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RESEARCH ARTICLE

A versatile Boc solid phase synthesis of daptomycin and analogues using site specific, on-resin ozonolysis to install the kynurenine residue

Buzhe Xu, Yann Hermant, Sung-Hyun Yang, Paul W. R. Harris,* and Margaret A. Brimble*

Abstract: A *de novo* solid-phase synthesis of the cyclic lipodepsipeptide daptomycin *via* Boc chemistry was achieved. The challenging ester bond formation between the nonproteinogenic amino acid kynurenine was achieved by esterification of a threonine residue with a protected tryptophan. Subsequent late-stage on-resin ozonolysis, inspired by the biomimetic pathway, afforded the kynurenine residue directly. Synthetic daptomycin possessed potent antimicrobial activity (MIC_{100} = 1.0 μg/mL) against *S. aureus*, while five other daptomycin analogues containing (*2R,3R*)-3-methylglutamic acid, (*2S,4S*)-4-methylglutamic acid or canonical glutamic acid at position twelve prepared using this new methodology were all inactive; clearly establishing that the (*2S,3R*)-3-methylglutamic acid plays a key role in the antimicrobial activity of daptomycin.

Introduction

The emergence of multidrug-resistant (MDR) bacteria has become one of the greatest threats to global public health, and infectious diseases rank among as the leading causes of mortality worldwide. Without urgent action, death rates caused by incurable infections are expected to rise more than tenfold by 2050, to an extremely disturbing 10 million every year (exceeding the number of deaths caused by cancer today).[1] This situation is further aggravated by the fact that only two new major classes of antibiotics, typified by linezolid (oxazolidinones) and daptomycin (**1**) (lipopeptides), have been approved for clinical use in the past 40 years, both of which have a narrow spectrum for Gram-positive bacteria.[2] This has created a pressing need for the discovery and development of new antibiotics with ~~novel chemical scaffolds and~~ different mechanisms of action.

Naturally occurring antimicrobial peptides (AMPs), a diverse class of molecules produced by most living organisms, have shown great potential as antibiotic candidates.[3] Daptomycin (**1**)[4], a cyclic lipodepsipeptide natural product, exhibits potent antimicrobial activity against highly resistant Gram-positive pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and several species of *Streptococci*.[5] It is a non-ribosomally synthesised peptide consisting of thirteen amino acids, six of which are non-proteinogenic residues: Kyn^{13}, (*2S,3R*)-$MeGlu^{12}$, *D*-Ser^{11}, *D*-Ala^{8}, Orn^{6} and *D*-Asn^{2}. Ten of the thirteen amino acids form a 31-membered depsipeptide ring, with an ester bond between the side-chain of Thr^{4} and the carboxylic acid of Kyn^{13}. The remaining tripeptide is exocyclic and is capped at the *N*-terminus by a decanoyl lipid tail (Figure 1). As a typical calcium-dependent lipopeptide antibiotic (CDA), the conserved Asp-Ala-Asp-Gly motif in the macrolactone ring of daptomycin (**1**, pink residues) is thought to facilitate calcium binding,[6] resulting in the aggregation of **1** to form micelles in the bacterial cell membrane, which subsequently induces membrane dissociation and cell death.[7] In 2003, daptomycin (**1**) was approved by the FDA as the first CDA for the treatment of complicated skin and soft tissue infections caused by Gram-positive pathogens.

Inspired by its potent antimicrobial activity and unique mode of action, daptomycin (**1**) is a promising structural motif

