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Solid-Phase Total Synthesis of Daptomycin and Analogs

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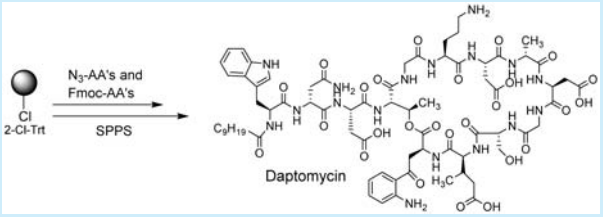
Supporting Information

ABSTRACT: An entirely solid-phase synthesis of daptomycin, a cyclic lipodepsipeptide antibiotic currently in clinical use, was achieved using a combination of α-azido and Fmoc amino acids. This methodology was applied to the synthesis of several daptomycin analogs, one of which did not contain kynurenine or the synthetically challenging amino acid (2*S*,3*R*)-methylglutamate yet exhibited an MIC approaching that of daptomycin.



Daptomycin, a natural product isolated from fermentations of *Streptomyces roseosporus*, is the first approved member of a novel class of antibiotics known as cyclic lipopeptides.[1a−d] Daptomycin was approved in the USA in 2003, and in Europe in 2006, for the treatment of complicated skin and skin structure infections caused by Gram-positive bacteria.[1b,2] In 2006 it was approved in the USA for the treatment of bacteremia and right-sided endocarditis caused by *Staphylococcus aureus* including methicillin resistant *S. aureus* (MRSA).[2] It is not effective against community-acquired pneumonia due to inhibition by pulmonary surfactant.[3]

Daptomycin is a branched, cyclic lipodepsipeptide consisting of a 10-amino acid macrolactone ring to which is attached an exocyclic tripeptide bearing a decanoyl lipid tail (Figure 1). It

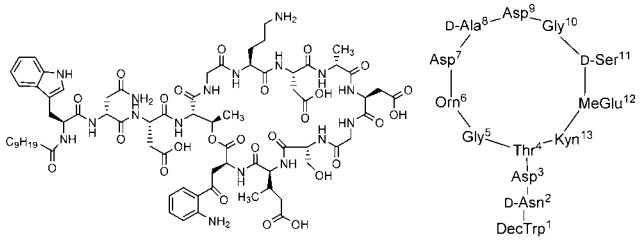


Figure 1. Structure of daptomycin. DecTrp = *N*-decanoyltryptophan.

contains six nonproteinogenic amino acids: D-Asn2, Orn6, D-Ala8, D-Ser11, (2*S*,3*R*)-methylglutamate (MeGlu12), and kynurenine (Kyn13). The depsi (ester) bond in the macrolactone ring is between the side chain of Thr4 and the α-COOH of Kyn13.

Although daptomycin was first discovered in the early 1980s, fundamental aspects of its mechanism of action (MOA) remain unknown. Its activity is Ca^{2+}-dependent. Some studies suggest that daptomycin forms oligomeric pores in bacterial membranes, which results in dissipation of membrane potential and cell death.[4a,b] However, this MOA has yet to be firmly established, and other MOAs have been proposed.[1d]

Daptomycin's unresolved MOA, its lack of activity in the presence of lung surfactant and the appearance of daptomycin-resistant strains,[5] emphasizes the need for establishing detailed structure−activity relationships (SARs). This requires a methodology for the ready preparation of numerous daptomycin analogs and the interrogation of most or all residues. Although daptomycin analogs have been prepared using chemoenzymatic[6] and combinatorial biosynthetic approaches,[7] these methods have produced less than two dozen daptomycin analogs since they were first described almost a decade ago. One approach that has the potential to produce large numbers of daptomycin analogs is total chemical synthesis using solid-phase methodologies; however, the chemical synthesis of daptomycin and its analogs has proven to be very challenging.[8] Lam et al. recently reported the total synthesis of daptomycin.[9] They were unable to form the crucial ester linkage using FmocKyn(CHO,Boc)OH as a building block either in solution or on a solid support. Hence they resorted to a 12-step solution-phase synthesis of a branched tetrapeptide, during which formation of the ester linkage between Thr4 and Kyn13 was accomplished by ozonolysis of a suitably protected Trp residue. This tetrapeptide was used as a building block for the preparation of a linear daptomycin precursor using solid-phase methods. A solution-phase serine ligation procedure was used to achieve macrocyclization. This combination of solid- and solution-phase chemistry, along with the need for HPLC purification immediately before and after cyclization, makes this approach very labor-intensive. Very recently, Martin et al. reported the synthesis of two daptomycin analogs and their enantiomers.[10] Their initial attempts at performing the entire synthesis on the solid phase were unsuccessful. Therefore, they developed a combined solid- and solution-phase approach which involved the initial preparation of linear precursors using solid-phase chemistry, followed by cleavage of the peptides from the support, cyclization in solution, and then side chain deprotection. To avoid the difficulties that were encountered by Lam et al. in forming the ester linkage, Martin opted to substitute Thr4 with L-diaminopropionic acid, thus replacing the ester linkage with an amide linkage.

