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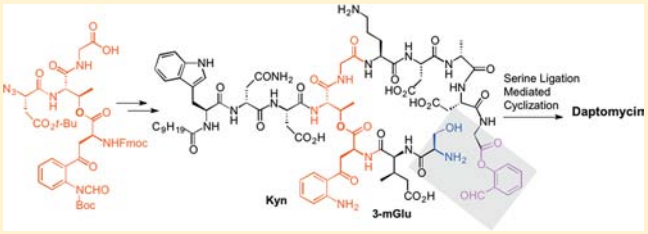
Total Synthesis of Daptomycin by Cyclization via a Chemoselective Serine Ligation

Hiu Yung Lam, Yinfeng Zhang, Han Liu, Jianchao Xu, Clarence T. T. Wong, Ci Xu, and Xuechen Li*

Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong, People's Republic of China

Supporting Information

ABSTRACT: A total synthesis of daptomycin, the first natural product antibiotic launched in a generation, was achieved. This convergent synthesis relies on an efficient macrocyclization via a serine ligation to assemble the 31-membered cyclic depsipeptide. The difficult esterification by the nonproteinogenic amino acid kynurenine was accomplished via the esterification of a threonine residue by a suitably protected Trp ester, followed by ozonolysis. This synthesis provides a foundation and framework to prepare varied analogues of daptomycin to establish its structure–activity profile.



INTRODUCTION

The emergence of multidrug resistance against bacterial pathogens has created an urgent need for the development of effective antibiotics with new modes of action against such resistant strains. Daptomycin is a lipodepsipeptide isolated from *Streptomyces roseoporus* that was obtained from a soil sample from Mount Ararat (Turkey) by scientists at Eli Lilly (Figure 1).

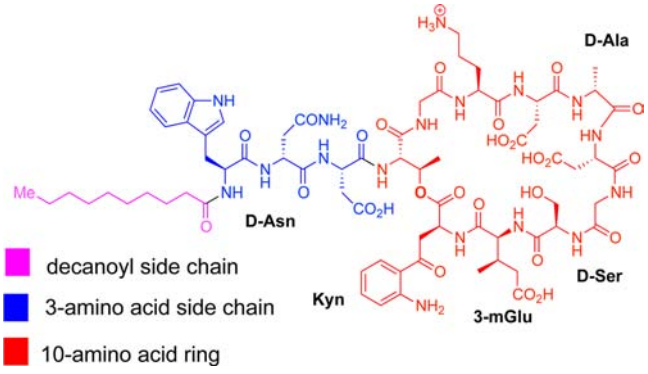


Figure 1. Structure of daptomycin.

Daptomycin has potent bactericidal activity with a unique mode of action against otherwise antibiotic-resistant Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and vancomycin-resistant *S. aureus*.[1] Daptomycin undergoes a conformational change on binding to calcium ions, which enables it to insert into bacterial membranes and induce membrane leakage as its apparent mode of action.[2] In 2003, daptomycin was officially approved by the FDA for the treatment of skin infections caused by Gram-positive pathogens. The development of drug resistance to daptomycin in pathogenic bacteria has so far not been observed.[3] The primary structure of daptomycin was established by Debono et al. in 1968.[4] It is a 13-amino acid cyclic lipodepsipeptide that belongs to the nonribosomal peptide family. It contains a 31-membered ring made up of 10 amino acids and a linear 3-amino acid side chain modified with an *n*-decanoyl lipid at the N-terminus. Within the sequence, there are two unnatural amino acids, kynurenine (Kyn) and 3-methyl glutamic acid (3-mGlu), along with D-Asn, D-Ser, and D-Ala (Figure 1).

Daptomycin is the first natural product antibiotic launched in a generation. Its distinct mechanism of action renders daptomycin a new structural motif for the development of new antibiotics. In this regard, the establishment of the structure–activity relationship (SAR) of daptomycin will be invaluable in the search for daptomycin-based next-generation antibiotics for additional clinical applications and in preparation for future waves of the development of bacterial resistance.[5] However, only a limited number of daptomycin analogues with but a few variations have been produced via genetic engineering of the nonribosomal peptide synthetase (NRPS) in the daptomycin biosynthetic pathway[6] and by chemoenzymatic synthesis[7] and semisynthesis.[8] The daptomycin analogues produced from biosynthesis include mutations at D-Ala, D-Ser, 3-mGlu, and Kyn.[6] Additional chemical modifications to improve the activity of daptomycin were confined to the lipid chain and the δ-amino group of ornithine.[1,8] An efficient route to chemically synthesize daptomycin that allows the assembly of the peptide sequence with precision and flexibility will make it possible to prepare a wider variation of daptomycin analogues, thereby enabling the establishment of a comprehensive SAR of daptomycin to kickstart the search for optimized analogues. However, a de novo chemical synthesis of daptomycin has not yet been reported. The presence of two nonproteinogenic amino acids,