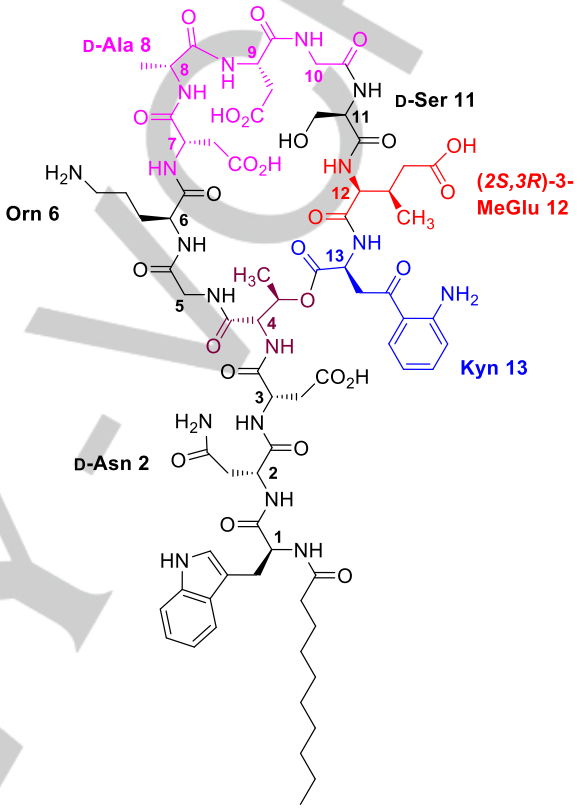

Figure 1. Structure of daptomycin (**1**) and its non-proteinogenic residues.

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for the development of novel antibiotics. Thus a general synthetic method to establish the structure-activity relationships (SARs) of daptomycin (**1**) is of paramount importance to enable discovery of novel daptomycin-based antibiotics such as the lipopeptide surotomycin, currently in phase III clinical trials for *Clostridium difficile*.[8] Previous chemoenzymatic synthesis,[9] semi-synthesis,[10] and combinatorial biosynthesis[11] of daptomycin only produced a very limited number analogues, leaving much of the chemical space unexplored. As a complementary strategy to biosynthesis, chemical synthesis is a powerful platform for the construction of native daptomycin (**1**) and its analogues, enabling greater structural variation. However, chemical synthesis of native daptomycin (**1**) has proven to be historically difficult due to the depsipeptide ester bond and non-canonical amino acids. For example, the enantiopure (*2S*,*3R*)-3-MeGlu building block is not readily accessible and its synthesis is labour-intensive.[12]

The first total chemical synthesis of daptomycin (**1**) was achieved by Cubist Pharmaceuticals in 2006, involving a tedious fragment-coupling approach wherein an azidobenzene on the side chain of kynurenine was used for the pivotal esterification step.[13] Li's group subsequently reported[14] the functionally critical,[15] but challenging depsipeptide bond formation between the hydroxyl group on the side chain of Thr^4 and Fmoc-Kyn^{13}(CHO, Boc)-OH could not be accomplished either on solid support or in solution phase, owing to the low reactivity of the Kyn residue or the steric hindrance of the linear peptide precursor.[14] As such, an alternative strategy was employed, which avoided using the Kyn^{13} for direct coupling, but necessitated a step-intensive, internal-ester fragment synthesis in solution phase.[14] More recently, Taylor's group overcame this problem by using *α*-azido amino acids, enabling direct on-resin esterification of a Kyn^{13} building block at Thr^4 using a truncated linear peptide that contained an *N*-terminal azido-masked Asp^3 residue (N_3-Asp^3). The synthesis could then be completed *via* Fmoc-SPPS following reduction of the azide to the amine.[16] However, α-azido amino acids must be prepared *in house* and suffer from elimination[17] and triazole formation[18] during unmasking of the azide. Additionally, the peptide ester fragment linked to Ser/Thr side chains in depsipeptides may undergo base-mediated diketopiperazine formation, or elimination to form dehydrobutyrine residues, based on the previous findings reported by our group[19] and others.[20]

Boc/Bn-solid-phase peptide synthesis (Boc-SPPS) is considered to possess several advantages over Fmoc-SPPS such as reducing peptide aggregation and suppressing aspartimide formation when making "troublesome" peptides.[21] In addition, Boc-SPPS may be preferred when synthesising peptides containing base-sensitive moieties such as depsipeptides. Herein we report an entirely Boc-based SPPS approach to the total synthesis of daptomycin (**1**) employing an on-resin ozonolysis of the corresponding Trp to install the Kyn residue. Moreover, several daptomycin analogues were also prepared using this newly-developed method to probe their biological activities and establish the contribution of several individual amino acid residues.

