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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.7b01241 • Publication Date (Web): 09 Apr 2018

Developing Equipotent Teixobactin Analogues Against Drug-Resistant Bacteria and Discovering A Hydrophobic Interaction Between Lipid II and Teixobactin

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KEYWORDS. Teixobactin, Antibacterial, Convergent Synthetic Route, Structure-Activity Relationship.

ABSTRACT. Teixobactin, targeting lipid II, represents a new class of antibiotics with novel structures and has excellent activity against Gram-positive pathogens. We developed a new convergent method to synthesize a series of teixobactin analogues and explored structure-activity relationships. We obtained equipotent and simplified teixobactin analogues, replacing the L-*allo*-enduracididine with lysine, substituting oxygen to nitrogen on threonine, and adding a phenyl group on the D-phenylalanine. Based on the antibacterial activities that resulted from corresponding modifications of the D-phenylalanine, we propose a hydrophobic interaction between lipid II and the N-terminal of teixobactin analogues, which we map out with our analogue **35**. Finally, a representative analogue from our series showed high efficiency in a mouse model of *Streptococcus pneumoniae* septicemia.

INTRODUCTION.

In 2014, the World Health Organization declared that the post-antibiotic era - a time in which people could die from ordinary infections and minor injuries - could begin this century. The emergence of multidrug resistant bacterial pathogens has created an urgent need for the development of effective antibiotics against those drug-resistant strains to prevent this era from arising[1-12]. Novel antibacterial lead structures are essential to guarantee future therapeutic efficacy. Recently, Lewis[13] and coworkers reported a new antibiotic named teixobactin, which targets lipid II[14-16]. Teixobactin demonstrated excellent activity against Gram-positive pathogens, such as MRSA, vancomycin-resistant enterococci and even against *M. tuberculosis*. Importantly, it is difficult for pathogens to develop resistance against teixobactin as teixobactin binds to a lipid substrate of an enzyme, rather than the mutable protein enzyme itself. This new antibiotic filled the discovery void and has generated excitement. Up to date, the Payne group reported the first

total synthesis of teixobactin[17]. Simultaneously, the Li group accomplished an elegant total synthesis of teixobactin using Ser/Thr ligation[18, 19]. Nowick[20-22], Su[23], Albericio[24, 25], Singh[26-28], and other groups[29, 30] reported the synthesis of teixobactin analogues using SPPS (solid phase peptide synthesis). However, to the best of our knowledge, just Singh group reported two analogues Leu10-teixobactin and Ile10-teixobactin with identical potency to teixobactin recently[31]. Simultaneously, teixobactin analogues were reported based on Leu10-teixobactin and Ile10-teixobactin[32]. In this regard, the exploration of SAR of teixobactin will be invaluable to develop new-generation teixobactin-based antibiotics for potential clinical applications.

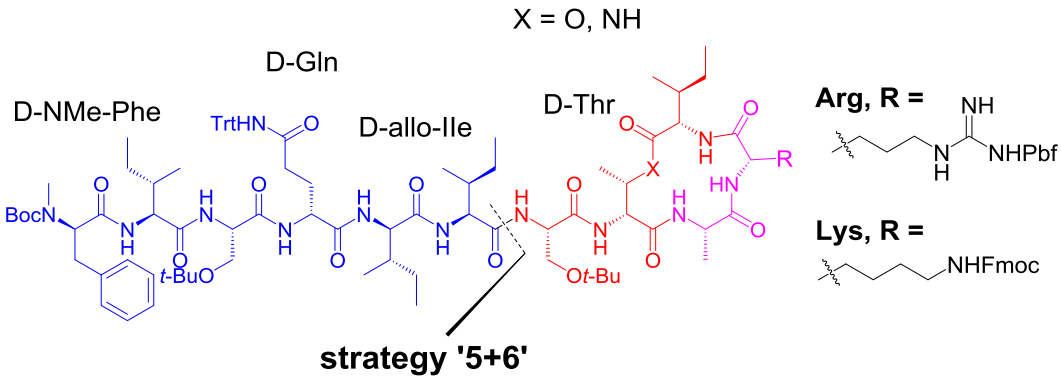
Considering relatively complicated structure of L-*allo*-enduracididine and six hydrophobic amino acids, undeniably, synthetic teixobactin has a few limitations including gelation in PBS due to poor solubility and high cost due to tedious synthesis of L-*allo*-enduracididine[33], which have hindered teixobactin's clinical application.Therefore, our group wished to hunt for analogues, which had simplified structures but exhibited comparable potency and stronger hydrophilicity to teixobactin. From the aspect of synthetic strategy, compared with the fully solid phase peptide synthesis (SPPS), we envisioned a highly convergent and efficient synthesis method to obtain diverse analogues of teixobactin.

RESULTS AND DISCUSSION.

At the beginning of our investigation, we planned to replace L-*allo*-enduracididine with natural amino acids, such as Arg or Lys, to simplify the structure of analogues and synthetic route. These strategies were applied in teixobactin analogues synthesis previously. As shown in **Scheme 1**, two additional major synthetic challenges were addressed based on our retrosynthetic analysis, which included: 1) site of macrolactamization, and 2) condensation of linear and cyclic peptide motifs.

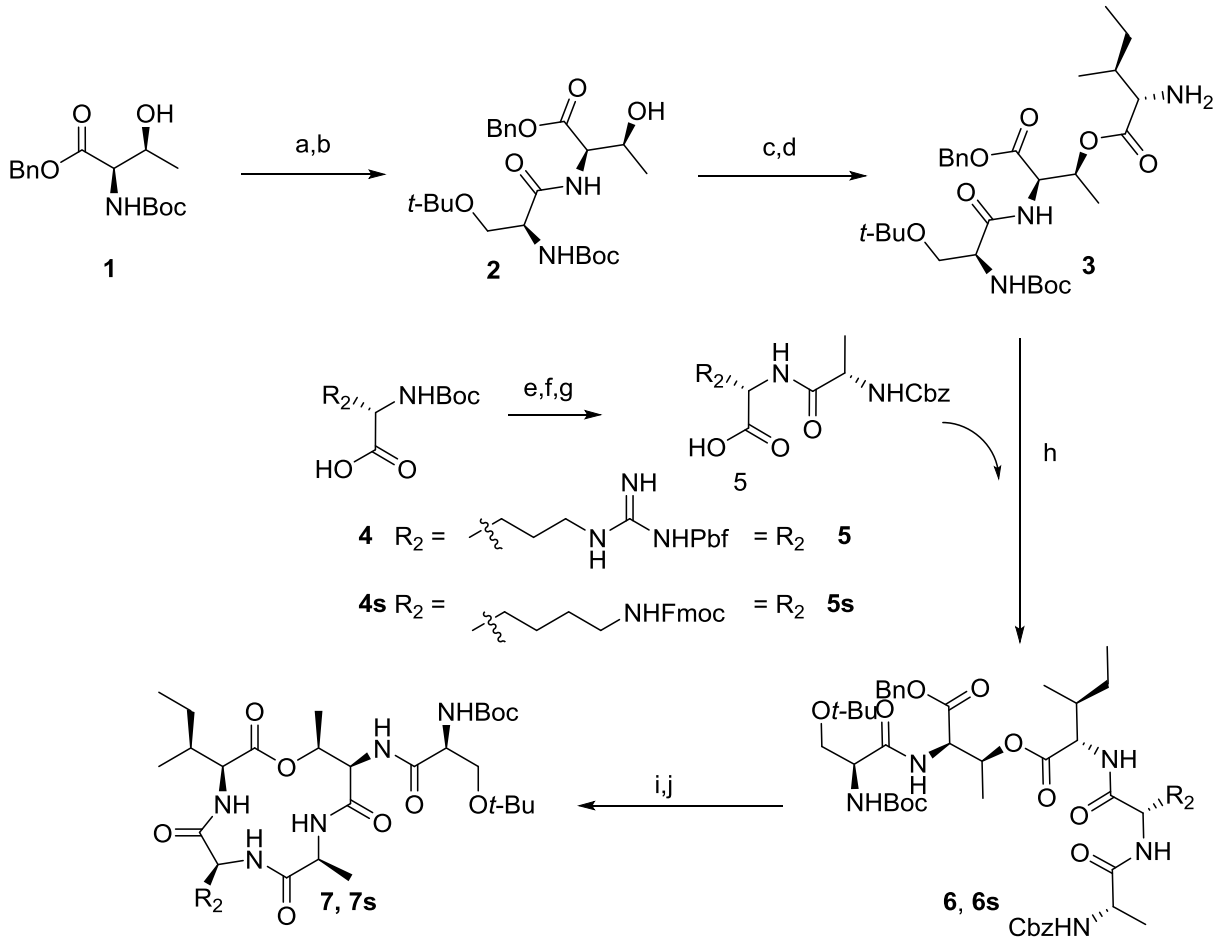
For the first, the relatively less sterically hindered site of Thr-Ala was selected to overcome ring strain and realize the cyclization. For the second, teixobactin analogues could be synthesized from a cyclic pentapeptide and a linear hexapeptide ('5+6'). A hybridized synthesis strategy combining solution-phase synthesis with solid-phase synthesis was employed to obtain teixobactin analogues. Following this design, the cylic and linear fragment were constructed via solution-phase and solid-phase synthesis respectively.