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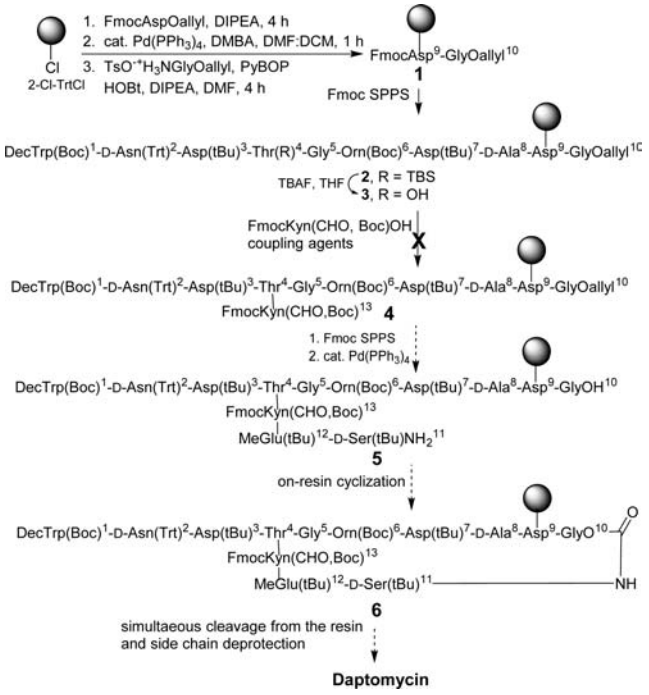
Unfortunately, this substitution was very detrimental to biological activity.

The development of a solid-phase approach to the synthesis of daptomycin and it analogs would greatly facilitate SAR studies, which would in turn expedite MOA investigations and the search for cyclic lipodepsipeptide antibiotics with improved activity and decreased bacterial resistance. Here we report a solid-phase approach to the total synthesis of daptomycin. We also apply this methodology to the synthesis of several daptomycin analogs.

In our initial approach to daptomycin (Scheme 1), dipeptide **1**, attached to the resin by the side chain of the Asp9 residue, would

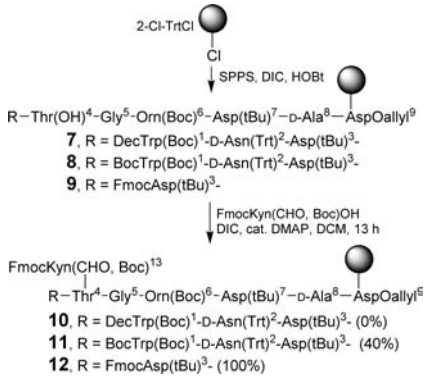
Scheme 1. First Attempted Synthesis of Daptomycin



be prepared and then converted into decapeptide **2** using standard Fmoc solid-phase peptide synthesis (SPPS). After removal of the Thr4 side chain protecting group in peptide **2** to give peptide **3**, the ester linkage would be established with Kyn13 to give peptide **4**. Residues 12 and 11 would be added, and the allyl group in Gly10 removed to give peptide **5**. An on-resin cyclization between Ser11 and Gly10 would enable us to avoid racemization and minimize the formation of oligomers during this procedure. Simultaneous resin cleavage and side-chain deprotection of the resulting peptide **6** would give daptomycin. The synthesis of resin-bound peptide **3** proved to be straightforward using standard SPPS methodology (diisopropyl carbodiimide (DIC)/HOBt as coupling agents). However, attempts to form the ester bond between FmocKyn(CHO,-Boc)OH and Thr4 in peptide **3** using a wide variety of coupling conditions (see the Supporting Information (SI)) were unsuccessful. This result was consistent with the report by Lam et al. that this ester bond is very challenging to form on a solid support or even in solution.[9]

Rather than turning to a solution-phase approach, we examined whether the decanoyl tail or peptide length had any effect on the esterification reaction. Three peptides, **7**–**9**, starting from Asp9, were prepared (Scheme 2) and used as model substrates for the on-resin esterification reaction. During the synthesis of these model peptides, it was found that protection of