Results and Discussion

Synthesis of (*2S,3R*)-3-MeGlu building block

At the outset, we required (*2S,3R*)-3-MeGlu in a suitably protected form for use in Boc SPPS protocols. The reported synthesis of enantiopure (*2S,3R*)-Fmoc-3-MeGlu(O^tBu)-OH is a step-intensive process, employing extremely sensitive, explosive and hazardous diazomethane as a methylating reagent.[12] Thus, we explored an alternative synthetic strategy for preparing this key building block, which involved a temperature-dependent asymmetric Michael addition for accessing (*2S,3R*)-3-MeGlu (see Supporting Information).[22] However, Michael addition of camphor-based tricyclic iminolactone to crotonate to form the crucial (*2S,3R*) stereocentres of 3-methyl glutamic acid, could only be carried out on 50 mg scale in our hands, with moderate to low yields and poor diastereoselectivity. Hence an inseparable diastereoisomeric mixture (d.r. = 2/1) of the desired (*2S,3R*)-Boc-3-MeGlu(OBn)-OH and (*2R,3R*)-Boc-3-MeGlu(OBn)-OH building blocks was obtained. Nonetheless, we postulated that using the diastereoisomeric mixture for SPPS assembly may enable separation of daptomycin (**1**) from its Dap-(*2R,3R*)-MeGlu analogue **16** at the completion of the synthesis (see Supporting Information).

Initial synthetic strategy toward daptomycin (1)

In our initial efforts toward the preparation of daptomycin (**1**) (Scheme 1), we attached the first residue, Fmoc-Asp-O^tBu to aminomethyl polystyrene resin equipped with the 4-(hydroxymethyl)phenylacetic acid (PAM) linker, *via* its side chain and obtained an acceptable loading of 0.35 mmol/g based on the Fmoc-release assay (see Supporting Information). The resulting PAM handle **2** was treated with TFA/H_2O to remove the *tert*-butyl (^{t}Bu) group on the *C*-terminal of Asp^9, followed by coupling of $TFA^{-+}H_3N$-Gly-OAllyl to afford the resin-bound dipeptide **3**, which would then be elongated to give the linear decapeptide **4** *via* standard Boc-SPPS (Scheme 1). Interestingly, it was found that protection of the side chain of Thr^4 was not necessary even though HATU/DIPEA were used as coupling reagents. Inspired by the superior advantages of Boc-SPPS over Fmoc-SPPS for the synthesis of "difficult" peptides, it was envisaged that the ester bond of daptomycin (**1**) (resin-bound peptidyl **5**) could be established directly between the free hydroxyl group of a resin-bound ~~threonine residue~~ Thr^4 **4** and Boc-$Kyn(CHO)_2$-OH, followed by chain elongation to afford the fully assembled sequence **6**. Subsequent on-resin macrocyclization then affords resin-bound peptidyl **7**, and HF cleavage affords the final product daptomycin (**1**) (Scheme