Scheme 1 Retrosynthetic Analysis of Teixobactin Analogues



In the initial stage, the preparation of the cyclic pentapeptide started with Boc-D-Thr-OBn. Firstly, Boc was removed under 50% TFA in DCM, followed by coupling with Boc-Ser(*t*Bu)-OH to yield the dipeptide Boc-Ser(*t*Bu)-D-Thr-OBn. Then the dipeptide was coupled with Fmoc-IIe-OH using EDCI/DMAP, followed by removal of Fmoc with 33% Et_2NH to provide compound **3** in 69% yield. Starting from Boc-Arg(Pbf)-OH, removal of Boc and esterification were accomplished simultaneously under 2 M HCl in MeOH. Without purification, the intermediate was reacted with Cbz-Ala-OH to give dipeptide Cbz-Ala-Arg(Pbf)-OH, followed by hydrolysis of ester bond to access compound **5** in 86% yield for 3 steps. Next, '3+2' coupling between **3** and **5** was achieved in 80% yield. Subsequently, the two protecting groups (Cbz, Bn) were removed under reductant conditions ($Pd(OH)_2/C$, H_2). The final cyclization was achieved with PyAOP/HOAt/DIEA[17, 24] in an isolated yield of 66%.

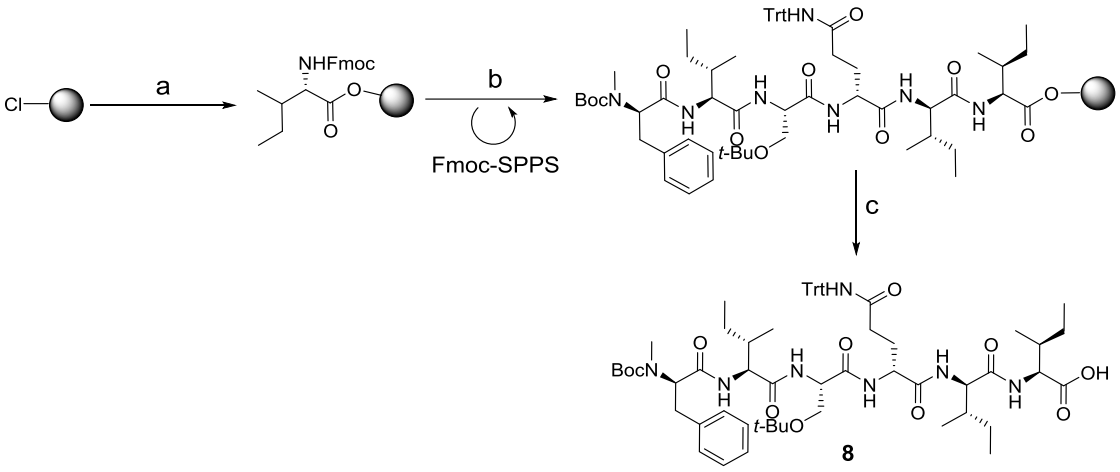
Scheme 2 Synthesis of Precursory Linear Pentapeptide



Conditions: a) 50 % TFA in DCM 30 min; b) 1.1 equiv. HCTU, 1.1 equiv. Boc-Ser(tBu)-OH, 2.5 equiv. DIEA, DCM/DMF, 2 h, 85% over 2 steps for compound **2**; c) 1.2 equiv. EDCI, 1.2 equiv. Fmoc-Ile-OH, 0.15 equiv. DMAP, DCM, rt, overnight; d) 33% Et_2NH in MeCN, 10 min, 69% over 2 steps for compound **3**; e) 2 M HCl in MeOH, 5 h, quantivative; f) 1.1 equiv. HATU, 1.1 equiv. Cbz-Ala-OH, 1.2 equiv. DIEA, DCM/DMF, 2 h; g) 2 equiv. LiOH, THF/H_2O (3:1), 5 min, 86% over 3 steps; h) 1.2 equiv. DEPBT, 1.2 equiv. compound **5**, 1.0 equiv. compound **3**, 1.4 equiv. DIEA, THF/DMF, overnight, 80%; i) 20% $Pd(OH)_2/C$, H_2, MeOH, 30 min; j) 4 equiv. PyAop, 4 equiv. HOAT, 8 equiv. DIEA, DCM:DMF/ 9:1, 24 h, 66% over 2 steps.

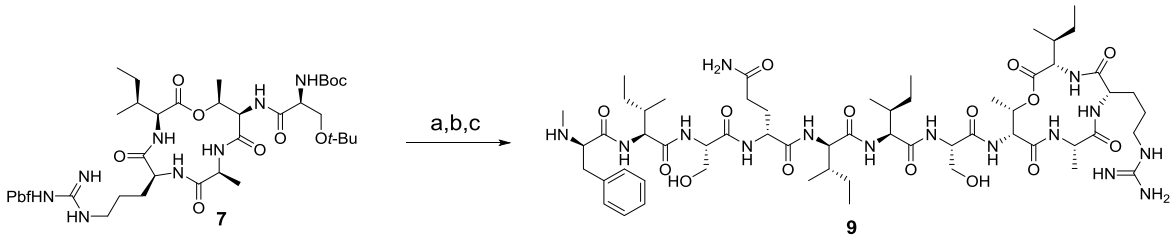
Synthesis of Linear Precursor. Fmoc-L-Ile-OH was first attached to 2-Cl-Trt resin in the presence of DIEA in DCM, followed by addition of MeOH for capping the unreacted Cl group on the resin. The peptide-bond formation was carried out using Fmoc-protected amino acids (3 equiv.) and HATU/DIEA (3:6 equiv.) in DMF for 50 minutes. Finally, the linear peptide **8** was cleaved from the resin with TFE/DCM (1:4) (**Scheme 3**).

Scheme 3 Synthesis of Linear Precursor



Conditions: (a) 5 equiv. Fmoc-Ile-OH, 10 equiv. DIEA, DMF:DCM/1:1, 4 h; Then MeOH/DIEA/DMF (1:1:8), 5 minutes (b) (i) 20% piperidine in DMF 15 min; (ii) 3 equiv. Fmoc-AA-OH, 3 equiv. HATU, 6 equiv. DIEA, DMF, 50 min; (c) TFE:DCM/1:4, 3 h.

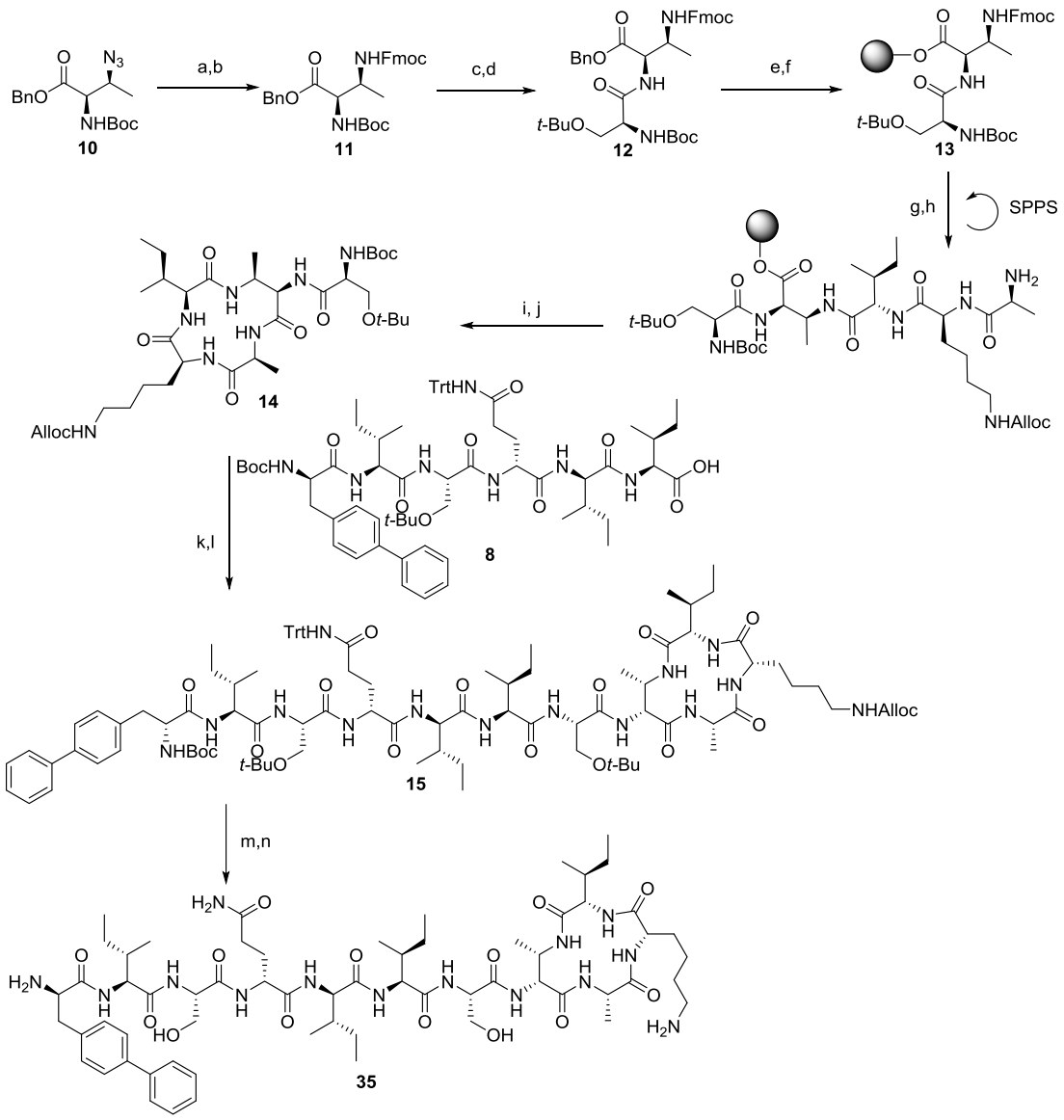
Scheme 4 Synthesis of Teixobactin Lactone Analogues via '5+6' Strategy



Conditions:a) 1.0 equiv. compound **7**, 4 M HCl in dioxane, 15 min; b) 0.9 equiv. compound **8**, 1 equiv. DEPBT, 1 equiv. DIEA, THF:DMF/9:1,12 h, 76%; c) TFA:TIPS:H_2O/ 95:2.5:2.5, 1 h. 51% over 3 steps.