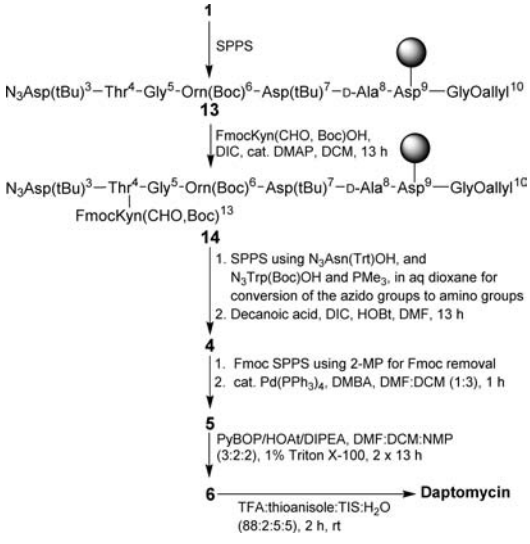
Scheme 2. Attempted Esterification Reactions with Model Peptides



the side chain of Thr4 was not necessary so long as DIC/HOBT were used as coupling agents and DMF was used as the solvent. We were unable to obtain any esterification product (peptide **10**) using 9-mer **7** as the substrate under a wide variety of conditions. However, when the decanoyl tail in **7** was replaced with a Boc group (peptide **8**), 40% of peptide **7** was converted into branched peptide **11** using DIC/cat. DMAP as coupling agents and dichloromethane (DCM) as the solvent, as determined by HPLC. When these conditions were applied to heptapeptide **9**, almost quantitative conversion into peptide **12** was achieved with no evidence of epimerization. The use of DCM as solvent was crucial, as the use of other solvents, such as DMF, resulted in significantly reduced yields.[11] The reason for the lack of ester bond formation in peptide **7** is not clear. It is possible that the decanoyl tail is interacting with the hydrophobic resin and making the hydroxyl group on the Thr residue less accessible.

The above findings prompted us to redesign our approach to daptomycin which involved using a combination of α-azido[12] and α-Fmoc-protected amino acids (Scheme 3). Octapeptide **13** was prepared using standard SPPS techniques, employing Fmoc amino acids for residues ten to four and N_3Asp(tBu)OH for residue three. Coupling of FmocKyn(CHO,Boc)OH to Thr4 in peptide **13** to give peptide **14** was accomplished using DIC/cat. DMAP in DCM. Peptide **14** was converted into peptide **4** by installing N_3Asn(Trt) and then N_3Trp(Boc) and using PMe_3 in

Scheme 3. Synthesis of Daptomycin



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aq dioxane for reduction of the α-azido groups to α-amino groups.[12b] The decanoyl tail was installed using decanoic acid and DIC/HOBt. It has been shown that the ester bonds in depsipeptides can be unstable toward piperidine, the most common reagent used for Fmoc removal.[13] We had previously reported that S−O bond cleavage in sulfate-protected sulfotyrosine peptides by piperidine can be avoided by using 2-methylpiperidine (2-MP) for Fmoc removal.[14] Hence, the Fmoc group in peptide **4** and subsequent peptides was removed using 2-MP as a precautionary measure against ester bond cleavage. The allyl group on Gly10 was removed using cat. $Pd(PPh_3)_4$ and dimethylbarbituric acid (DMBA). Attempts to cyclize peptide **5** using PyBOP/DIPEA in DMF or PyBOP/HOBt/DIPEA in DMF gave only very small amounts of cyclic peptide **6**. Using PyBOP/HOAt/DIPEA in DMF, the cyclization efficiency was about 30%. The cyclization efficiency was increased to 40% using PyBOP/HOAt/DIPEA and DMF/DCM/NMP (3:2:2 (v/v)) containing 1% Triton X-100 (Magic mixture[15]) as the solvent. Although this cyclization efficiency is modest, attempts by Lam et al. to perform a similar macrocyclization in solution during daptomycin synthesis using HATU/DIPEA/DMF or DEPBT/DIPEA/DMF gave only a trace amount of cyclized product.[9] Simultaneous cleavage of peptide **6** from the support and side chain deprotection using TFA/thioanisole/triisopropylsilane (TIS)/H_2O (88:2:5:5) gave daptomycin in a 9% overall yield. The synthetic daptomycin was identical to authentic daptomycin in every respect (mass spectrum, ^1H NMR spectrum, HPLC retention time; see the SI) including biological activity (MIC of 0.75 μg/mL against *Bacillus subtilis* 1046 and *B. subtilis* PY79 under commonly used conditions of 5 mM $CaCl_2$).

The approach to daptomycin outlined in Scheme 3 was applied to the synthesis of three daptomycin analogs (**15**−**17**, Table 1). The efficiency of the cyclization step during the synthesis of these analogs varied. Peptides **15** and **16** exhibited cyclization efficiencies slightly lower or greater than that experienced during the synthesis of daptomycin (35−45%), while, for peptide **17**, the cyclization efficiency was >95% even when DIC/HOBt was used to promote the cyclization.