Received: February 4, 2013
Published: April 5, 2013

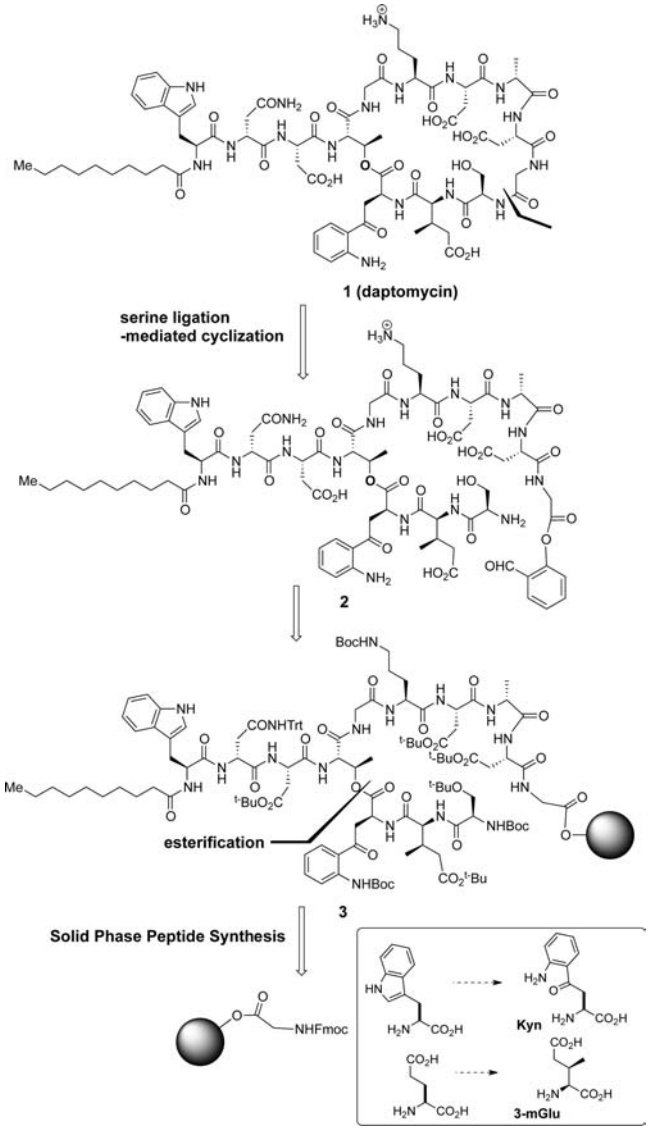
   dx.doi.org/10.1021/ja4012468 | J. Am. Chem. Soc. 2013, 135, 6272–6279

Kyn and 3-mGlu, embedded in the cyclic peptide backbone, and the macrolactamization of a 31-membered depsipeptidic ring render daptomycin a challenging target for total synthesis. Herein, we describe the first total synthesis of daptomycin, featuring a macrolactamization based on a serine ligation developed in our group.

RESULTS AND DISCUSSION

Retrosynthesis. Our retrosynthetic analysis for daptomycin synthesis is presented in Scheme 1. An anticipated challenge in the synthesis of the cyclic depsipeptide is the macrolactamization.[9,10] The development of new methods of peptide cyclization has attracted extensive research efforts because the conventional methods suffer significant drawbacks.[11] Prior to macrocyclization, the limited solubility of the side-chain-protected peptides in most solvents makes the handling and purification of the linear peptide precursor very difficult. Then the cyclization typically has to be performed under highly dilute conditions (typically submillimolar concentrations) to minimize dimerization and oligomerization, thereby limiting the scale of each preparation.[11]

Scheme 1. Retrosynthetic Strategy of Daptomycin



Furthermore, epimerization at the C-terminus often accompanies slow peptide cyclizations.

Chemical ligation-mediated cyclization presents an alternative protocol which is more efficient than the traditional head-to-tail lactamization methods. Chemical ligation-mediated peptide cyclization is less prone to epimerization and is able to accommodate side-chain-unprotected peptides. For example, native chemical ligation (NCL) has already been applied to the synthesis of cysteine-containing cyclic peptides.[12,13] To extend the application of this method to the preparation of non-cysteine-containing cyclic peptides, the use of thiol-containing unnatural amino acids as cysteine surrogates, followed by effective desulfurization techniques,[14] has overcome this limiting requirement of NCL.[11] Alternatively, the Staudinger ligation has been demonstrated by Kleineweischede and Hackenberger to be applicable to the synthesis of an array of cyclic heptapeptides.[15] Yudin and co-workers have utilized unprotected NH aziridine aldehyde, isocyanide, and linear peptides to achieve peptide cyclization efficiently.[16]

Recently, we have reported that an *O*-salicylaldehyde ester could react with a 1,2-hydroxylamine moiety as found in serine and threonine to form an *N*,*O*-benzylidene acetal-linked intermediate. The resultant acetal group was readily removed by acidolysis to afford an amide bond at the juncture to restore the natural peptidic bond, resulting in an overall peptide ligation.[17]

We further hypothesized that the C-terminal salicylaldehyde ester of a side-chain-unprotected peptide may also be able to react with the N-terminal serine/threonine of the same peptide intramolecularly, to realize a head-to-tail peptide macrocyclization. Indeed, we were well aware that, in an intramolecular context, the presence of side-chain-unprotected functional groups (e.g., amines) could complicate the chemoselectivity and efficiency of the ligation. Thus, we sought to examine experimentally whether the peptide cyclization of the side-chain-unprotected linear daptomycin precursor could be achieved via the *O*-salicylaldehyde ester-mediated intramolecular chemical ligation at the serine/threonine site. Daptomycin serves as an ideal target molecule to demonstrate the effectiveness or constraints of this approach because of its representative amino acid composition, structural complexity, and its therapeutic value.