With the cyclic pentapeptide and linear hexapeptide in hand, desired compound was obtained in the presence of DEPBT/DIEA[34] with 76% isolated yield. Final global deprotection of the peptide was carried out with TFA-H_2O-TIPS (95:2.5:2.5) in 1 h. The crude peptide was purified by semi-preparative reversed-phase high-performance liquid chromatography (HPLC) to furnish compound **9** in 12% isolated yield from starting material. (**Scheme 4**).

Scheme 5 Synthesis of Teixobactin Lactam Analogue Via ' 5+6 ' Strategy

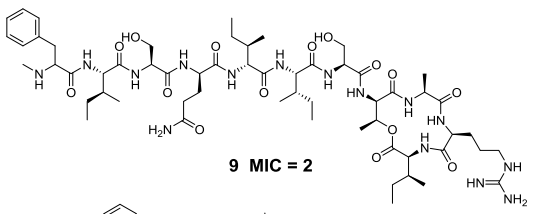
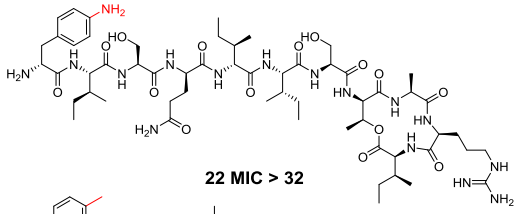
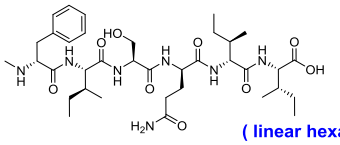
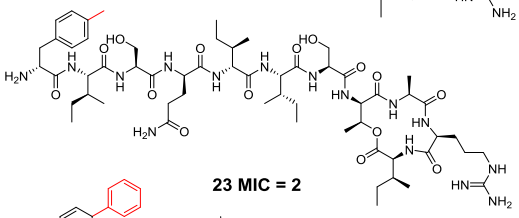
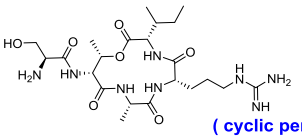
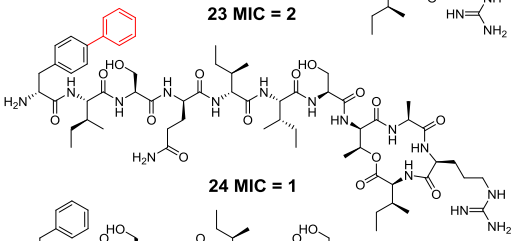
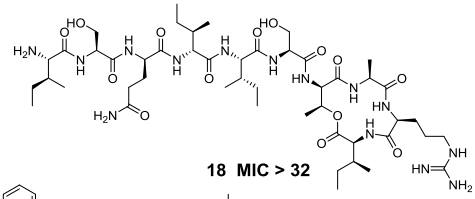
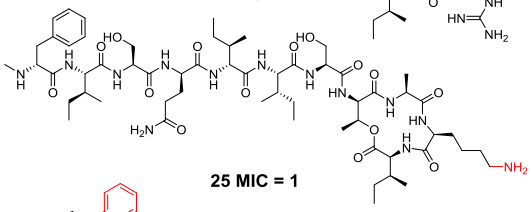
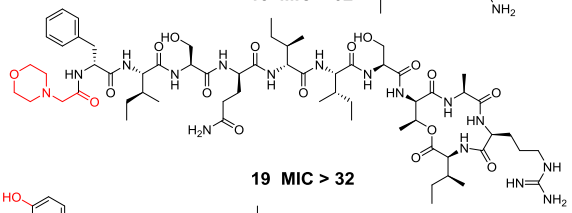
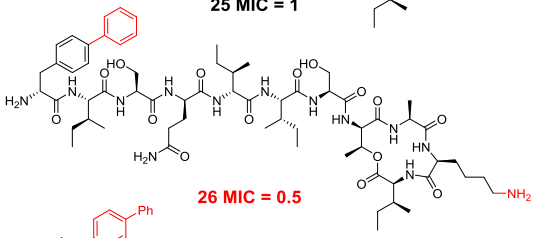
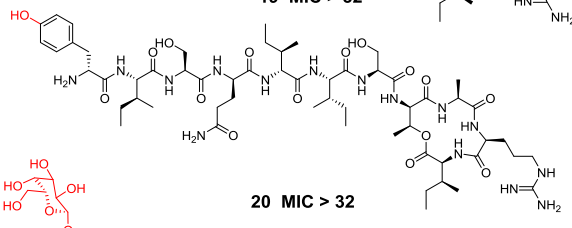
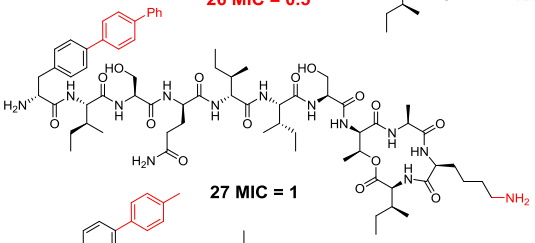
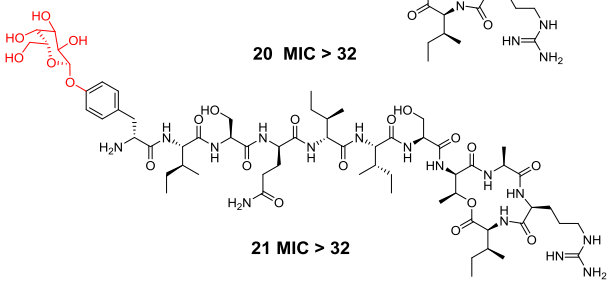
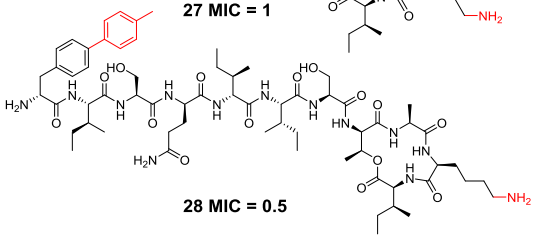


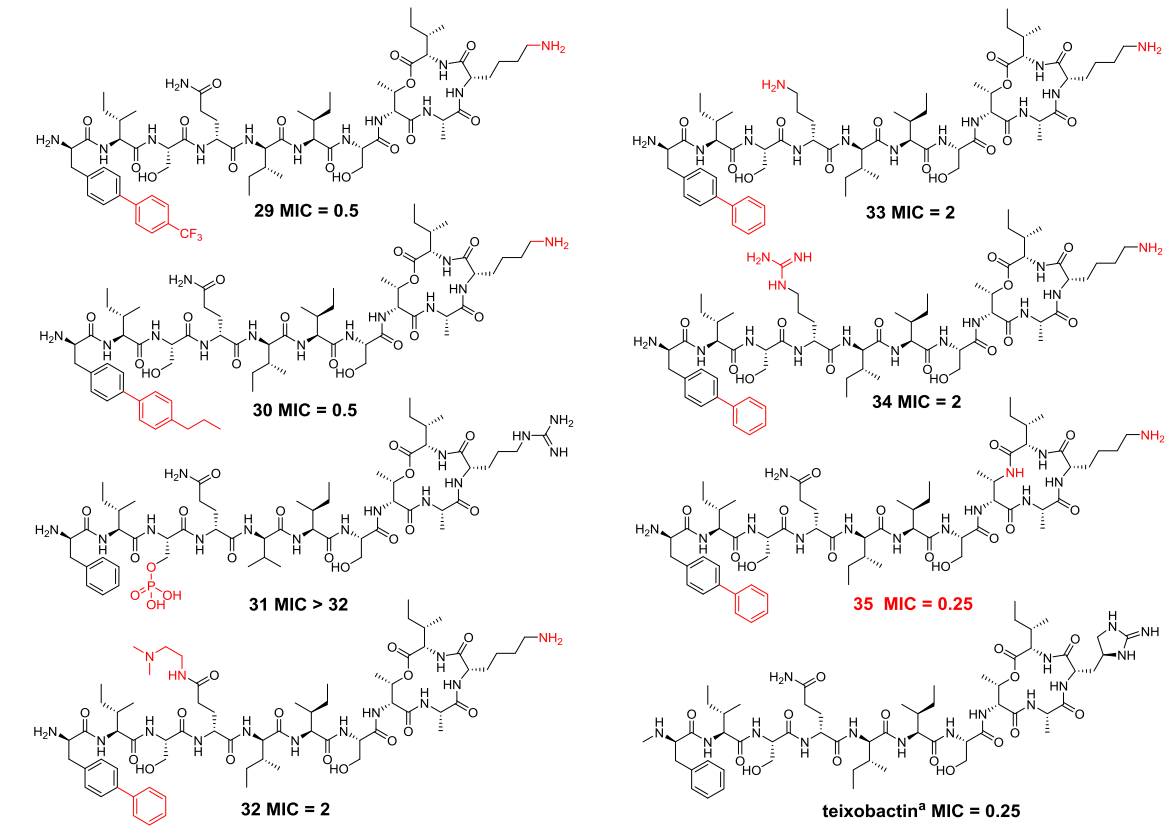
Conditions: a) 3 equiv. PMe_3, THF:H_2O/9:1, rt, 5 h; b) 2 equiv. FmocOSu, 4 equiv. $NaHCO_3$, dioxane/H_2O, RT, overnight, 60% over 2 steps; c) 4 M HCl, dioxane, 15 min; d) 1.1 equiv. Boc-Ser(tBu)-OH,1.1 equiv. HATU, 1.2 equiv. DIEA, DCM/DMF, 1 h, 84%; e) 10% $Pd(OH)_2$/C, H_2, MeOH, 20 min; f) 2-Cl resin, 10.0 equiv. DIEA, DCM/DMF;Then MeOH/DIEA/DMF (1:1:8) for 5 minitues g) 20% piperidine in DMF; h) 3 equiv. Fmoc-AA-OH, 3 equiv. HATU, 6 equiv. DIEA, DMF; i) 20% TFE in DCM, 3 h; j) 3 equiv. HATU, 3 equiv. HOAT, 6 equiv. DIEA, DCM:DMF/9:1, rt, 24 h; 42% from compound **12** k) 4 M HCl in dioxane, 15 min; l) 1 equiv. compound **8**, 1 equiv. DEPBT, 1.5equiv. DIEA, THF:DMF/8:2, rt, 12 h, 71%; m) 10% $Pd(PPh_3)_4$, 2 equiv. 1,3-Dimethylbarbituric acid, anhydrous DCM, 1 h; n) TFA: TIPS:H_2O/ 95:2.5:2.5,1 h.