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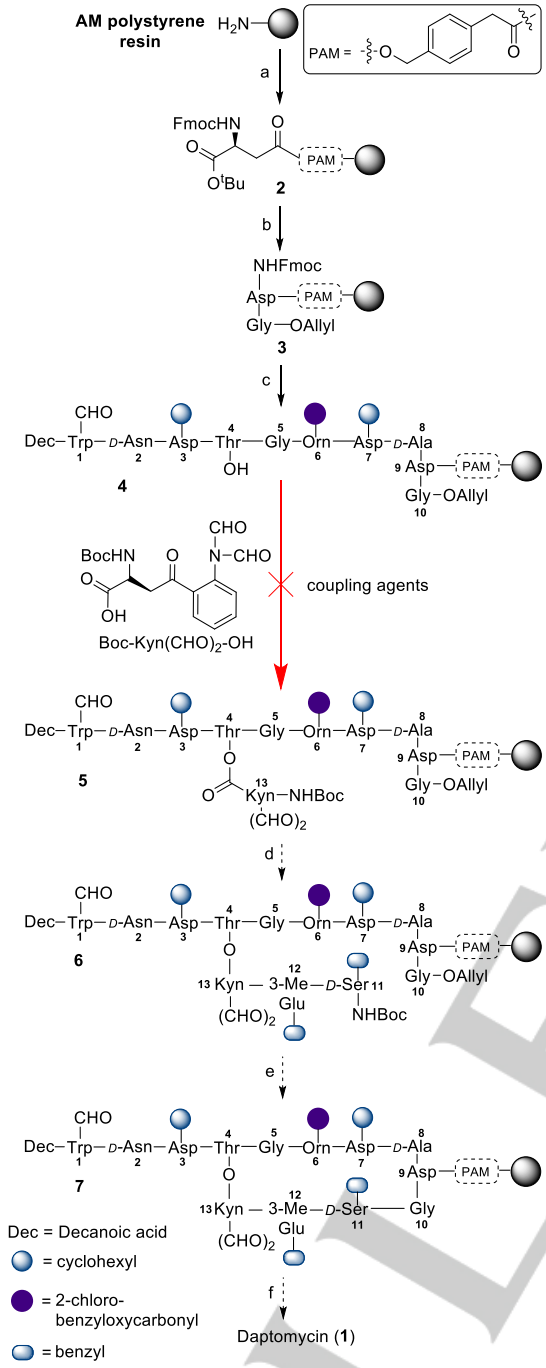
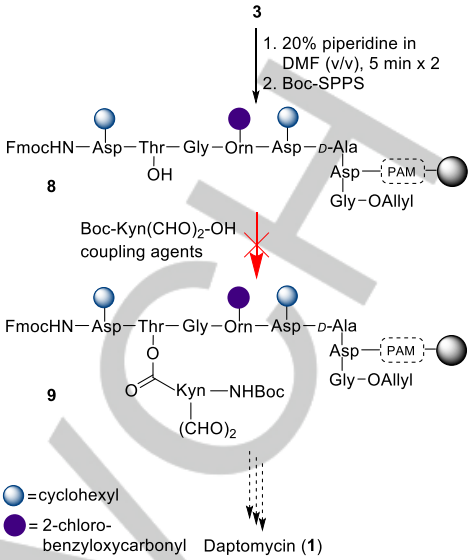


Scheme 1. Initial synthetic strategy toward daptomycin (**1**). Reagents and conditions: (a) i. PAM linker, DIC, 6-Cl-HOBt, DMF/DCM (1/1, *v/v*), 12 h; ii. FmocAspO^{t}Bu, DIC, DMAP, DMF, 12 h. (b) i. TFA/H_2O (95/5, *v/v*), 1 h; ii. TFA$^-$ $^+H_3$NGlyOAllyl, PyBOP, 6-Cl-HOBt, DIPEA, DMF, 3 h. (c) i. 20% piperidine in DMF (*v/v*), 5 min x 2; ii. standard Boc-SPPS: 1) HATU, DIPEA, DMF, 5 min; 2) TFA, 2 min x 2. (d) standard Boc-SPPS. (e) i. cat. $Pd(PPh_3)_4$, phenylsilane, DCM; ii. TFA, 2 min x 2; iii. PyBOP, 6-Cl-HOBt, DIPEA, DMF. (f) HF/*p*-cresol (95/5, *v/v*).

1). However, numerous attempts to form the crucial ester linkage (Thr^4-Kyn^{13}) including using carbodiimide chemistry, acyl fluoride, modified Yamaguchi conditions,[23] and other conditions, failed (see Supporting Information). This was purported to caused either by the low reactivity of the Kyn residue, or the steric hindrance of the bulky lipid chain and



Scheme 2. Second synthetic strategy toward daptomycin (**1**).

the three peptide residues at the *N*- terminus making the hydroxyl group of Thr^4 less accessible. This result was consistent with those reported by the Li and Taylor groups.[14,16]

Second synthetic strategy toward daptomycin (1)

Lohani *et al.* overcame the difficult Kyn^{13}-Thr^4 esterification using an orthogonal protecting group strategy employing an azide protected building block Asp^3 (N_3-Asp-(Trt)-OH) that was coupled to the resin-bound Thr^4 to afford a resin-bound octapeptide. In this case, esterification proceeded readily on the octapeptide **8**.[16] Similarly, we anticipated that the esterification reaction between the sterically less hindered, truncated N^{α}-Fmoc protected octapeptide **8** and Boc-Kyn(CHO)$_2$-OH would be more favourable (Scheme 2). Disappointingly, in our case, no desired product **9** was formed after HF cleavage and analysis by LC-MS (see Supporting Information).