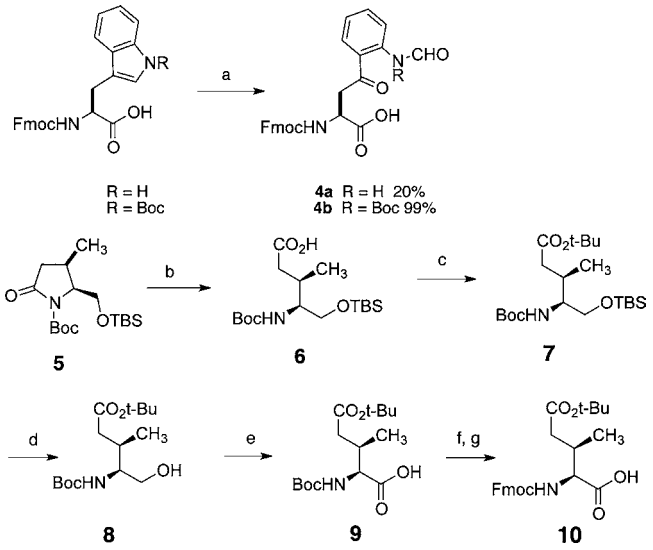
Upon the inspection of the daptomycin structure, we decided to effect cyclization at Gly-DSer juncture using the serine ligation (Scheme 1). We anticipated that the requisite linear peptide precursor (cf. **2**) could be obtained via Fmoc-SPPS. Optimally, the depsipeptide ester bond would be formed via on-resin esterification of Kyn (cf. **3**).

Synthesis of Kyn and (2*S*,3*R*)-3-mGlu Building Blocks. Daptomycin contains two nonproteinogenic amino acids: kynurenine (Kyn) and 3-methylglutamic acid (3-mGlu), which are critical for bioactivity. Studies have revealed that the Kyn residue is a key factor for enzymatic recognition during daptomycin biosynthesis, and 3-mGlu residue is essential for antibiotic activity.[3,8,18]

The preparations of Kyn and 3-mGlu are presented in Scheme 2. Although Kyn derivatives have previously been prepared from the corresponding Trp compounds by ozonolysis in one step, this approach suffered from low yields (20−33%) and the generation of a number of impurities.[19] Due to these limitations, a multistep synthetic route to L-kynurenine via β-3-oxindolylalanines was recently developed.[20]

dx.doi.org/10.1021/ja4012468 | *J. Am. Chem. Soc.* 2013, 135, 6272−6279

Scheme 2. Synthesis of Fmoc-Kyn-OH and Fmoc-3-mGlu-OH[a]



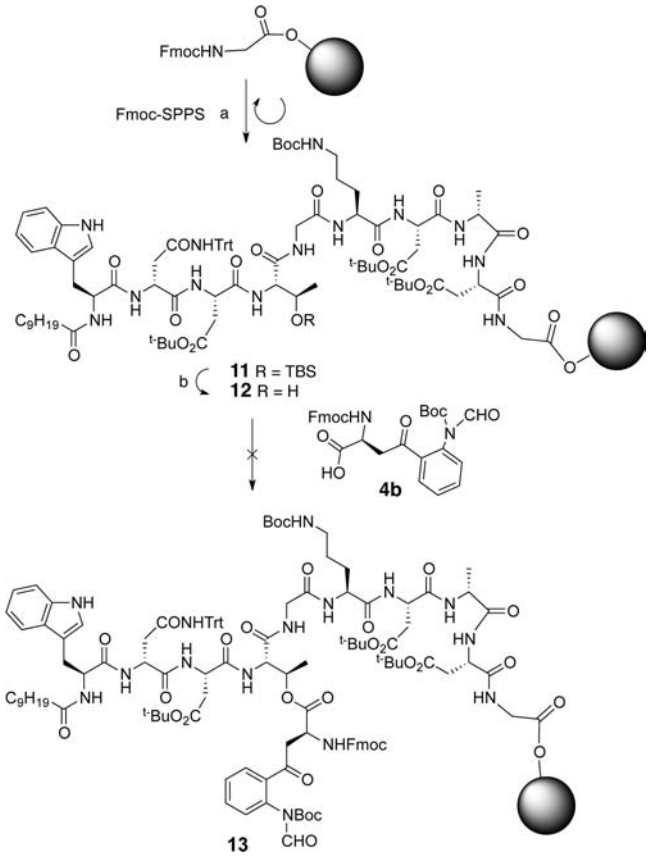
[a]Conditions: (a) (i) O_3, −78 °C, CH_2Cl_2, 10 min; (ii) Me_2S, −78 °C − rt, 2 h; (b) LiOH, H_2O/THF, 18 h; (c) *t*Bu-Br, BTEAC, DMAC, K_2CO_3, 55 °C, 24 h, 65% over two steps; (d) TBAF, THF, 1 h, 84%; (e) $NaIO_4$, $RuCl_3$, MeCN/CCl_4/H_2O, 1/1/2, 2 h, 82%; (f) 4 M HCl in dioxane, 0 °C to rt, 50 min; (g) Fmoc-OSu, Na_2CO_3, dioxane/H_2O, 18 h, 54% over two steps.

We suspected that the electron-rich indole moiety suffered decomposition during ozonolysis of Trp and resulted in poor yields. To verify this hypothesis, we subjected both Fmoc-Trp-OH and Fmoc-Trp(Boc)-OH to ozonolysis. Indeed, Fmoc-Kyn(CHO)-OH **4a** was obtained from Fmoc-Trp-OH in only 20% yield, a result similar to that reported in the literature. However, Fmoc-Trp(Boc)-OH was ozonolyzed to Fmoc-Trp(Boc,CHO)-OH **4b** in quantitative yield (99%). This approach is scalable and provides ready access to the valuable Kyn moiety.