An alternative pathway was developed to synthesize analogue **35** with a lactam scaffold. Starting material **10** was synthesized from D-allo-threonine according to the literature[35]. The azide

group was reduced by trimethyl phosphine to an amine, which was protected with FmocOSu to get compound **11**. After treatment with 4 M HCl in dioxane, compound **11** was coupled with Boc-Ser(*t*Bu)-OH to form intermediate **12**. Along with removal of the benzyl group under reductant conditions ($Pd(OH)_2/C$, H_2), the dipeptide was immobilized onto the 2-Cl resin with the aid of DIEA to generate intermediate **13**. Subsequently, Fmoc-Ile-OH, Fmoc-Lys(Alloc)-OH and Fmoc-Ala-OH was linked onto the resin under standard conditions to form the linear precursor for cyclization. After cleavage from the resin under mild acidic conditions, the intermediate was treated with HATU/HOAT/DIEA[35] at the concentration of 2 mM to accomplish the lactam with 42% isolated yield from compound **12**. Compound **15** was synthesized via a similar '5+6' strategy. After removal of Alloc group under $Pd(PPh_3)_4$/ 1,3-dimethylbarbituric conditions, all the other acid-labile protecting groups were cleaved with the aid of TFA-H_2O-TIPS (95:2.5:2.5) to provide desired lactam analogue **35** with 2.6% isolated yield from starting material **10**. (**Scheme 5)**.

Scheme 6 MIC (μg/mL) for MRSA (BAA-1695）

compound	compound
 9 MIC = 2	 22 MIC > 32
 (linear hexapeptide) 16 MIC > 32	 23 MIC = 2
 (cyclic pentapeptide) 17 MIC > 32	 24 MIC = 1
 18 MIC > 32	 25 MIC = 1
 19 MIC > 32	 26 MIC = 0.5
 20 MIC > 32	 27 MIC = 1
 21 MIC > 32	 28 MIC = 0.5



Biological Tests and SAR Study. Based on our newly developed synthetic route, a series of analogues were synthesized, and we evaluated their antibacterial activity against MRSA BAA-1695 (**Scheme 6**). Firstly, compound **9** showed modest inhibition with MIC 2 μg/mL. The linear (compound **16**) and cyclic fragments (compound **17**) of compound **9** were assessed to determine which motif was essential. However, **16** and **17** showed no inhibition at all with MIC greater than 32 μg/mL. Previously, Su group[23] has demonstrated that the methyl group on N-Me-D-phenylalanine was not essential, so a series of analogues that lacked the methyl group were synthesized. To explore the minimum effective structure and increase solubility, the last hydrophobic N-Me-D-phenylalanine was removed to form decapeptide **18**. However, it exhibited no inhibition either. Given that the peptide could not be shortened, hydrophilic functional groups such as morpholine were linked on the N-terminal to improve the solubility. Unfortunately,

compound **19** with morpholine was still ineffective. Furthermore, a series of compounds were synthesized linking hydrophilic groups to side chains of compound **9** to improve aqueous solubility. Firstly, D-Phe was replaced with D-Tyr to form compound **20**. Galactose was installed on the hydroxyl group of D-Tyr to obtain compound **21**. To our disappointment, the MIC of compounds **20** and **21** was greater than 32 μg/mL. The simple addition of the hydroxyl group between D-Tyr on compound **20** and D-Phe on compound **9** resulted in 10-fold-potency loss. It was speculated that steric hindrance and negative charge may account for this phenomenon. The hydroxyl group was substituted by an amine to access compound **22**. It exhibited no inhibition at all. Interestingly, when instead of a hydroxyl a methyl group was added to D-Phe of compound **9,** the MIC of compound **23** was 2 μg/ml. The above results indicated that hydrophobic substitution of D-Phe would increase antibacterial activity while hydrophilic groups would decrease activity dramatically. So we speculated that there exists a hydrophobic interaction between lipid II and the N-terminal of teixobactin. To verify our hypothesis, compound **24**, possessing a phenyl group extended from the side chain of phenylalanine to enhance hydrophobic interaction, was prepared. Surprisingly, the potency was increased 2-fold compared with **23**. It confirmed the existence of hydrophobic interactions at the N-terminal of teixobactin. On the other end of the molecule, when the Arg was replaced by Lys, the potency was better than compound **9**. Combining the biphenyl and Arg to Lys modification, compound **26** was obtained. It demonstrated very strong potency (MIC was as low as 0.5 μg/mL), which was comparable to teixobactin. Next, compounds **27** - **30** were synthesized to modify the phenylalanine, which showed similar potency to compound **26**. Then serine was modified by phosphoric acid (compound **31**) to further improve solubility. Unfortunately, although solubility was improved, it lost activity. We further confirmed that compound **31** was not a good prodrug via an in vivo study. D-Gln was modified by N, N-

dimethylethane-1,2-diamine to get compound **32**, and was replaced by D-Orn and D-Arg to get compound **33** and **34** respectively. However, MIC of these three compounds showed 4-fold loss of potency. According to previous studies, the cyclic tetrapeptide may interact with pyrophosphoric acid[20]. Hence, the hydroxyl group in the Thr was replaced with amine to access compound **35** with lactam scaffold. Compound **35** showed 2-fold better potency than compound **26**. Considering this result, we speculate that when the oxygen is replaced by nitrogen, it may improve the interaction between the cyclic tetrapeptide and the lipid pyrophosphate. Finally, compounds **26** and **35** were selected for further antibacterial study. Compared with teixobactin against different pathogenic microorganisms, they showed similar potency to teixobactin in MRSA, MSSA, VRE, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (**Table 1)**.

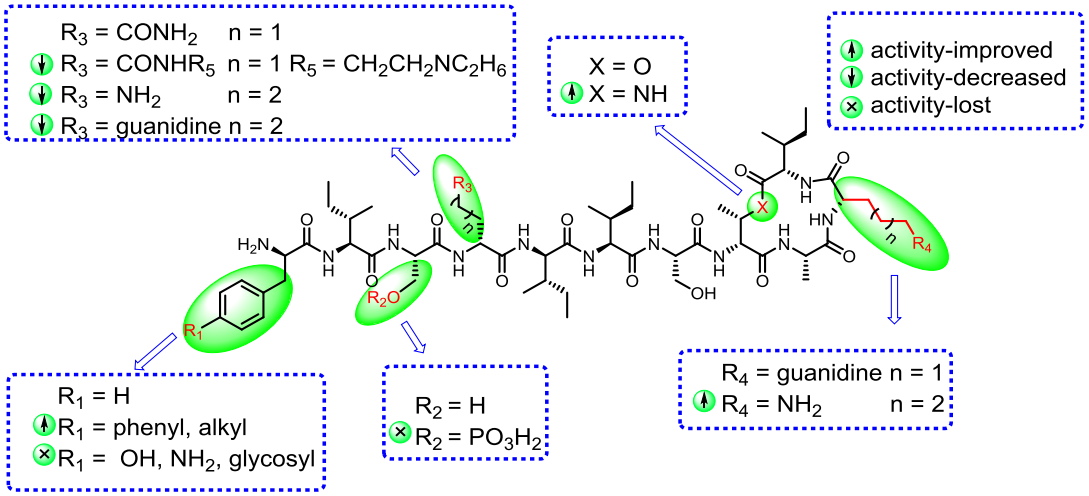
Table 1 Activity of Compound 26, 35 Against Pathogenic Microorganisms MIC (μ g/mL)