Synthesis of daptomycin (1)

An alternative synthetic route towards daptomycin (**1**) was devised to circumvent the direct esterification of the Kyn building block. Inspired by the effective synthesis of Kyn-containing peptide cyclomontanin B *via* on-resin ozonolysis of the corresponding Trp residue[24] to a Kyn residue

Scheme 3. On-resin ozonolysis of Trp to install Kyn residue. AA = amino acid.[24]

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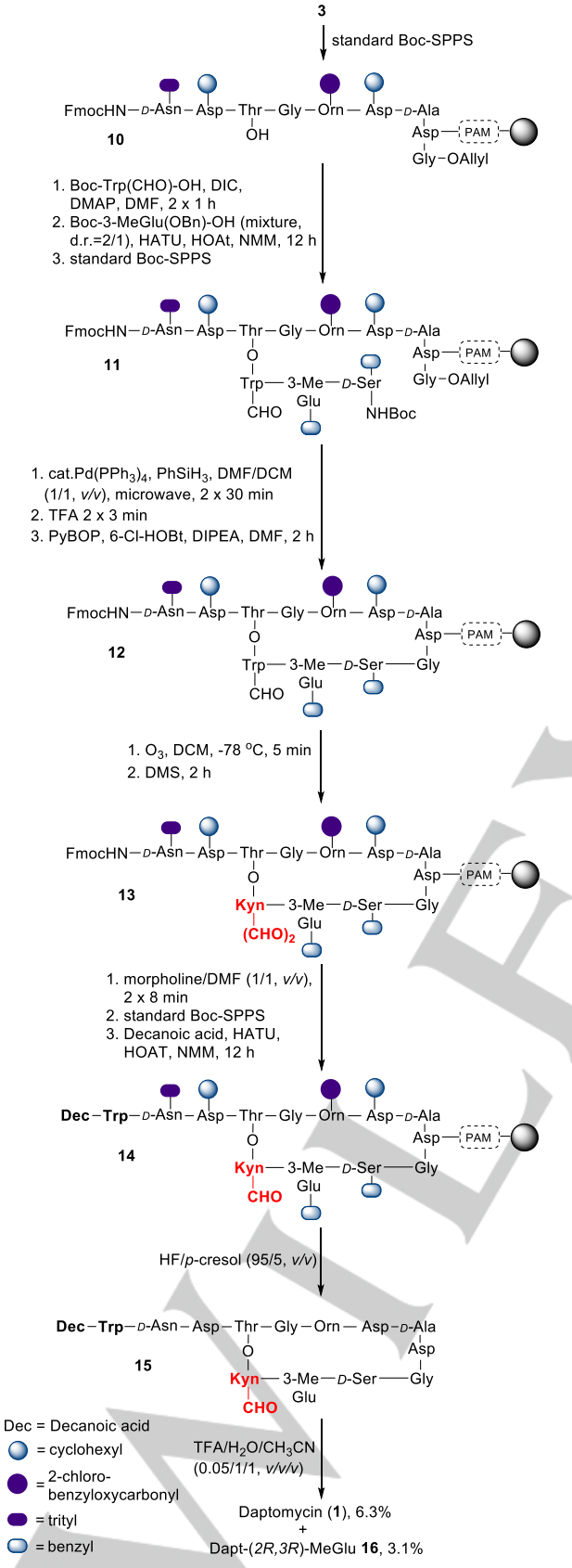

Scheme 4. Synthesis of daptomycin (**1**) and Dapt-(*2R,3R*)-MeGlu analogue **16**

(Scheme 3), and a previous report on the high efficiency of the esterification reaction between Fmoc-Trp(Boc)-OH and a resin-bound threonine-containing decapeptide[25], we adopted this combined strategy for the solid-phase synthesis of native daptomycin (**1**) (Scheme 4).