The preparation of Fmoc-3-mGlu(*t*Bu)-OH started with compound **5**.[21] Ring opening of **5** with LiOH generated compound **6**. The subsequent esterification of the γ-carboxylic acid of **6** was at first problematic using *t*BuOH, but compound **7** was finally obtained via the *tert*-butyl bromide method[22] in 65% yield over two steps. Subsequently, the TBS group of compound **7** was removed with TBAF, followed by oxidation using sodium periodate and ruthenium(III) chloride, to afford Boc-3-mGlu-(*t*Bu)-OH **9** in good yield. The Boc group of **9** was selectively removed in the presence of the *t*-butyl ester by treatment with 4 M HCl in dioxane. After reprotection with an Fmoc group, the desired (2*S*,3*R*)-methyl glutamic acid building block **10** suitable for Fmoc/*t*Bu-SPPS was obtained with an overall yield of 24% over six steps (Scheme 2).

Initial Synthetic Strategies toward Daptomycin. Having prepared the Kyn and 3-mGlu building blocks, we proceeded to synthesize the daptomycin linear precursor **3**. According to the retrosynthetic plan (Scheme 1), **3** was expected to be readily constructed via standard SPPS on a 2-chlorotrityl chloride resin. After the resin-Gly-Asp(*t*Bu)-DAla-Asp(*t*Bu)-Orn(Boc)-Gly-Thr(OTBS)-Asp(*t*Bu)-DAsn(Trt)-Trp(Boc)-C_9H_{19} **11** was assembled via standard Fmoc/*t*Bu-SPPS, and the threonine residue was desilylated to give **12** (Scheme 3), we encountered difficulties in the coupling reaction of Fmoc-Kyn(Boc,CHO)-OH **4b** with the resin-linked threonine-OH. A variety of coupling

Scheme 3. First Attempt toward Daptomycin[a]



[a]Conditions: (a) (i) Fmoc-AA-OH, HATU, DIEA, DMF; (ii) piperidine, DMF; (b) TBAF, THF, 1 h.

reactions, including the use of common coupling reagents, Mukaiyama esterification, and Yamaguchi esterification, were tried in vain. It became apparent that the on-resin esterification would be rather problematic. This was probably due to the low reactivity of the Kyn moiety or the steric hindrance of the resin-linked peptide chain.

To overcome the obstacles presented by the on-resin esterification of Kyn, we decided to adopt a hybrid synthesis strategy using both solid-phase and solution-phase synthesis. We envisaged that a fragment containing Kyn (i.e., **16**, Scheme 4) could be preassembled via solution-phase synthesis, and then joined with the resin-linked pentapeptide (NH_2-Orn(Boc)-Asp(*t*Bu)-DAla-Asp(*t*Bu)-Gly) **17**, to afford compound **13** (Scheme 4). Using this strategy, we anticipated that the esterification reaction conducted in the solution phase between compound **15** and Fmoc-Kyn(Boc,CHO)-OH **4b** would be more favorable. To construct compound **15**, NH_2-Gly-OAll was first coupled with Fmoc-Thr(*t*Bu)-OH with the aid of HATU. The *t*Bu group was removed under acidic conditions, followed by reprotection with a TBS group. The Fmoc group of the resultant dipeptide was removed with diethylamine (DEA). Sequentially, the peptide was homologated with Fmoc-Asp(*t*Bu)-OH, Fmoc-DAsn(Trt)-OH, Fmoc-Trp(Boc), and decanoic acid. After each coupling and de-Fmoc step, the product was purified by flash chromatography. After the desilylation of **14**, we attempted the coupling of compound **15** with Fmoc-Kyn(Boc,CHO)-OH **4b** in solution. However, again, the results were very disappointing.

Modified Route to Daptomycin. Having tried a range of coupling strategies and methods to engage **4b**, we conceded that

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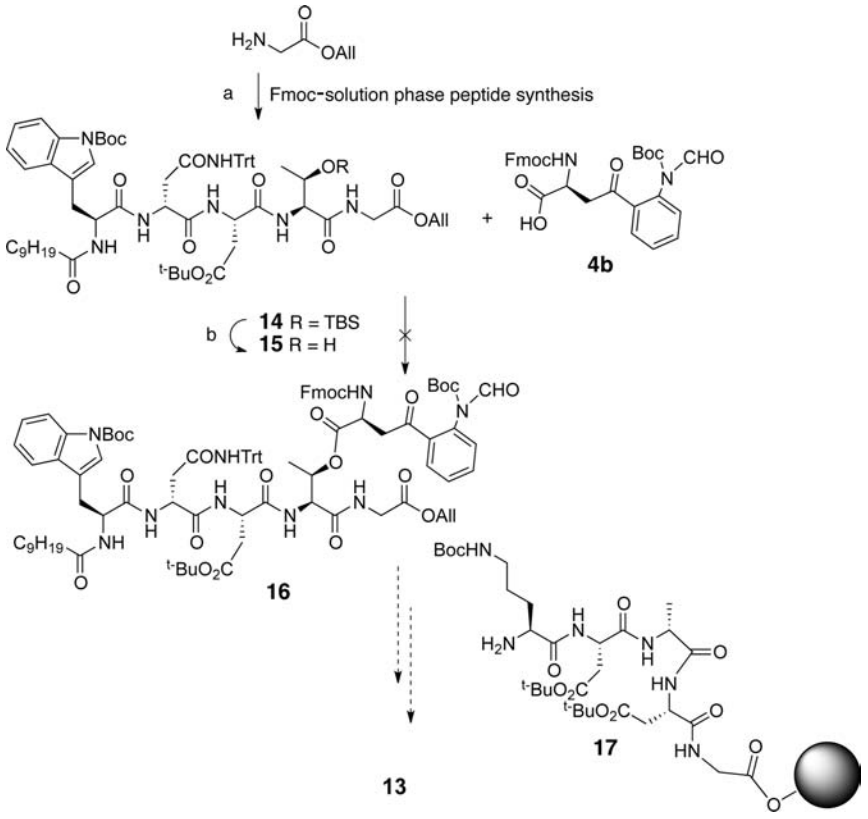
Scheme 4. Second Attempt toward Daptomycin[a]