Organism and genotype	**Teixobactin** MIC	compound **26** MIC	compound **35** MIC
MSSA (TH 4806)	0.25	0.5	0.25
MRSA (BAA-1695)	0.25	0.5	0.25
VRE (ATCC 29212)	1	0.5	0.5
Streptococcus pneumoniae (D39)	< 0.032	< 0.032	< 0.032
Streptococcus pyogenes (ATCC 19615)	< 0.25	< 0.25	< 0.25

From our structure-activity relationship study, we drew the following conclusions. Firstly, L-*allo*-enduracididine could be replaced by simplified Lys. Secondly, N-terminal could not be shortened or elongated with hydrophilic groups. Previously, Nowick confirmed that the N-terminal linear pentapeptide could be replaced by a lipid chain. Other than demethylation, acetylation and dimethylation of amine in phenylalanine at N-terminal induced inactivation. The amine group and

phenyl ring may interact with phosphate and lipid chain of membrane respectively. Thus, we speculated that the N-terminal phenylalanine played an essential role in anchoring on the membrane. Thirdly, when the hydrophobic effect was strengthened, stronger antibacterial potency emerged. It further confirmed our hypothesis that hydrophobic interactions exist between lipid II and the N terminal of the analogues. Lastly, when the lactone was substituted by lactam, it increased activity, perhaps through the binding affinity to pyrophosphate (**Scheme 7**).

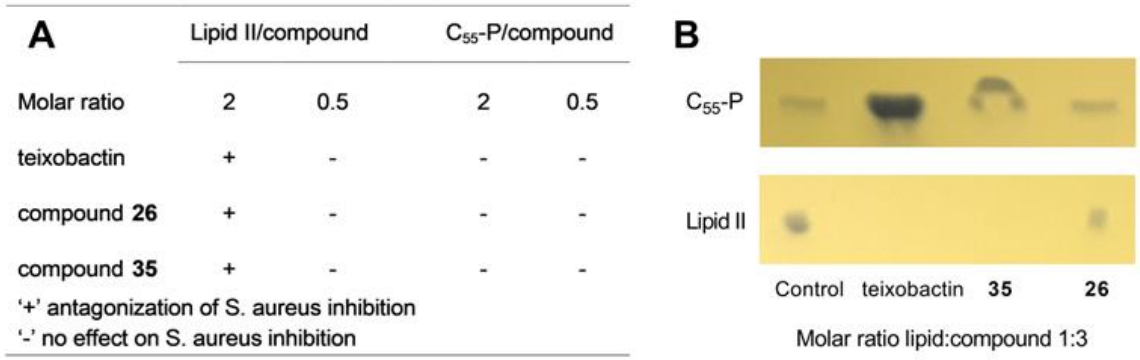
Scheme 7 Structure-Activity Relationship of Teixobactin Analogues



The mechanism of action of compounds **26** and **35** was compared to teixobactin through lipid II antagonization and binding assays[13]. Addition of lipid II eliminated the activity of compounds **26** and **35** against *S. aureus* at the same ratios that antagonized teixobactin. For all three compounds, lipid II was more effective at antagonizing activity than undecaprenyl-phosphate (C_{55}-P) (**Figure 1A**, **Supplementary Figure 1**). Likewise, as observed with teixobactin, compound **35** prevented in vitro butanol extraction of lipid II but not C55-P. Compound **26** did not completely prevent lipid II extraction at the ratios tested(lipid II: compound 26/1:4), perhaps reflecting its 2-fold lower potency than teixobactin and compound **35** and weaker binding to the lipid pyrophosphate (**Figure**

1B, Supplementary Figure 2). Both compounds have comparable mechanism of action to teixobactin. Compared with compound **35**, compound **26** could be available in large scale easily, so compound **26** was selected to conduct the ITC and in vivo study.

Figure 1. Lipid II Antagonization and Binding.



(A) Lipid II antagonized the effect of teixobactin, compound **26**, and compound **35** against S. aureus at a ratio of 2:1 lipid:compound. At a lower ratio of 0.5:1 lipid II no longer antagonized activity. Undecaprenyl-phosphate (C55-P) did not antagonize the activity of teixobactin or compounds **26** and **35**. n=3 for compound **35**, and n=2 for compound **26**. (B) On incubation with purified lipid II or C55-P, teixobactin and compound **35** prevented extraction of lipid II but not C55-P, as observed by the lack of a lipid II band by TLC analysis. Molar ratio lipid:compound /1:3. Cropped TLC images shown, adjusted for brightness and contrast, n=2.

Considering the bad solubility of lipid II stemming from the long lipid chain, Park's nucleotide (UDP-MurNAc-L-Ala- γ -D-Glu-meso-Dap-D-Ala-D-Ala) was selected as the target to validate binding. Using ITC, we evaluated the binding affinity of compound **26** to Park's nucleotide[36] which was isolated from *Bacillus subtilis*. Compound **26** bound to Park's nucleotide with a Kd of 23 μM. The loss of long lipid chain could account for more than 10-fold binding affinity decrease (**Figure 2**). Based on all of our results, we propose a pattern diagram of the interaction between lipid II and compound **35** (**Figure 3**).

In Vivo Efficacy. Finally, compound **26** was evaluated with an in vivo study. An animal efficacy study was performed in a mouse sepsis protection model[13]. Mice were infected intraperitoneally with *Streptococcus pneumonia* at a dose that leads to 90% death. B6 female mice were infected with 0.2 ml of bacterial suspension (1×10^4 c.f.u. per mouse). An hour post-infection, compound **26** was introduced i.v. at single doses ranging from 1 to 5 mg per kg. Vancomycin which was selected as the positive-control drug and was introduced i.v. at a dose of 5 mg per kg. As expected, compound **26** revealed excellent results within 48 hours after infection. The survival rate of all animals was 100%. Then B6 female mice were infected with a higher inoculum of *Streptococcus pneumoniae* suspension (5×10^4 c.f.u. per mouse, 0.2 mL). Animals were dosed 1 h post infection, and blood c.f.u was evaluated at 12, 18, and 22 h. Compound **26** caused over a 6 $\log_{10}$ reduction of c.f.u. in blood at 22 h post infection.. (**Figure 4, Supplementary Figure 3**).

