R*-ethyl-Glu, 3*R*-methyl-Gln or 3,3-dimethyl-Glu and the study by Brimble's group[22] with the 2*S*,3*R*-mGlu substituted by 2*R*,3*R*-mGlu and 2*S*,4*S*-mGlu. These results showed that both the carboxylate and the methyl group of 3-mGlu are crucial for antibacterial activity. As a result, without the 3-mGlu, all Taylor's analogues exhibited much reduced antibacterial activity.

Herein, we report our structure-activity relationship studies based on the *bona fide* daptomycin structure. We have synthesized five classes of daptomycin analogues which cover a wide span of possible modifications on daptomycin by employing the total synthesis method (Scheme 1).[23] Site-specific modification was achieved by incorporating the protected form of amino acids that was compatible with Fmoc-SPPS protocol at the designated position. The obtained analogues were studied with antibacterial assays to evaluate their antimicrobial activity and to establish the structure-activity relationship.

Scheme 1. General scheme for chemical synthesis of daptomycin.



The first class of analogues was based on alanine scanning strategy in which each amino acid residue within daptomycin was individually replaced with the alanine residue. Amino acids at position 5 and 10 were not examined due to concern of epimerization during the synthesis, while position 11 has been examined previously by Cubist scientists. Changing D-Ser to D-Ala in position 11 had little effect on the antibacterial activity of the analogue, suggesting this position could allow structural alteration.[11] Biological evaluations of this class of analogues (Table 1) showed that only the analogue modified at position Orn6 (**4**) showed similar activity as daptomycin. A surge in MIC was observed when amino acids at other positions were substituted by alanine.

Based on the result of the alanine scanning, we next prepared a series of analogues bearing modification at the Orn6 site. Ornithine is the only residue carrying a positive charge in daptomycin under physiological conditions. The role of ornithine in daptomycin is not known, but the alanine scanning results indicated that the positive charge is not necessary for antibacterial activity. Cubist scientists have prepared a large set of analogues by installing different groups via the side chain amino group of Orn by *N*-acylation or *N*-alkylation.[16,17] Most of their analogues retained antibacterial activity, which is consistent with our alanine-scanning results showing that modifications at position 6 were well tolerated. As Cubist scientists did not produce any analogues with activity superior to daptomycin, we adopted a different approach for the modification. Instead of adding moieties onto the side chain of Orn, we switched Orn to other amino acids. Since the 20 proteinogenic amino acids have side chains with different size, functional groups and polarity, we replaced Orn with the 20 natural amino acids to see to what extent the modification at this site can be tolerated and also to gain insight on the role of Orn. The MICs of these analogues are listed in Table 2. When Orn was changed to acidic residues such as Asp (**11**) or Glu (**12**), the bioactivity was lost totally. Loss of activity was also observed when Orn was changed to Asn (**20**) or Gln (**22**). These results suggested the presence of the negative charge or amide group demolishes the antibacterial effect of daptomycin. When Orn was changed to Gly (**14**) or

Table 1 MICs[a] of analogues for alanine-scanning

#	Amino acid replaced	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
	daptomycin	0.5	0.5	0.5	0.25	0.25	4	2	2	4
1	Trp1	≥32	≥32	≥32	≥32	≥32	≥64	≥64	≥64	≥64
2	D-Asn2	≥32	≥32	≥32	16	≥32	16	64	32	64
3	Asp3	≥32	≥32	16	16	16	≥32	≥32	≥32	≥32
4	Orn6	0.5	0.25	0.5	1	0.5	4	2	1	4
5	Asp7	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64
6	D-Ala8[e]	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64
7	Asp9	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64
8	3-mGlu12	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64	≥64
9	Kyn13	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32

[a]units in μg/mL. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*. [e]replaced with L-Ala.

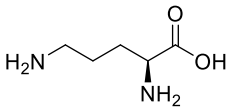
Table 2 MICs[a] of analogues with substitution at Orn.