[a]Conditions: (a) (i) Fmoc-AA-OH, HATU, DIEA, DMF, 80−95%; (ii) DEA, CH_2Cl_2, 85−95%; (b) TBAF, THF, 90%.

the Kyn building block was not suitable for direct esterification. Hence, an alternative strategy had to be devised. At this point, we planned to take recourse to using Trp for the esterification of the threonine residue, after which Trp would be converted to Kyn at a later stage. We were well aware that the structural complexity presented by a large peptide fragment could complicate the effort to achieve a clean transformation of Trp to Kyn via ozonolysis. Therefore, a suitable Kyn-containing peptide fragment of a minimal and robust structure was required.

First, we considered the simplest dipeptide Fmoc-Thr[O-Trp(Boc)]-OH as a building block amenable to ozonolysis. The Fmoc-Thr[O-Kyn(Boc,CHO)]-OH obtained after ozonolysis could be coupled with the resin-linked growing peptide. In this plan, however, an O→N acyl transfer[23] may be a potential side reaction during the subsequent SPPS from the threonine N-terminus. If the tripeptide Fmoc-Asp(*t*Bu)-Thr[O-Kyn-(Boc,CHO)]-OH were used as a preassembled building block, epimerization at the threonine residue would be likely in the coupling of this tripeptide to the resin-bound growing peptide chain. At last, we settled on N_3-Asp(*t*Bu)-Thr-[O-Kyn-(Boc,CHO)-Fmoc]-Gly-OH **26** (Scheme 5) as the coupling fragment via solution-phase synthesis. The azide group was selected to protect the N-terminus of Asp in order to achieve orthogonality with Fmoc-SPPS.

Our synthesis of ester **26** started with glycine **18** (Scheme 5). Acidolysis of the Boc group of **18**, followed by coupling with Fmoc-Thr(*t*Bu)-OH afforded the dipeptide Fmoc-Thr(*t*Bu)-Gly-OAll. Removal of the *t*Bu group, followed by silylation of the hydroxyl group gave rise to dipeptide **19**. This compound, after removal of the N-terminal Fmoc group with DEA, was coupled with Fmoc-Asp(*t*Bu)-OH in 62% yield over two steps. The resultant tripeptide **20** underwent Fmoc removal, diazo transfer,[24] and TBS deprotection to give tripeptide **23** in an overall 58% yield over three steps. Esterification of **23** with Fmoc-Trp(Boc)-OH, with the aid of PyBOP, gave rise to ester **24** without epimerization, in an excellent yield (87%). After palladium-catalyzed deallylation, ester **25** was subjected to ozonolysis to convert Trp to Kyn. Gratifyingly, the reaction proceeded without incident, providing ester **26** in an overall 81% yield (Scheme 5). Overall, this late-stage ozonolysis strategy could be a general route for the preparation of Kyn-containing peptides.

Next, the trityl-resin-linked pentapeptide (Fmoc-Orn(Boc)-Asp(*t*Bu)-DAla-Asp(*t*Bu)-Gly) **17** was assembled via standard Fmoc-SPPS, which was then successfully coupled with N_3-Asp(*t*Bu)-Thr-[O-Kyn(Boc,CHO)-Fmoc]-Gly-OH **26**, using HATU as the coupling reagent. The resultant resin-linked peptide was subsequently coupled with Fmoc-3-mGlu(*t*Bu)-OH **10** and Boc-DSer(*t*Bu)-OH under standard SPPS conditions to produce **28**. After reduction of the N-terminal azide group in **28** with dithiothreitol (DTT), further peptide homologation via Fmoc-SPPS afforded the whole linear sequence **29** successfully (Scheme 6).

Completing the Synthesis of Daptomycin via Serine Ligation. The side-chain-protected linear peptide was released from the trityl resin with AcOH/TFE/CH_2Cl_2 to afford the peptide acid **30** with the side-chain-protecting groups intact. To set the stage for the final macrocyclization via serine ligation, a salicylaldehyde ester needed to be installed at the C-terminal carboxyl group of compound **30**. The requisite peptide salicylaldehyde ester was obtained through direct coupling between the peptide acid and α,α-dimethoxysalicylaldehyde