Figure 2 ITC of Park's Nucleotide + Compound 26

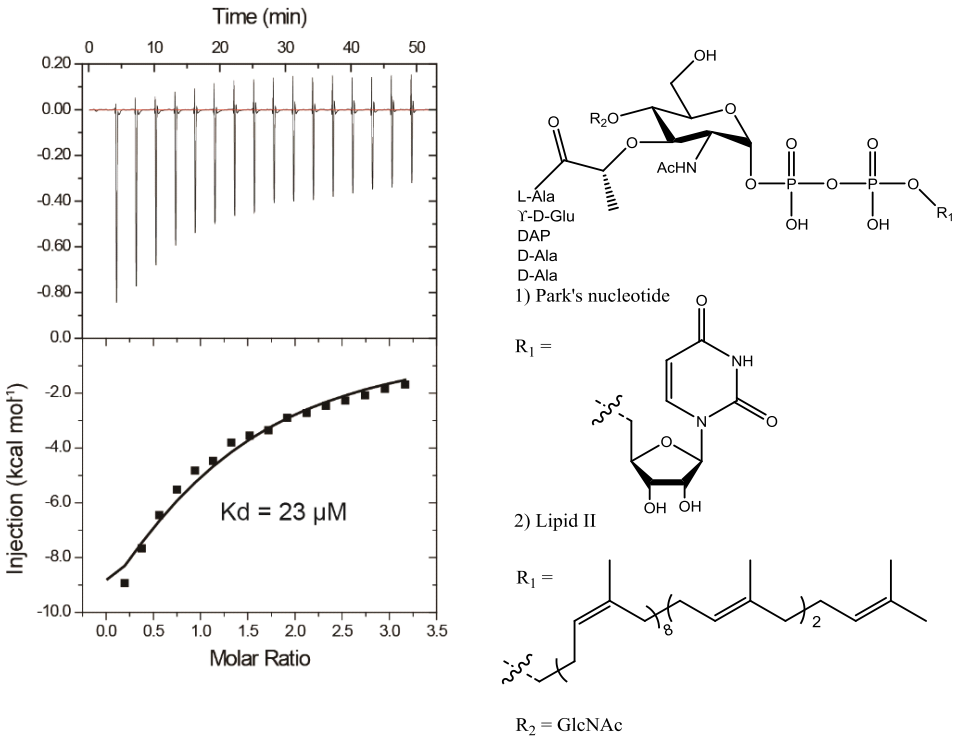


Figure 3 Pattern Diagram of the Interaction of Lipid II and Compound 35

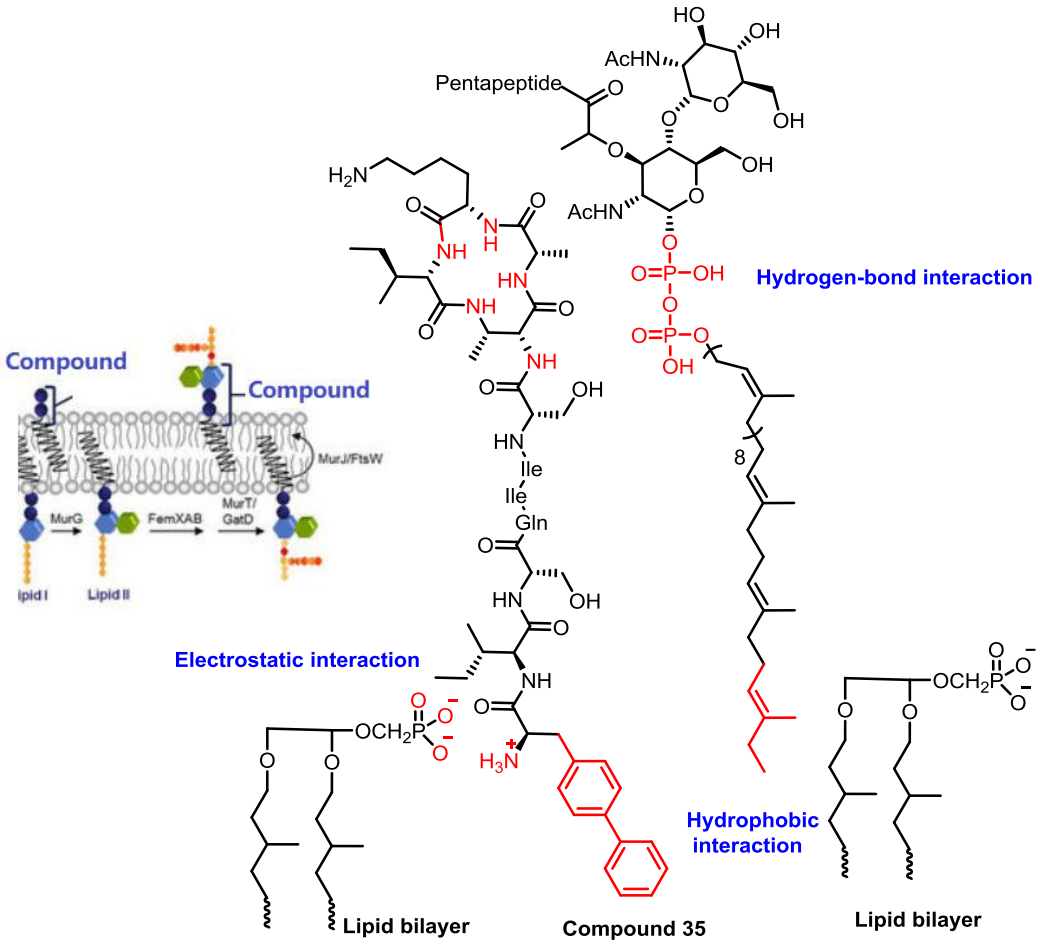
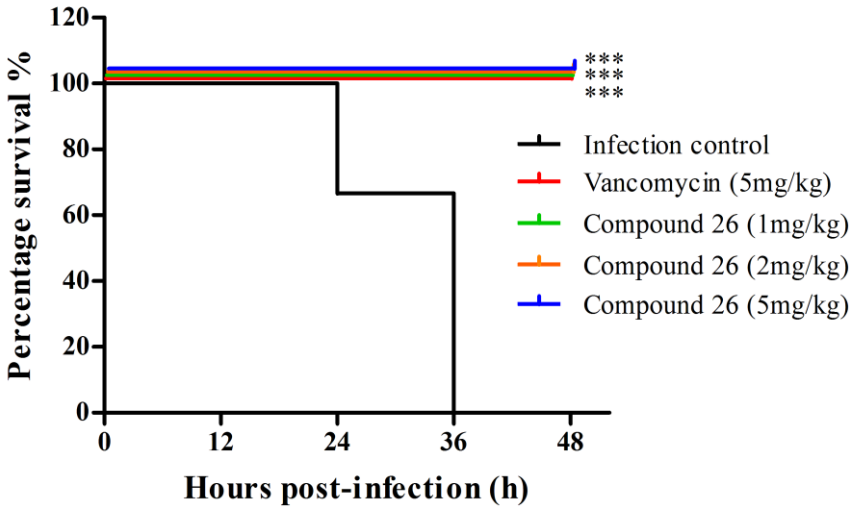


Figure 4 Compound 26 Efficacy in Mouse Models of Infection.



Single dose treatment (i.v., 1 h post-infection, 6 female mice per group) with compound **26** and vancomycin in septicemia protection model using *Streptococcus pneumoniae* (1×10^4 c.f.u. for each mouse). Survival is depicted 48 h after infection. *** $P < 0.0001$ (determined by non-parametric log-rank test).

CONCLUSION

Compared with the linear synthesis strategy, we have developed a new highly convergent method ('5+6' strategy) to prepare diverse teixobactin analogues to explore the SAR more efficiently. Based on our study, we drew the following conclusions: Firstly, L-*allo*-enduracididine could be replaced by more simplified lysine. Secondly, there may exist a hydrophobic interaction between lipid II and the N terminal of teixobactin analogues. Properly increasing the hydrophobic groups was beneficial to the interaction. We proposed that the biphenyl group could anchor to the membrane and the free amine of N-terminal interacts with the phosphate of the lipid bilayer. Thirdly, the increased potency of the lactam analog was perhaps due to the additional amide bond contributing to the binding affinity to the pyrophosphate of lipid II. The most potent compound, **35,** demonstrated comparable potency to teixobactin in various Gram-positive species. Finally,

compound **26** showed effectiveness in in vivo studies. Further studies on increasing solubility and analyzing pharmacokinetic properties are ongoing in our lab.

EXPERIMENTAL SECTION

Materials. All commercial materials (Alfa Aesar, Aladdin, J&K Chemical LTD.) were used without further purification. All solvents were analytical grade. The 1H NMR spectra were recorded on a Bruker 400 MHz spectrometer. Low-resolution mass spectral analyses were performed with a Waters AQUITY UPLCTM/MS. Analytical HPLC was performed on a SHIMADZU system, using a Vydac 218TP C18 column (5 μm, 4.6×250 mm). Semi-preparative HPLC was performed on a SHIMADZU system, using a Vydac 218TP C18 column (10 μm, 10×250 mm, A column), Dr. Maisch GmbH Reprosil 100 C18 column (5 μm, 10×250 mm, B column). Buffer A: 0.1% TFA in acetonitrile; buffer B: 0.1% TFA in H2O. Analytical TLC was performed on Yantai Chemical Industry Research Institute silica gel 60 F254 plates and flash column chromatography was performed on Qingdao Haiyang Chemical Co. Ltd silica gel 60 (200-300 mesh). The rotavapor was BUCHI's Rotavapor R-3. MicroCal iTC200 system (GE Healthcare). Mice was from Beijing Vital River Laboratory Animal Technology Co., Ltd..

Synthesis of compound 2 Compound **1** (2.874 g, 9.3 mmol) was dissolved in 15 mL 50% TFA in DCM. The resulting solution was stirred at room temperature for 30 min to remove the Boc group. The TFA was blown off under a stream of condensed air. Boc-Ser(tBu)-OH (2.613 g, 10 mmol), HCTU (4.13 g, 10 mmol), and DIEA (3.63 mL, 22 mmol) were mixed in 50 mL anhydrous DCM. The resulting solution was added to the above TFA salt. The reaction mixture was stirred at room temperature for 3 h. After completion, the reaction mixture was diluted with DCM (250 mL) and

washed with 1N HCl (100 mL x 3) and brine (100 mL x 1). The organic phase was dried over Na_2SO_4, concentrated under vacuum and purified by flash column chromatography on silica gel (hexane/EA, 4:1) to give compound **2** (3.573 g, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.40-7.30 (m, 5H), 7.26-7.20 (m, 1H), 5.44 (s, 1H), 5.25-5.15 (m, 2H), 4.65 (d, *J* = 8.76 Hz, 1H), 4.35-4.25 (m, 1H), 4.22 (s, 1H), 3.80-3.70 (m, 1H), 3.43 (t, *J* = 8.00 Hz, 1H), 1.45 (s, 9H), 1.19 (d, *J* = 6.40 Hz, 3H), 1.18 (s, 9H). LRMS (ESI) calculated for $C_{23}H_{36}N_2O_7$ $[M + H]^+$ 453.25, found 453.34.

Synthesis of compound 3 Fmoc-Ile-OH (0.424 g, 1.2 mmol,), EDCI (0.23 g, 1.2 mmol), DMAP (18.3 mg, 0.15 mmol) was dissolved in 10 mL DCM and the mixture was stirred for 20 min. Then compound **2** (0.452 g, 1 mmol) was added to the solution and stirred at room temperature for 12 h. The reaction mixture was washed with 1 N HCl (10 mL x 1) and brine (10 mL x1). The organic phase was dried over Na_2SO_4, concentrated under vacuum and purified by flash column chromatography on silica gel (EA/PE, 1:1) to give intermediate. Then the intermediate was dissolved in 10 mL of a mixture of MeCN/diethylamine (2/1, v/v) and stirred at room temperature for 10 min to remove the Fmoc group. The reaction mixture was concentrated under vacuum and purified by flash column chromatography on silica gel (DCM/MeOH, 20:1) to give the free amine **3** (0.389 g, 69% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.36-7.30 (m, 5H), 7.26-7.20 (m, 1H), 5.52-5.46 (m, 1H), 5.46 (s, 1H), 5.15 (d, *J* = 12.0 Hz, 1H), 5.04 (d, *J* = 12.0 Hz, 1H), 4.86 (dd, *J* = 9.2 Hz, *J* = 2.0 Hz, 1H), 4.24 (s, 1H), 3.79 (s, 1H), 3.44 (t, *J* = 8.0 Hz, 1H), 3.18 (d, *J* = 5.2 Hz,1H), 1.70-1.60 (m, 1H), 1.45(s, 9H), 1.40-1.10 (m, 7H) 1.19 (s, 9H); 0.89-0.86 (m, 6H). LRMS (ESI) calculated for $C_{29}H_{47}N_3O_8$ $[M + H]^+$ 566.34, found 566.62.