Table 1. MICs of Daptomycin and Daptomycin Analogs 15−17

peptide	MIC (μg/mL)	
	B. subtilus 1046	*B. subtilus* PY79
daptomycin	0.75,[a] 0.5[b,c]	0.75[a]
15 (Dap-(2*S*,3*S*)-MeGlu)	40,[a] 5.0,[b] 5.0[c]	>40[a]
16 (Dap-E12/W13)	1.0[a]	3.0[a]
17 (Dap-E12/Y13)	35,[a] 3.0,[b] 1.3[c]	ND

[a]5 mM $CaCl_2$. [b]25 mM $CaCl_2$. [c]100 mM $CaCl_2$

Since the synthesis of enantiomerically pure (2*S*,3*R*)-Fmoc-3-MeGlu(*t*Bu)OH is a labor-intensive process (see the SI), it would be advantageous if this residue could be replaced with an amino acid that is more readily accessible. However, replacing this residue with Glu results in an 11-fold loss of activity.[7a] Since the synthesis of (2*S*,3*S*)-Fmoc-3-MeGlu(*t*Bu)OH is slightly less labor-intensive than the synthesis of the 2*S*,3*R* isomer (see the SI), daptomycin analog **15** was prepared in which (2*S*,3*R*)-MeGlu was replaced with (2*S*,3*S*)-MeGlu (Dap-(2*S*,3*S*)-MeGlu) (7.9% yield). However, analog **15** was found to be over 40-fold less active than daptomycin (5 mM $CaCl_2$, Table 1), revealing that the stereochemistry of the methyl group has a significant effect on biological activity.

Marahiel et al. reported that a daptomycin analog bearing L-Asn at position 2, Glu at position 12, and Trp at position 13 (Dap-L-N2/E12/W13) was 33-fold less potent than daptomycin.[6b] At the time their results were reported, it was not known that daptomycin contains D-Asn rather than L-Asn at position 2.[16] Consequently, these workers interpreted the reduced activity of the Dap-L-N2/E12/W13 analog as being due to only the Glu12/Trp13 double substitution when, in fact, the presence of L-Asn may also have contributed to this loss of activity, as it has been shown that daptomycin-L-N2 is 10-fold less active than daptomycin.[16] To determine what effect a Glu12/Trp13 double substitution has on activity, we prepared Dap-E12/W13 (**16**, 3.7% yield). The MIC of peptide **16** was almost the same as the MIC of daptomycin when assayed against *B. subtilis* 1046 and only 3-fold greater when assayed against *B. subtillis* PY79 (Table 1). These results indicate that the significant reduction in activity of the triply substituted analog reported by Marahiel was mainly due to the presence of the L-Asn residue. We also prepared a Glu12/Tyr13 analog (Dap-E12/Y13, peptide **17**, 16% yield). The MIC of peptide **17** was over 35-fold greater than the MIC of daptomycin (5 mM $CaCl_2$, Table 1).

The MICs of daptomycin and peptides **15** and **17** were also determined at high $CaCl_2$ concentrations against *B. subtillis* 1046. Surprisingly, at 25 and 100 mM $CaCl_2$ the MIC of **17** decreased dramatically and was only 6- and 2.5-fold greater than the MIC of daptomycin respectively (Table 1). The MIC of peptide **15** also decreased significantly at high Ca^{2+} concentrations but not to the same extent as peptide **17**. In contrast to peptides **15** and **17**, the MIC of daptomycin decreased only marginally over this range of $CaCl_2$ (Table 1). These findings indicate that residues 12 and 13 play a role in determining the calcium affinity of daptomycin.

To summarize, we have developed a solid-phase approach to the synthesis of daptomycin and daptomycin analogs. This opens the door for the rapid synthesis of other daptomycin analogs to establish SARs and for the solid-phase synthesis of other branched, cyclic lipodepsipeptide antibiotics. We have shown that the methyl group in the MeGlu residue in daptomycin must be of the *R*-configuration for potent activity. The low potencies exhibited by peptides **15** and **17** under standard conditions could, to a large extent, be remedied by supplementation of Ca^{2+} at high concentrations suggesting that these modifications mainly affect calcium affinity but have little effect on other aspects of the MOA. Finally, we have shown that a Glu12/Trp13 analog (peptide **16**) has an MIC approaching that of daptomycin. This result is significant, as it reveals that highly active daptomycin analogs that lack both Kyn and 3-MeGlu can be obtained. The synthesis of other daptomycin analogs and the preparation of combinatorial libraries based on Dap-E12W13 are in progress.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures for (2*S*,3*R*)-Fmoc-3-MeGlu(*t*Bu)OH, (2*S*,3*S*)-Fmoc-3-MeGlu(tBu)OH, daptomycin, and daptomycin analogs and characterization data for these compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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