#	modification	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
	daptomycin	0.5	0.5	0.5	0.25	0.25	4	2	2	4
4	Ala	0.5	0.25	0.5	1	0.5	4	2	1	4
10	Cys[e]	8	4	8	16	16	4	16	16	16
11	Asp	≥32	≥32	16	8	16	≥32	≥32	≥32	≥32
12	Glu	≥32	≥32	≥32	16	16	≥32	≥32	≥32	≥32
13	Phe	≥32	≥32	16	16	16	≥32	≥32	≥32	≥32
14	Gly	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
15	His	16	16	8	8	8	≥32	≥32	≥32	≥32
16	Ile	≥32	≥32	8	4	8	≥64	≥64	32	≥64
17	Lys	16	≥32	1	1	1	8	≥32	16	≥32
18	Leu	1	0.5	4	2	4	≥32	≥32	16	≥32
19	Met	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
20	Asn	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
21	Pro	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
22	Gln	≥32	≥32	4	4	4	≥32	≥32	≥32	≥32
23	Arg	8	16	2	1	2	8	≥32	16	≥32
24	Ser	4	4	2	1	2	4	≥32	≥32	≥32
25	Thr	16	16	2	2	2	≥32	≥32	≥32	≥32
26	Val	≥32	≥32	8	8	8	≥32	≥32	≥32	≥32
27	Trp	2	1	4	2	4	2	≥32	≥32	≥32
28	Tyr	1	0.5	4	4	1	≥32	≥32	16	≥32

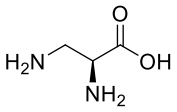
[a]units in μg/mL. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*. [e]in dimeric form via disulfide bond.

Pro (**21**), no activity was observed in the tested concentration range (0.03-16 μg/ml, or 32 μg/ml in some cases), either. This may indicate that a flexible side chain is essential for the antimicrobial activity. While analogues changing Orn to aromatic or hydrophobic amino acids showed antibacterial activity towards *S. aureus*, these analogues were inactive towards *Enterococcus* spp. We also examined the effect of the length of the side chain at this position on the antibacterial activity (Table 3). From these results, it is obvious that there exists an optimal length of the side chain at this position. Reducing or increasing the chain length both increased the MIC, with MIC against *Enterococci* being more affected. Since change in the Orn position yielded analogues with different sensitivities to different bacteria, Orn may have a role in the membrane binding/insertion on Gram-positive bacteria. Nevertheless, further investigation is needed to find out the underlying cause of the difference before a conclusive explanation of the role of Orn in daptomcyin could be drawn.

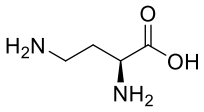
Table 3 MICs[a] of analogues with side-chain amine of different length at position 6



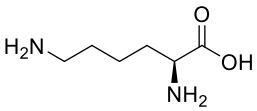
Orn
(in daptomycin)



Dap
2,3-diaminopropanoic acid



Dab
2,4-diaminobutanoic acid



Lys

#	modification	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
	Orn	0.5	0.5	0.5	0.25	0.25	4	2	2	4
29	Dap	8	1	1	0.5	1	ND[e]	≥32	8	≥32
30	Dab	2	0.5	0.25	0.25	0.25	ND	16	8	16
17	Lys	16	≥32	1	1	1	8	≥32	16	≥32

[a]units in μg/mL. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*. [e]not determined.

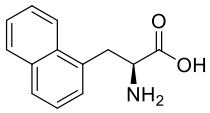
Table 4 MIC[a] of analogues with modification at the DXDG conserved motif

#	modification	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
	daptomycin	0.5	0.5	0.5	0.25	0.25	4	2	2	4
31	Asp9 to Asn9	≥32	≥32	32	16	16	≥32	≥32	≥32	≥32
32	Asp9 to Glu9	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
33	Asp9 to D-Asp9	≥32	≥32	16	16	16	≥32	≥32	≥32	≥32
34	Asp9 to 3-mGlu9	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
35	Gly10 to Sar10	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
36	Asp7, Asp9 to Asn7, Asn9	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
37	Asp7, Asp9 to Glu7, Glu9	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
38	Asp9, 3-mGlu12 to 3-mGlu9, Asp12	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32

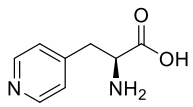
[a]units in μg/mL. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*.