Nonapeptide **10** was prepared *via* standard Boc-SPPS conditions, employing Fmoc-*D*-Asn^2(Trt)-OH as residue two in order to achieve orthogonality to Boc-chemistry. Remarkably, Boc-Trp(CHO)-OH was attached to the side chain of the Thr^4 residue in peptide **10** to form the ester bond quantitatively using double coupling with DIC/cat. DMAP for 1 h. Subsequent peptide chain elongation from Trp^{13} was smoothly carried out by standard Boc-SPPS to afford resin-bound peptidyl **11**. The mixture (d.r. = 2/1) of (*2S,3R*)-Boc-3-MeGlu(OBn)-OH and (*2R,3R*)-Boc-3-MeGlu(OBn)-OH was used for the Glu^{12} coupling. The allyl group on Gly^{10} was then removed by cat. Pd(PPh$_3$)$_4$ and phenylsilane in DCM with microwave irradiation for 30 min (2 x), followed by treatment with TFA to remove the N^{α}-Boc group on Ser^{11}. On-resin macrolactamisation was then conducted using PyBOP, 6-Cl-HOBt, DIPEA in DMF for 2 h, affording resin-bound peptide **12** quantitatively.

Next, we attempted to construct the Kyn-containing peptide **13** *via* on-resin ozonolysis of the corresponding Trp residue in peptide **12**. The resin-bound peptide **12** was directly treated with ozone (O_3, -78 °C , DCM, 5 min; subsequently, Me_2S, 2 h, rt), followed by washing of the resin with DCM and DMF. Fortunately, LC-MS analysis of a cleaved resin sample showed that the Trp-containing peptide **12** was quantitatively converted into Kyn-containing peptide **13** without noticeable formation of side-products.

Having established a reliable site-specific method to convert the Trp to Kyn residue on-resin, the rest of the synthesis was expected to be straightforward requiring Fmoc deprotection of peptide **13,** followed by coupling of Boc-Trp-OH and finally acylation with decanoic acid to afford resin-bound peptide **14**. Unfortunately, utilization of 10-20% piperidine in DMF (*v/v*) for Fmoc-removal from the Fmoc-*D*-Asn^2 residue followed by the subsequent coupling of the final two residues (Trp, decanoic acid) and HF cleavage did not afford the desired product **15**. LC-MS analysis of the crude peptide indicated that unidentified, inseparable side products were the main component of the reaction mixture (Table S1, entry 1, see Supporting Information). This was not a surprising result as ester bonds in depsipeptides are susceptible to basic conditions.[19-20] Hence, deprotection of the *N*-terminal Fmoc group of peptide **13** needed further investigation (Scheme S4, see Supporting Information).

Direct HPLC analysis of the deprotected peptide **13** was difficult as it was not sufficiently retained on the HPLC column, hence we decided to complete the synthesis by installing the decanoyl lipid tail on the *N*-terminus, thereby enabling monitoring of the reaction by HPLC-MS. Several conditions were investigated for the Fmoc deprotection (Table S1, see Supporting Information). 2-Methylpiperidine

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(2-MP) has been reported to remove the Fmoc group mitigating the risk of ester bond cleavage.[16] 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), a non-nucleophilic base, has also been used as an alternative Fmoc-deprotection reagent for difficult or sensitive peptide sequences.[26] The milder base piperazine (5% in DMF) containing 0.1 M 6-Cl-HOBt as an acidic additive has been found to be effective at suppressing aspartimide formation on peptides containing aspartic acid and asparagine.[27] Disappointingly, use of these Fmoc-removal reagents presented above proved fruitless (Table S1, entry 1-4, see Supporting Information) although some of the desired product (ca. 40%) was observed with piperazine/6-Cl-HOBt (entry 4). This suggested that amine bases with a lower pKa (DBU, pKa = 13.5; piperidine, pKa = 11; piperazine, pKa = 9.8) would be beneficial. Pleasingly, we found that use of morpholine (pKa = 8.5) (50% in DMF, *v/v*) was able to deprotect the Fmoc group rapidly (10 mins, 90% complete; 16 mins, 100% complete) without formation of any notable side products (Table S1, entries 5-6, see Supporting Information). Subsequent coupling of Boc-Trp-OH and final capping with decanoic acid afforded resin-bound peptide **14** (Scheme 4). HF cleavage from the resin with simultaneous removal of side chain protecting groups gave peptide **15** without noticeable aspartimide formation or ester bond cleavage.