Synthesis of compound 5 Boc-Arg(Pbf)-OH (10 mmol, 5.27 g) was dissolved in 2 M HCl of 15 mL MeOH and stirred for 5 hours at room temperature. After completion, the solvent was removed and dissolved in ethyl acetate. Then the solution was washed with aqueous saturated $NaHCO_3$ and

brine, dried over anhydrous Na_2SO_4 and concentrated for next step without further purification. Cbz-Ala-OH (2.232 g, 10 mmol), HATU (3.8 g, 10 mmol) and DIEA (1.815 ml, 11 mmol) were mixed in 30 mL DMF:DCM (2:8). Then the intermediate was added to the above solution. The reaction mixture was stirred at room temperature for 2 h. The reaction mixture was diluted with DCM (250 mL), washed with 1N HCl (100 mL x 3) and brine (100 mL x 1). The organic phase was dried over Na_2SO_4, concentrated under vacuum and purified by flash column chromatography on silica gel (DCM/MeOH, 20:1) to give intermediate. Then it was dissolved in 30 mL THF/H_2O (3:1). LiOH (0.38 g, 15.84 mmol) was added to the above solution and stirred at room temperature for 5 min. After completion, the mixture was acidified to pH 3 with 10 % citric acid and extracted with EA (100 mL x 3). The combined organic phase was dried over Na_2SO_4 and concentrated under vacuum to give compound **5** without further purification (5.425 g, 86% over 3 steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.56 (s, 1H), 7.28-7.20 (m, 5H), 7.10-6.90 (s, 1H), 6.80-6.20 (m, 3H) ,6.13 (s, 1H), 5.04 (d, *J* = 12.0 Hz, 1H), 4.95 (d, *J* = 12.0 Hz, 1H), 4.53-4.40 (m, 1H), 4.38-4.28 (m, 1H), 3.20-3.10 (m, 2H), 2.90 (s, 2H), 2.51 (s, 3H), 2.45 (s, 3H), 2.04 (s, 3H), 1.96-1.65 (m, 2H), 1.65-1.50 (m, 2H), 1.43 (s, 6H), 1.33 (d, *J* = 6.56 Hz, 3H). LRMS (ESI) calculated for $C_{30}H_{41}N_5O_8S$ $[M + H]^+$ 632.27, found 632.88.

Synthesis of compound 5s Followed the synthesis of compound **5** to obtain compound **5s**. ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.02 (d, *J* = 7.56 Hz, 1H),7.88 (d, *J* = 7.48 Hz, 2H), 7.68 (d, *J* = 7.4 Hz, 2H), 7.43-7.39 (m, 3H), 7.34-7.30 (m, 7H), 5.0 (s, 2H), 4.30-4.27 (m, 2H), 4.21-4.03 (m, 3H), 2.9-3.0 (m, 2H), 1.73-1.62 (m, 1H), 1.61-1.5 (m, 1H), 1.42-1.35 (m, 2H), 1.35-1.24 (m, 2H), 1.20-1.19 (d, *J* = 7.12 Hz, 3H). LRMS (ESI) calculated for $C_{32}H_{35}N_3O_7$ $[M + H]^+$ 574.25, found 574.83.

Synthesis of compound 6 Compound **5** (0.379 g, 0.6 mmol) and compound **3** (0.283 g, 0.5 mmol) were mixed in 6 mL DCM/DMF(9:1). DEPBT (0.24 g, 0.6 mmol) and DIEA (119 μl, 0.72 mmol) were added at 0 ℃. Then the reaction was stirred at room tempreture over night. The reaction mixture was washed with 1 N HCl (10 mL x 1) and brine (10 mL x 1). The organic phase was dried over Na_2SO_4, concentrated under vacuum and purified by flash column chromatography on silica gel (DCM/MeOH, 20:1) to give compound **6** (0.471 g, 80 %). ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.14 (d, *J* = 9.04 Hz, 1H), 7.06 (d, *J* = 7.80 Hz, 1H), 7.88 (d, *J* = 7.88 Hz, 1H), 7.47 (d, *J* = 7.20 Hz, 1H), 7.35-7.31 (m, 10H), 6.82-6.38 (m, 3H), 5.36-5.34 (m, 1H), 5.13-4.96 (m, 4H), 4.81 (d, *J* = 9.08 Hz, 1H), 4.33-4.32 (m, 1H), 4.25-4.21 (m, 2H), 4.08-4.04 (m, 1H), 3.47 (d, *J* = 5.76 Hz, 2H), 3.05 (m, 2H), 2.94 (s, 2H), 2.47 (s, 3H), 2.42 (s, 3H), 1.99 (s, 3H), 1.78 (m, 1H), 1.66 (m, 1H), 1.51-1.37 (m, 18H), 1.20-0.95 (m, 18H), 0.81-0.76 (m, 6H). LRMS (ESI) calculated for $C_{59}H_{86}N_8O_{15}S$ $[M + H]^+$ 1179.59, found 1179.62.

Synthesis of compound 6s The procedure followed the synthesis of compound **6**. LRMS (ESI) calculated for $C_{61}H_{80}N_6O_{14}$ $[M + H]^+$ 1121.57, found 1121.97.

Synthesis of compound 7 To a solution of compound **6** (0.118 g, 0.1 mmol) in MeOH was added $Pd(OH)_2/C$ (20% w/w, 23 mg), and the reaction mixture was stirred at room tempreture under H_2 (1 atm) for 30 min. Then the reaction mixture was filtrated and concentrated in vacuum. To a solution of the residue and DIEA (132μ L, 0.8 mmol) in mixed solvents of DCM/DMF (50 mL, 9:1, 0.002 M) was added PyAOP (0.208 g, 0.4 mmol), HOAT (55 mg, 0.4 mmol) at 0 ℃, and then the resulting reaction mixture was stirred at rt for 24 h. After completion, the reaction mixture was washed with 1N HCl (100 mL x 3) and brine (100 mL x1). The organic phase was dried over Na_2SO_4, concentrated under vacuum and purified by flash column chromatography on silica gel (DCM/MeOH, 20:1) to give compound **7** (62 mg, 66 %). ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm)

8.24 (m, 1H), 7.87-7.86 (m, 2H), 7.71 (m, 1H), 7.23 (m, 1H), 6.38 (m, 1H), 5.30-5.29 (m, 1H), 4.64 (d, *J* = 9.48 Hz, 1H), 4.26-4.24 (m, 1H), 4.17 (m, 1H), 4.09-3.96 (m, 3H), 3.58-3.52 (m, 2H), 3.05 (m, 2H), 2.96 (s, 3H), 2.47 (s, 3H), 2.42 (s, 3H), 2.00 (s, 3H), 1.68 (m, 3H), 1.40-1.34 (m, 21H), 1.16-1.11 (m, 13H), 0.84-0.79 (m, 6H). LRMS (ESI) calculated d for $C_{44}H_{72}N_8O_{12}$ $[M + H]^+$ 937.50, found 937.61.

Synthesis of compound 7s The procedure followed the synthesis of compound **7**. ^{1}H NMR (400 MHz, CD_3OD-d4) δ (ppm) 7.78 (d, *J* = 7.6 Hz, 2H), 7.63 (d, *J* = 7.2 Hz, 2H), 7.38 (t, *J* = 7.6 Hz, 2H), 7.30 (t, *J* = 7.2 Hz, 2H), 5.60-5.58 (m, 1H), 4.45 (t, J = 8.0 Hz, 1H), 4.32-4.30 (m, 1H), 4.24 (d, J = 8.0 Hz, 1H), 4.18-4.06 (m, 4H), 3.66-3.61 (m, 1H), 1.85-1.68 (m, 3H), 1.54-1.43 (m, 18H), 1.29 (d, J = 6.8 Hz, 4H), 1.27-1.16 (m, 11H), 0.90-0.86 (m, 6H). LRMS (ESI) calculated for $C_{46}H_{66}N_6O_{11}$ $[M + H]^+$878.48, found 880.18.

Synthesis of compound 8 0.2 g of 2-Cl-Trt resin (0.5 mmol/g) was placed in a 10 mL polypropylene syringe. The resin was activated with DCM/DMF (1:1), washed with DMF (2 ×10 mL, 1 min) and DCM (2 × 10 mL, 1 min) followed by addition of the first amino acid Fmoc-L-Ile-OH (0.177 g, 0.5 mmol) and DIEA (165 µ l, 1 mmol) in 2 ml DCM/DMF (1:1) and shacked for 4 h. Then the unreacted Cl group was blocked with MeOH/DIEA/DMF (1:1:8) for 5 minutes. Next, the resin was washed with DMF and DCM. Then, Fmoc removal was achieved by 20 % piperidine in DMF (2 × 10 mL, 15 min). The next amino acids were added by using the following coupling condition: For coupling conditions: Fmoc-AA-OH/HATU/DIEA (3:3:6) in 1 mL DMF for 50 min. The following amino acids were coupled one by one: Fmoc-D-allo-Ile-OH, Fmoc-D-Gln(Trt)-OH, Boc-Ser(tBu)-OH, Fmoc-Ile-OH, Boc-NMe-D-Phe-OH. For Fmoc removal: 20 % piperidine in DMF (2 × 10 mL, 15 min). Washing: DMF (2 × 10 mL, 1 min), DCM (2 × 10 mL, 1 min) and DMF (2 × 10 mL, 1min). Boc-NMe-D-Phe-OH. At last, the resin was washed with 20%

TFE (in DCM) for 4 h, and then washed with dry DCM (3 x). The filtrates were combined and concentrated under reduced pressure to afford compound **8** (0.107 g, 95%).