It has been proposed that the interaction between daptomycin and calcium ion is realized via the DXDG motif[25] within the cyclic backbone of daptomycin and the two carboxylates of two aspartic acid residues are involved in direct interaction with the calcium ion. We prepared a class of analogues with modifications at the DXDG region by replacing the relevant aspartic acid residue with other similar amino acids (e.g. Glu, Asn), and replacing the Gly with sarcosine (*c.f. N*-methylated Gly). The MIC results showed that this region is not amendable for variation, as a slight change in this region yielded inactive analogues (Table 4). We next conducted conformational studies to investigate the reason for the loss of the activity of these analogues. Daptomycin was shown to interact with model membrane containing phosphatidylglycerol (PG) in the presence of calcium ions resulting conformational changes/oligomerization by fluorescence and circular dichroism (CD) spectroscopy.[26,27] Förster resonance energy transfer (FRET) between Trp and Kyn in daptomycin was observed in the presence of PG and Ca^{2+} ions (Figure S2a), indicating that the two residues are in close proximity. The dramatic change in the molar ellipticity from positive to negative at around 232 nm (Figure S2e) also suggested daptomycin exhibits conformational changes or oligomerizes in the presence of PG and Ca^{2+} ions. Our results showed that upon changing the two Asp to Asn, the analogue lost its ability to interact and insert to the model membrane in the same fashion as daptomycin (Figure S2b) and no FRET between Trp and Kyn was observed (Figure S2f). However, changing Asp at position 9 to Glu, the analogue was still able to interact with the model membrane and/or undergo conformational changes, but probably the interaction was decreased when compared to the case of daptomycin as both the increase in fluorescent intensity and the change in molar ellipticity were less than that of daptomycin (Figure S2c and S2f). This reduced interaction with the membrane may account for the reduced antibacterial activity in the MIC assay. From this class of analogues, we confirmed that the two Asp residues in DXDG motif are essential for interacting with calcium ions and both the length and the presence of the carboxylic group are important for daptomycin interacting with the membrane in the presence of calcium.

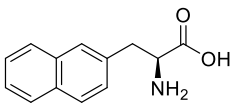
Table 5 MIC[a] of analogues with modification at Trp1



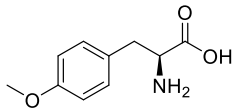
1-Nap
-(1-naphthyl)-L-alanine



Pyr
-(4-pyridyl)-alanine



2-Nap
-(2-naphthyl)-L-alanine

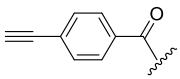
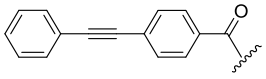
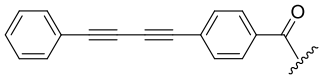
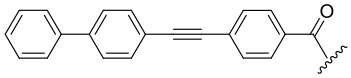


TyrOMe

#	modification	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
	daptomycin	0.5	0.5	0.5	0.25	0.25	4	2	2	4
39	1-Nap	1	0.25	0.25	0.125	0.25	0.5	2	2	2
40	Pyr	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
41	2-Nap	0.5	0.125	0.06	0.125	0.25	0.25	4	0.5	1
42	TyrOMe	16	4	2	2	2	8	≥32	≥32	≥32

[a]units in μg/mL. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*.

Table 6 MIC[a] of analogues with modification of the lipid tail

#	Structure of lipid substituted	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
43		≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32	≥32
44		0.25	0.5	0.5	0.5	0.5	1	8	2	8
45		1	0.5	0.5	0.5	0.5	1	4	1	1
46		1	1	2	1	2	2	0.5	2	0.5

[a]units in μg/mL. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*.

At this point, all these SAR studies, together with previous studies[11,19] have indicated that replacing amino acid residues in daptomycin with other "natural amino acid" residue could not yield any daptomycin analogue equally potent or more potent than daptomycin; since the building block is amino acids, daptomycin is likely the optimized structure by natural evolution.

To surpass nature evolution mostly limited to the natural amino acids, we turned to introduce noncanonical amino acids into the daptomycin analogues in an attempt to produce analogues with enhanced antibacterial activity to aid the SAR study. We started using unnatural aromatic amino acids that share structural features with Trp and generated four analogues (Table 5). To our delight, theses analogues, except **40,** showed similar or even slightly improved *in vitro* antibacterial activity compared to daptomycin. It appears that the presence of a phenyl ring is necessary for the antibacterial activity, as the replacement with a pyridyl moiety at position 1 caused a significant drop in the antibacterial activity.