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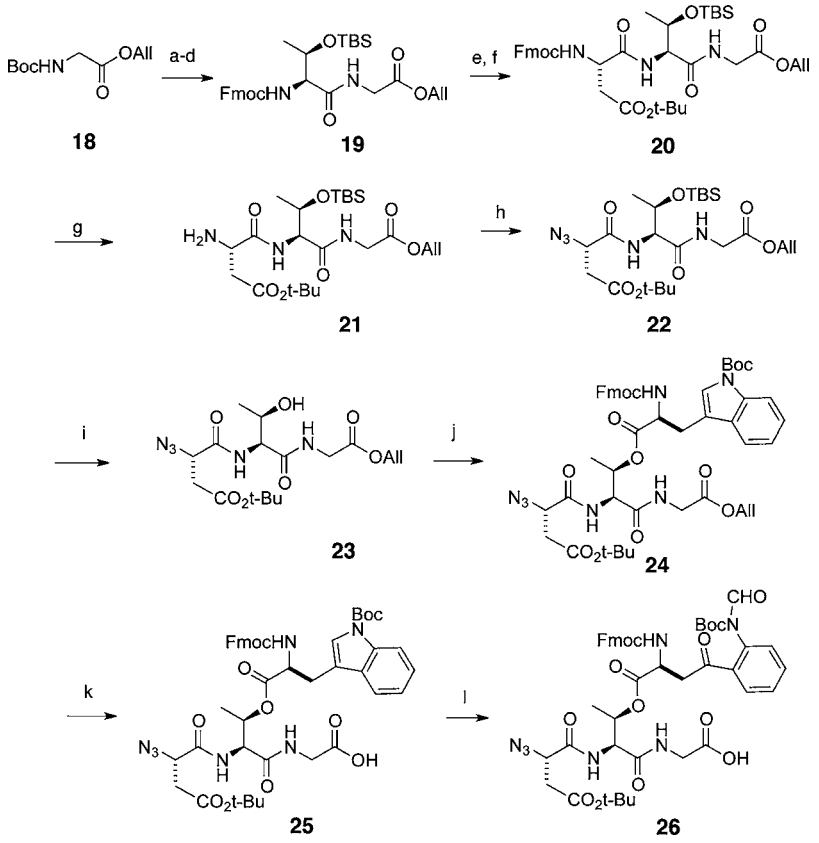
Scheme 5. Solution-Phase Synthesis of a Kyn Containing Fragment[a]



[a]Conditions: (a) TFA, 30 min; (b) Fmoc-Thr(*t*Bu)-OH, EDCI, HOBt, DIEA, CH_2Cl_2, 8 h, 74% over two steps; (c) 95% TFA, 30 min; (d) TBSCl, imidazole, DMF, 12 h, 70% over two steps; (e) DEA, CH_2Cl_2, 2 h, 80%; (f) Fmoc-Asp(*t*Bu)-OH, HATU, DIEA, DMF, 8 h, 77%; (g) DEA, CH_2Cl_2, 2 h, 100%; (h) imidazole-1-sulfonyl azide, $CuSO_4{\cdot}5H_2O$, $NaHCO_3$, MeOH, H_2O, 2 h; (i) TBAF, AcOH, THF, 4 h, 58% over two steps; (j) Fmoc-Trp(Boc)-OH, PyBOP, DIEA, CH_2Cl_2, 12 h, 87%; (k) $Pd(PPh_3)_4$, *N*-methylaniline, THF, 4 h, 85%; (l) (i) O_3, −78 °C, CH_2Cl_2; (ii) Me_2S, −78 °C to rt, 2 h, 95%.

mediated by PyBOP. Then, global deprotection of all side-chain-protecting groups with TFA/H_2O/PhOH afforded peptide salicylaldehyde ester **31** in 17% from the resin loading. Notably, the formyl group on Kyn was also removed during the acidolysis.

With the linear side-chain-unprotected peptide salicylaldehyde ester **31** in hand, we were in a position to investigate the peptide cyclization via serine ligation (Scheme 7). To this end, peptide **31** was dissolved in pyridine acetate buffer (mol/mol, 1:1) at a concentration of 5 mM. The desired macrolactamization proceeded smoothly, attaining a full conversion within 4 h at room temperature, and no side products were observed. Without isolation,[25] the *N*,*O*-benzylidene acetal-linked cyclic product was treated with TFA/H_2O for 10 min, whereupon daptomycin (**1**) was obtained in 67% yield after purification by semipreparative reverse-phase HPLC. It is worthwhile to note that the cyclization was tried at a range of concentrations from 5 to 50 mM, and at all concentrations, the monomeric cyclic peptide was furnished as the only product. This enables the effective scaling up of the synthesis of cyclic peptides and makes this strategy highly practical. To compare, we have also tried the conventional lactamization approach to cyclize the daptomycin linear precursor at the same Gly-DSer juncture, using a side-chain-protected linear peptide precursor.[32] However, under all conditions attempted (HATU, DIEA, DMF; DEPBT, DIEA, DMF) at a concentration of 5 mM, only a trace amount of cyclization, together with dimerized and oligomerized products, was observed by LC-MS analysis.

That the cyclization of **31** was successful indicated that the functional groups of the unprotected side chain, in particular, the amine group, did not interfere with the serine ligation-mediated cyclization. Although the competition between the free amine of the side chain and the N-terminal serine for reacting with the aldehyde group of the salicylaldehyde ester cannot be precluded, and in fact could very well occur, the Schiff base of the free amine is produced reversibly and represents a transient and non-productive intermediate species whose formation does not consume the aldehyde and inhibit the desired macrolactamization.