Notably only one formyl protecting group on the aniline nitrogen of the Kyn residue of peptide **13** was removed upon treatment with morpholine, leaving the other one intact during peptide elongation and HF cleavage. Pleasingly, deformylation of peptide **15** proceeded quantitatively under acidic conditions TFA/H_2O/CH_3CN (0.05/1/1, *v/v/v*) to afford native daptomycin and its (*2R,3R*)-MeGlu congener. Fortunately, daptomycin (**1**) could be readily separated by RP-HPLC from its Dapt-(*2R,3R*)-MeGlu analogue **16**, giving a 9.4 % overall yield (Dapt **1**, 2.5 mg, 6.3%; Dapt-(*2R,3R*)-MeGlu analogue **16**, 1.3 mg, 3.1%). Synthetic daptomycin (**1**) was identical to authentic daptomycin (HRMS, co-injection HPLC, ^{1}H NMR spectrum; see Supporting Information).

Synthesis of daptomycin analogues 17-20

We next adapted our successful synthesis of daptomycin (**1**) to prepare four daptomycin analogues (Figure 2). The presence of (*2S,3R*)-3-MeGlu at position twelve of daptomycin (**1**) is crucial to its bioactivity,[11, 16, 28] however the biological significance of this particular amino acid has yet to be further investigated. Analogue **17** containing a (*2S,4S*)-4-MeGlu residue was therefore synthesized *via* this approach (Scheme 4, 5.0 mg, overall yield, 12.3 %) in order to probe the effect of substitution at this position on the antimicrobial activity. The MICs of a daptomycin analogue containing the substitutions of Lys at position six, Glu at position twelve and Trp at position thirteen (Dapt-Lys^6/Glu^{12}/Trp^{13}) were reported to be quite close to native daptomycin when assayed against *B. subtilis* and *S.aureus* strains,[25] indicating that

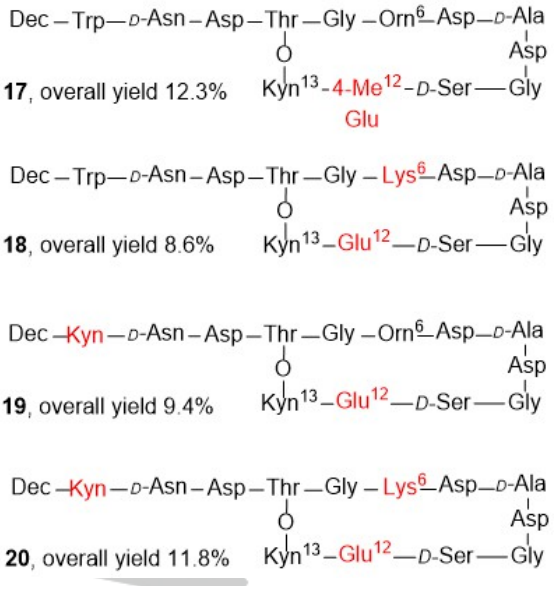

Figure 2. Structures of daptomycin analogues **17-20**.

replacement of Orn^6 by Lys^6 was beneficial to the antimicrobial activity. A daptomycin analogue Dapt-Lys^6/Glu^{12} **18**, containing Lys at position six, Glu at position twelve but conserving the Kyn amino acid was thus also prepared (3.5 mg, overall yield, 8.6 %). Due to the fact that mutation of Kyn^{13} to Trp^{13} in daptomycin (**1**) caused decreased antibacterial activity,[11] the double Kyn substituted analogues Dapt-Kyn^1/Glu^{12} **19** containing substitution of Kyn^1 for Trp^1 (3.8 mg, overall yield, 9.4 %) and Dapt-Kyn^1/Lys^6/Glu^{12} **20** ~~which contained an additional substitution, Lys^6 for Orn^6~~ (4.8 mg, overall yield, 11.8 %) were also prepared.