The last class of analogues was modified on the lipid chain. A hydrophobic aliphatic chain is present in daptomycin, which may induce daptomycin to interact and insert into the bacterial cell membrane. Although modifications at the lipid tail have been extensively studied, the structural variety of the lipid tails modified was limited to mostly saturated alkyl chains.[12,13] A more recent study included lipid tails with aromatic moieties.[15] We have substituted the lipid tail with a series of alkyne-containing lipid chains with different lengths, which have a 'straight-line' geometry (Table 6). From this method, we obtained several analogues with improved *in vitro* activity against MRSA compared to daptomycin. The antibacterial activity of the analogues increased with lipid chain length, consistent with the results from previous studies.[12] These analogues exhibited very similar activity compared to daptomycin against *S. aureus*, but higher growth inhibitory effect on *Enterococci* tested. This analogue may be the potential lead candidate for the further development as new antibiotics in the future to combat the multidrug resistance in Gram-positive bacterial pathogens.

We also substituted some amino acids with their methylated derivates, namely, N-methylated Trp, N-methylated Kyn and sarcosine (Sar, i.e. *N*-methylglycine) to replace Trp, Kyn and Gly, respectively (analogues **47-49**).[23] These methylated analogues showed increased bactericidal activity compared to daptomycin (Table 7). The Kyn methylated daptomycin, termed kynomycin, showed improved *in vivo* antibacterial activity, which has been recently reported.[23] These studies indicated that generation of analogues by substituting the original amino acid present in daptomycin using unnatural amino acid is an

effective strategy to produce analogues that retain its antibacterial activity.

Table 7 MIC[a] of analogues with modification by using methylated derivatives

#	modification	MRSA[b]				SA[c]	SF[d]	*Enterococcus*		
		SA11	SA14	SA86	SA88	ATCC29213	SF	ET6	ET60	ATCC29212
	daptomycin	0.5	0.5	0.5	0.25	0.25	4	2	2	4
47	Kyn-13 to Me-Kyn-13 (kynomycin)	0.5	0.25	0.25	0.25	0.25	0.5	1	1	1
48	Trp-1 to 1-Me-Trp-1	2	0.5	0.5	0.25	0.5	0.5	1	1	2
49	Gly-5 to Sar-5	0.5	0.25	0.5	0.5	0.5	2	8	4	8

[a]units in μg/mL, data from ref. 23. [b]Methicillin-resistant *S. aureus*. [c]*S. aureus*. [d]*Streptococcus faecalis*.

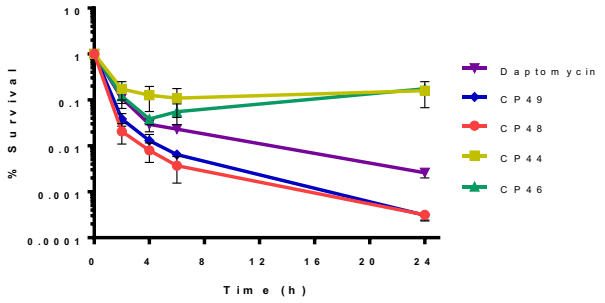


Figure 3. Bactericidal effects of selected daptomycin analogues against *S. aureus* SA86 (a clinical isolated strain) determined by time kill assays (mean± SD).

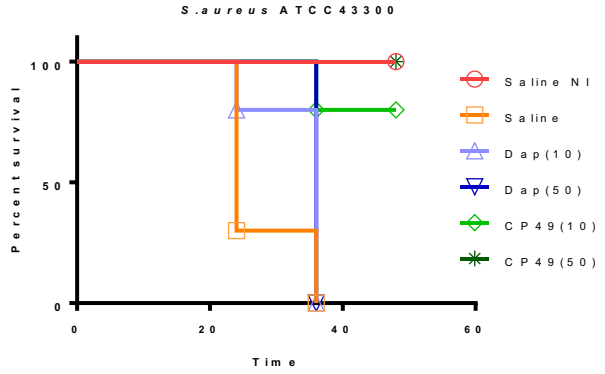


Figure 4. In vivo study of analogue **49** using *Galleria mellonella*. Dap: daptomycin, CP49: analogue **49**, the value in brackets denotes the dosage in mg/kg. Significant difference (P<0.0001) was observed between Dap (50) and CP45 (50); Significant difference (P<0.0004) was observed between Dap (10) and CP45 (10).