The clean cyclization of **31** also indicated that the direct aminolysis by the side chain amine group in the Orn residue of the peptide salicylaldehyde ester to afford a head-to-side chain-cyclized product did not occur.[31] The serine/threonine-mediated peptide cyclization reported herein is a two-step process. The first step is hemiaminal formation between the C-terminal salicylaldehyde ester and the N-terminal serine/threonine to give an *N*,*O*-benzylidene acetal cyclic peptide. If a head-to-side chain cyclization via direct aminolysis were to occur under these conditions, this product would have a different molecular weight and different functional groups compared with the desired head-to-tail *N*,*O*-benzylidene acetal product and would be likely to be distinguishable and separable by chromatography. Gratifyingly, we carefully analyzed the crude cyclization mixture and did not observe any undesired head-to-side chain cyclic product. Therefore, the second step in the

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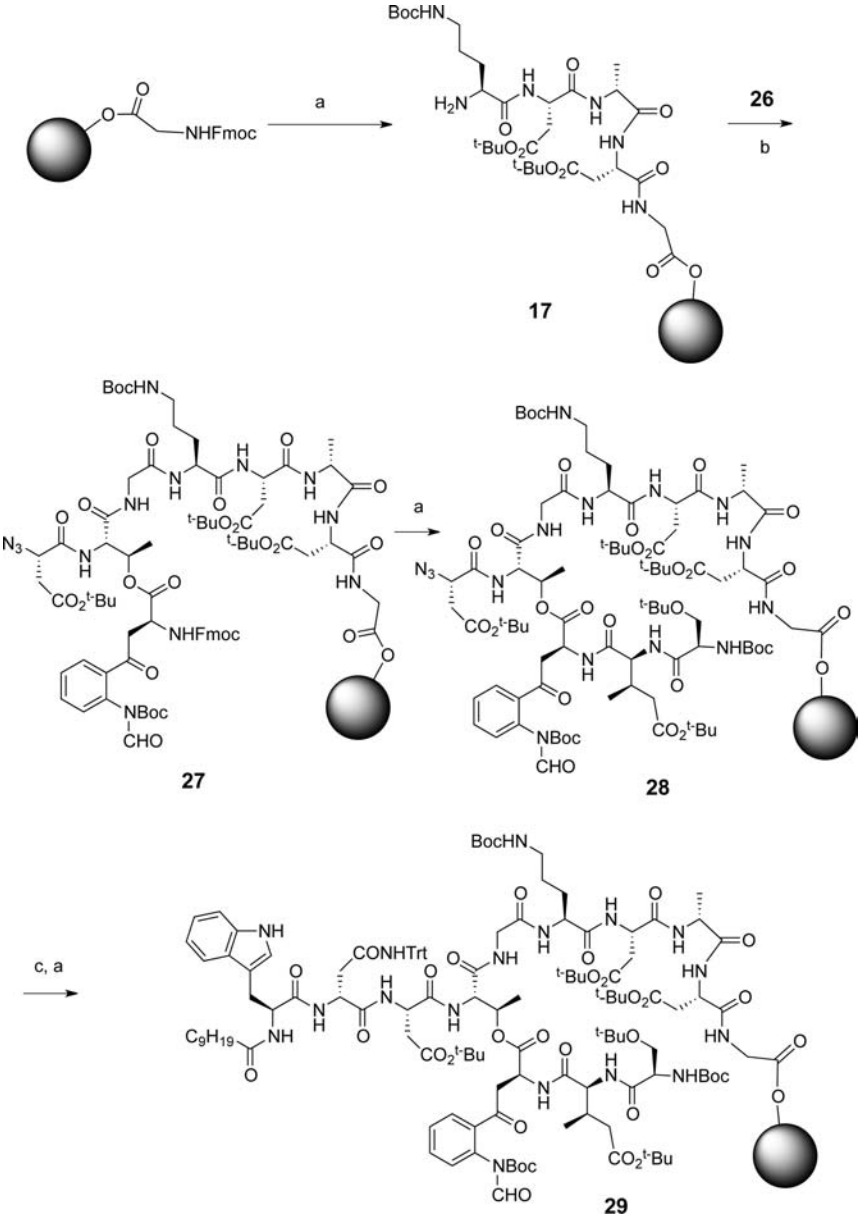
Scheme 6. Synthesis of the Daptomycin Linear Precursor[a]



[a]Conditions: (a) Fmoc-SPPS; (b) HATU, DIEA, DMF, 45 min; repeat; (c) DTT, DIEA, DMF, 2 h, 100% conversion as analyzed by LC-MS after the peptide was released from the resin.

ligation which restores the natural peptidic bond at the coupling site by removing the acetal group by acidolysis yielded daptomycin as the only product. Therefore, we have demonstrated that the presence of unprotected side chain functional groups does not adversely impact the cyclization process. The success of the daptomycin synthesis serves to showcase this chemical ligation with N-terminal serine/threonine as an alternative tool for synthesizing cyclic peptides. Indeed, many naturally occurring cyclic peptides with promising bioactivities, such as phakellistatin, lysobactin, and kawaguci-peptin,[9,10,29,30] contain serine or threonine, and these targets could be disconnected for synthesis based on this approach.