Biological evaluation of daptomycin (1) and analogues 16-20

Table 1. MICs of Daptomycin (**1**) and Dapt-analogues **16-20**

Peptides	MIC$_{100}$ (μg/mL)[a] *S. aureus ATCC 29213*
Authentic daptomycin	0.5
Synthetic daptomycin (**1**)	1.0
Dapt-(*2R,3R*)-MeGlu **16**	>128
Dapt-(*2S,4S*)-MeGlu **17**	>128
Dapt-Lys^6/Glu^{12} **18**	64
Dapt-Kyn^1/Glu^{12} **19**	>128
Dapt-Kyn^1/Lys^6/Glu^{12} **20**	>128

[a] 1.25 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

We next assessed the antimicrobial activities of **1** and its five analogues **16-20** (Table 1). Synthetic daptomycin (**1**) exhibited inhibitory activity against *S. aureus* (MIC$_{100}$ = 1.0

μg/mL), which was close to the MIC_{100} data (0.5 μg/mL) for authentic daptomycin. However, the analogue Dapt-(*2R,3R*)-MeGlu **16** that contains the opposite stereochemistry at the α-carbon and analogue Dapt-(*2S,4S*)-MeGlu **17** that relocates the methyl group from the *C*-3 to the *C*-4 position on the Glu side chain, displayed no antibacterial activity (>128 μg/mL) against *S.aureus*, which confirmed the finding that both the absolute stereo-configuration and the position of the methyl group in the MeGlu residue were critical to the activity of daptomycin (**1**). Evaluation of compounds **18**-**20** revealed that Dapt-Lys^6/Glu^{12} **18** displayed 128-fold less activity (64 μg/mL) than authentic daptomycin with *S.aureus* strain. While analogues **19** and **20** exhibited no antibacterial activity (>128 μg/mL) against *S.aureus*.

Conclusion

In summary, we have developed a robust and entirely solid-phase strategy for the synthesis of daptomycin (**1**) and analogues using Boc-chemistry. To the best of our knowledge, this is the first total synthesis of a cyclic lipodepsipeptide *via* Boc-SPPS. The crucial depsipeptide bond in the macrolactone ring of daptomycin (**1**) was established by esterification between the side chain of Thr^4 and Trp^{13}, followed by conversion of Trp^{13} to Kyn^{13} *via* site-specific on-resin ozonolysis, which circumvented the multistep solution-phase synthesis of a Kyn-containing tetrapeptide fragment. The preference to use Fmoc SPPS over Boc SPPS is largely due to the need to use hazardous HF, but HF-free Boc SPPS protocols have been reported[29] which should aid in preparing difficult peptide sequences such as daptomycin. This approach opens the door for the rapid preparation of various daptomycin analogues to establish SARs, and may be useful for the synthesis of other cyclic lipodepsipeptide natural products containing a Kyn residue such as taromycin A and B.[30] Through biological evaluation of our daptomycin compounds, we have determined that the stereochemistry of the α-carbon and the 3-substituted methyl group in the $MeGlu^{12}$ residue of daptomycin (**1**) must be (*2S,3R*) to confer activity. The preparation of other daptomycin analogues to further explore the SAR is currently being carried out and will be reported in due course.

Acknowledgements

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Keywords: solid-phase synthesis • daptomycin • Boc chemistry • on-resin ozonolysis • cyclic lipodepsipeptide

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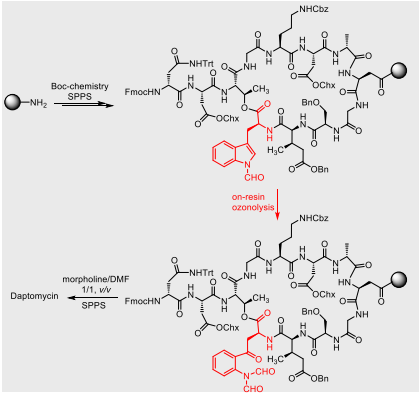
An entirely solid-phase peptide synthesis of daptomycin *via* Boc chemistry was achieved, which paves the way for rapid generation of daptomycin analogues containing the Thr(OKyn) fragment, as well as other cyclic lipodepsipeptide natural products featuring the non-proteinogenic Kyn residue.



Buzhe Xu, Yann Hermant, Sung-Hyun Yang, Paul W. R. Harris, and Margaret A. Brimble**

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A versatile Boc solid phase synthesis of daptomycin and analogues using site specific, on-resin ozonolysis to install the kynurenine residue

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