Among all the obtained analogues, compounds **44**, **46**, **48** and **49** were chosen for further evaluations. Time-kill assays were performed on these analogues in comparison with daptomycin. The data indicated that compounds **48** and **49** showed significantly higher bactericidal effects (Figure 3). Mutation prevention concentration (MPC) of these active analogues was also determined. Our data showed that compounds **48** and **49**, with the highest bactericidal effects, showed lower MPC than daptomycin (Table S1). Next, compound **49** was chosen to be the representative compound to study its *in vivo* antibacterial activity compared to daptomycin. *Galleria mellonella* ("wax" worm) larvae model was used for this purpose since this model has been proven to be a reliable and simple model for *S. aureus* infections [14,23,28]. Our data showed that compound **49** could protect *S. aureus* ATCC43300 infection at CFU of 7.4×10^6 for 48 h and longer, while all worms treated with daptomycin were dead at 36 h post-infection (Figure 4). This study suggested that compound **49** is potentially an improved antibiotic compared to daptomycin.

In this study, we reported 49 daptomycin analogues synthesized via total synthesis for the establishment of a comprehensive SAR of daptomycin. The results from alanine scanning indicates all the amino acids present in daptomycin are critical for its antibacterial activity except ornithine, where the MIC only increased one-fold against MRSA strain when ornithine was replaced by alanine. Replacing ornithine with each of the 20 natural amino acids showed that the position of Orn prefers hydrophobic side chain with neutral or positive charge over negative charge. Moreover, the position at Orn cannot accommodate a flexible (Gly) or a rigid (Pro) residue. The MIC results suggested that the interacting counterpart of Orn is probably in a hydrophobic environment and the interacting moieties bear negative charges. These results implied that Orn may have a role in the membrane binding.

Changing the residues that are believed to bind calcium ions (DXDG motif) to other highly similar residues, such as Asp to either the structurally similar Asn or functionally similar Glu or 3-mGlu, as well as Gly to sarcosine, abolished the ability of the analogues to kill bacteria. The fact that a slight change to each of the two aspartic acid residues and the Gly within the DXDG motif inactivated the antibacterial property projects the spatial requirement of the carboxylates binding to Ca^{2+} ions. The type and number of ligands present as well as the size of an ion binding pocket govern the ion recognition. Any change in the calcium binding site in daptomycin analogues disrupts the calcium ion binding interaction which prevents the analogues to undergo the essential conformational changes for bactericidal action.

The results of SAR studies directed us to search for better analogues utilizing unnatural amino acid for modification as natural evolution has probably selected the best "natural building block" for daptomycin. Our preliminary results showed that we could obtain more potent analogues by fine-tuning the structure of daptomycin through replacing the residues with their structurally similar unnatural amino acids.

Chemical synthesis provides a robust, flexible and effective way to prepare a library of analogues with modification at the sites that are rather difficult to access by bioengineering and semisynthesis. Through the medicinal studies on daptomycin, we have established a comprehensive structure-activity

relationship of daptomycin. In addition, we have obtained analogues with improved antibacterial activity as compared to daptomycin, which provides us new direction to search for the daptomycin analogues with improved potency.

ASSOCIATED CONTENT

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

Supporting Information
Details of chemical synthesis, biological assays and conformation analysis studies, LC-MS traces of analogues and supplementary figures and tables. (PDF)

AUTHOR INFORMATION

Corresponding Author

* Xuechen Li − Department of Chemistry, State Key Lab of Synthetic Chemistry, The University of Hong Kong, Pok Fu Lam, Hong Kong; orcid.org/0000-0001-5465-7727; Email: xuechenl@hku.hk; Sheng Chen, City University of Hong Kong, Kowloon, Hong Kong; Email: shechen@cityu.edu.hk.

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ABBREVIATIONS

FDA, Food and Drug Administration; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococci*; VRSA, vancomycin-resistant *S. aureus*; MOA, mechanism of action; Orn, ornithine; Kyn, kynurenine; 3-mGlu, 3*R*-methyl-glutamic acid; SAR, structure-activity relationship; MIC, minimal inhibitory concentration; SPPS, solid-phase peptide synthesis; PG, phosphatidylglycerol; CD, circular dichroism; FRET, Förster resonance energy transfer; Sar, sarcosine; MPC, mutation prevention concentration.

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