As daptomycin tends to aggregate, the ^{1}H NMR of the synthetic daptomycin (**1**) was recorded[26] using the reported optimal conditions[27] (5% D_2O in pH = 7.8 PBS buffer). The spectrum was in full accordance with those in the literature.[27] In addition, the identity and purity of the synthetic material was in full agreement with authentic daptomycin from a commercial source when compared by NMR spectroscopy and HPLC under the optimized HPLC conditions.[28]

■ CONCLUSION

In summary, we have described the first total synthesis of cyclic depsipeptide daptomycin. The synthesis involves a hybrid strategy using both solid-phase and solution-phase synthesis. The key features of the synthesis include a practical synthesis of Fmoc-3-mGlu(*t*Bu)-OH and a solution to the problem of Kyn preparation,[19,20] in which an N-Boc-protected Trp building block is ozonolyzed to provide Kyn in a quantitative yield. This strategy offers a large-scale and practical synthesis of Kyn moieties. Furthermore, the Boc-protected Trp residue embedded in a peptide fragment (e.g., **25**) could also be converted into

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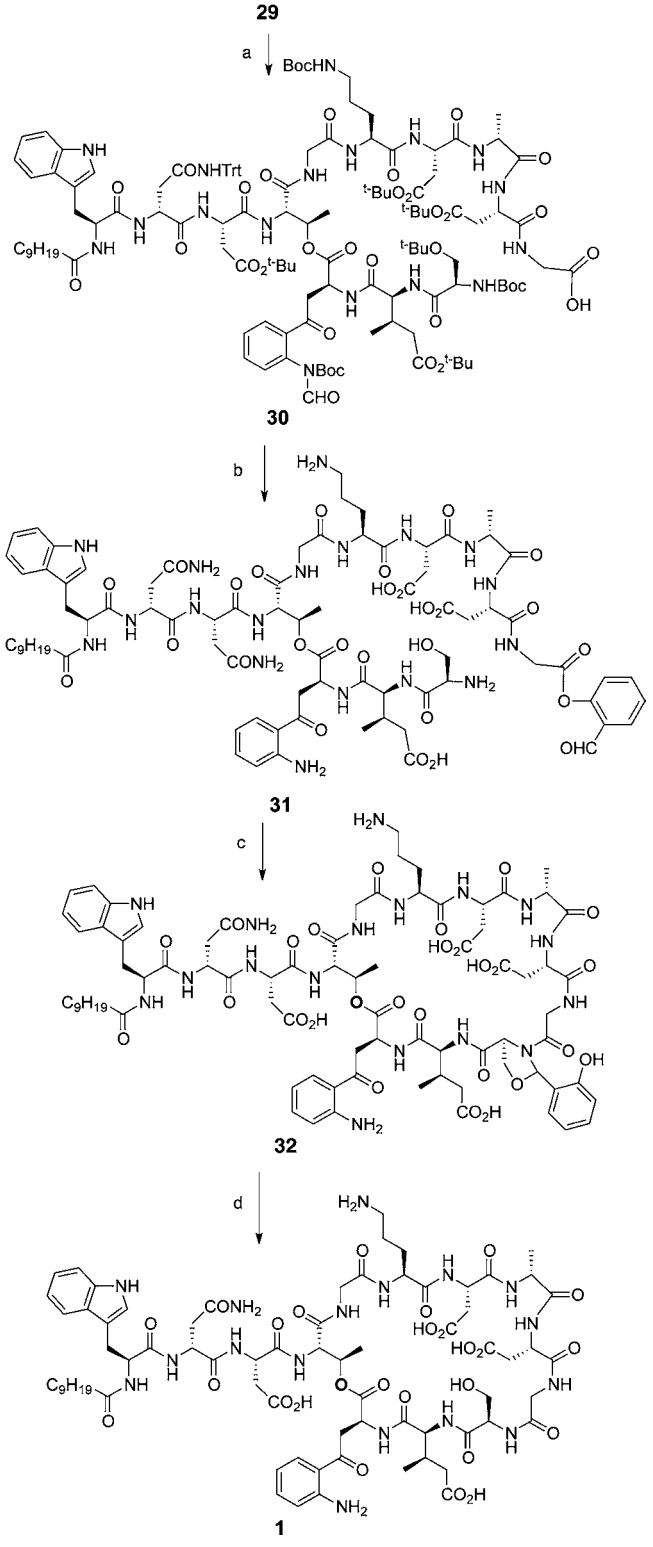
Scheme 7. Completion of Daptomycin via a Serine Ligation-Mediated Cyclization[a]



[a]Conditions: (a) AcOH/TFE/DCM, 1.5 h; (b) (i) α,α-dimethoxysalicylaldehyde, PyBOP, DIEA, CH_2Cl_2, 2 h; (ii) TFA/H_2O/PhOH, 1 h, 17% based on the resin loading; (c) pyridine acetate, rt, 4 h; (d) TFA/H_2O, 10 min, reverse-phase HPLC purification, 67%.

the Kyn (CHO, Boc) residue cleanly (e.g., **26**) via ozonolysis, providing a solution to the problem of the esterification of Kyn. Finally, the key cyclization step in the daptomycin synthesis was achieved via a chemoselective serine ligation, which could be accomplished at substrate concentrations of up to 50 mM. This scalable synthetic route to daptomycin should enable the preparation of various daptomycin analogues, which are difficult to obtain otherwise. Future studies are aimed at the investigation of the structure−activity relationship of daptomycin and the search for optimized analogues. We are also currently investigating the feasibility and scope of the serine/threonine ligation in synthesizing cyclic peptides of various ring sizes. The results of these studies will be reported in due course.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, spectral and other characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

xuechenl@hku.hk

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the General Research Fund (HKU703811P, 707412P) of the Research Grants Council of Hong Kong, the National Basic Research Program of China (973 Program, 2013CB836900), Peacock Program-Project Development Fund (KQC201109050074A) and Seed Funding from The University of Hong Kong (20101059015). We thank Prof. Chi-Ming Che for helpful discussions in the preparation of the manuscript, Prof. Pauline Chiu for suggestions on editing, and Dr. Eva Fung of the Department of Chemistry at the University of Hong Kong for ESI-HRMS work.

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