Synthesis of compound 9 Compound **7** (0.033 mmol, 31 mg) was dissolved in 3 ml 4 M HCl in dioxane and stirred for 15 min. when the reaction finished, the protecting group Boc was removed completely, then the mixture was diluted with EA and washed with aqueous saturated $NaHCO_3$. The organic phase was dried over Na_2SO_4 and concentrated under vacuum without purification. Then the intermediate and compound **8** (0.03 mmol, 33 mg) were dissolved in THF/DMF (2 mL). DEPBT (0.033 mmol, 10 mg) and DIEA (6 μ L, 0.033 mmol) were added at 0 ℃ and stirred at room tempreture overnight. The mixture was diluted with EtOAc (10 mL) and washed with 1M HCl (2 x 5 mL), aqueous saturated $NaHCO_3$ (2 x 5 mL), and brine (1 x 5 mL). Then the organic layer was dried with Na_2SO_4, filtered and concentrated. The product was purified by silica gel column with DCM/MeOH (20:1) to give intermediate **S9** (45 mg, 76%). Intermediate **S9** (35 mg) was treated with 5 ml TFA/H_2O/TIPS (*v/v/v* = 95:2.5:2.5) for 1 hour and the reaction was monitored by LC-MS. After completion, the reaction mixture was concentrated under low temperature, following by the addition of cold diethyl ether to precipitate the crude product. The crude product was purified by preparative HPLC (5-60% CH_3CN/H_2O over 30 min) to afford compound **9** (15 mg, 51%) ^{1}H NMR (400 MHz, MeOH-d4) δ (ppm) 7.40-7.25 (m, 5H), 5.61-5.52 (m, 1H), 4.53 (t, *J* = 8.60 Hz, 1H), 4.50-4.28 (m, 4H), 4.28-4.10 (m, 4H), 4.05 (d, *J* = 7.52 Hz, 1H), 4.00-3.88 (m, 2H), 3.88-3.78 (m, 2H), 3.33-3.28 (m, 3H), 3.18-3.08 (m, 1H), 2.68 (s, 3H), 2.38-2.30 (m, 2H), 2.20-2.10 (m, 1H), 2.10-1.95 (m, 2H), 1.94-1.76 (m, 4H), 1.76-1.45 (m, 9H), 1.32-1.10 (m, 8H), 1.02-0.72 (m, 25H). LRMS (ESI) calculated for $C_{58}H_{97}N_{15}O_{15}$ [$M+H^+$]: 1244.73, found:1244.69

Synthesis of compound **11** Compound **10** (33.5 mg, 0.1 mmol) was dissolved in THF (4 mL) and H_2O (0.4 mL) and kept at 0 °C, followed by addition of PMe_3 (1.0 M in THF, 0.3 mL, 0.3 mmol). After that, the reaction was warmed to room temperature and stirred for another 5 h. The solvent was removed in vacuo to afford the crude primary amine, which was dissolved in dioxane (3 mL) and H_2O (1 mL), followed by addition of $NaHCO_3$ (33.6 mg, 0.4 mmol) and FmocOSu (67.4 mg, 0.2 mmol). The reaction was stirred at room temperature overnight. After that, the reaction mixture was evaporated in vacuo and extracted with ethyl acetate (3 × 15 mL), the combined organic phases were washed with brine, dried with $MgSO_4$, filtered and concentrated in vacuo. The residue was purified by silica gel column chromatography to afford compound **11** (31.8 mg, 60 % for two steps). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.77 (d, *J* = 7.4 Hz, 2H), 7.58 (d, *J* = 5.2 Hz, 2H), 7.45-7.28 (m, 9H), 5.47 (s, 1H), 5.21-5.11 (m, 2H), 5.02 (s, 1H), 4.48-4.21 (m, 4H), 4.21-4.10 (m, 1H), 1.45 (s, 9H), 1.21 (d, *J* = 6.52 Hz, 3H). LRMS (ESI) calculated for $C_{31}H_{34}N_2O_6$ $[M + H]^+$ 531.24, found 531.70.

Synthesis of compound 12 Compound **11** (0.06 mmol, 31.8 mg) was dissolved in 3 mL 4 M HCl in dioxane and stirred for 15 min. when the reaction finished, the protecting group Boc was removed completely, then the mixture was diluted with EA and washed with aqueous saturated $NaHCO_3$. The organic phase was dried over Na_2SO_4 and concentrated under vacuum without purification to get intermediate. Then Boc-Ser(tBu)-OH (0.07 mmol, 18.3 mg), HATU (0.07 mmol, 26.6 mg), and DIEA (0.08 mmol, 14 μ L) were dissolved in 2 mL of DCM/DMF (9:1). The intermediate was added and stirred at room temperature for 1h. The mixture was diluted with DCM (10 mL) and washed with 1M HCl (2 x 5 mL), aqueous saturated $NaHCO_3$ (2 x 5 mL), and brine (1 x 5 mL). Then the organic layer was dried with Na_2SO_4, filtered and concentrated. The product was purified by silica gel column with DCM/MeOH (20:1) to give compound **12** (34 mg, 84%).

^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 7.75 (d, *J* = 7.52 Hz, 2H), 7.60-7.52 (m, 2H), 7.45-7.28 (m, 10 H), 5.45 (s, 1H), 5.21-5.11 (m, 2H), 5.02 (s, 1H), 4.74 (d, *J* = 8.72 Hz, *J* = 0.84 Hz, 1H), 4.40-4.30 (m, 2H), 4.25-4.15 (m, 2H), 3.78 (s, 1H), 3.42 (d, *J* = 8.92 Hz, *J* = 1.76 Hz, 1H), 1.45 (s, 9H), 1.20-1.10 (m, 12 H). LRMS (ESI) calculated for $C_{31}H_{34}N_2O_6$ $[M + H]^+$ 674.34, found 674.97.

Synthesis of compound 13 To a solution of compound **12** (34 mg, 0.05 mmol) in MeOH was added $Pd(OH)_2/C$ (20% w/w, 7 mg), and the reaction mixture was stirred at rt under H_2 (1 atm) for 20 min. Then the reaction mixture was filtrated and concentrated in vacuum to get the intermediate. 0.1 g of 2-Cl-Trt resin (0.5 mmol/g) was placed in a 10 mL polypropylene syringe. The resin was activated with DCM/DMF (1:1), washed with DMF (2 ×10 mL, 1 min) and DCM (2 × 10 mL, 1 min) followed by addition of the intermediate and DIEA (82 μ L, 0.5 mmol) in 2 ml DCM/DMF (1:1) and shacked for 4 h. Then the unreacted Cl group was blocked with MeOH/DIEA/DMF (1:1:8) for 5 min to get intermediate **13**.

Synthesis of compound 14 Fmoc-Lys(alloc)-OH, Fmoc-Ala-OH were linked onto the resin by using the following conditions in sequence. For coupling conditions: Fmoc-AA-OH/HATU/DIEA (3:3:6) in 2 mL DMF for 50 min. For Fmoc removal: 20 % piperidine in DMF (2 × 10 mL, 5 min). Washing: DMF (2 × 10 mL, 1 min), DCM (2 × 10 mL, 1 min) and DMF (2 × 10 mL, 1min). Then the free pentapeptide was cleaved from the resin and the solvent was evaporated. The following procedure complied with the synthesis of compound **7**. Isolated yield from compound **14** was 42%. ^{1}H NMR (400 MHz, MeOD-d4) δ (ppm) 5.95-5.88 (m, 1H), 5.28 (d, *J* = 17.2 Hz, 1H), 5.17 (d, *J* = 10.4 Hz, 1H), 4.58-4.50 (m, 4H), 4.18-4.15 (m, 2H), 4.04-4.00 (m, 1H), 3.76 (m, 1H), 3.65 (m, 1H), 3.15 (m, 2H), 1.90-1.88 (m, 3H), 1.56-1.54 (m, 3H), 1.47 (s, 9H), 1.41-1.24 (m, 5H), 1.21-1.17 (m, 12H), 0.91-0.89 (m, 6H). LRMS (ESI) calculated for $C_{31}H_{34}N_2O_6$ $[M + H]^+$ 740.45, found 741.09.

General Procedure for synthesis of substituted phenylalanine via Suzuki-Miyaura Couplings[37] Boc-4-iodophenylalanine (1 equiv), arylboronic acid (1.5 equiv), sodium carbonate (3 equiv), palladium acetate (0.05 equiv), and tri-o-tolylphosphine (0.1 equiv) were added to a degassed mixture of dimethoxyethane (6 mL/mmol of amino acid) and water (1 mL/mmol of amino acid). The reaction mixture was kept under argon and heated to 80 °C for 4-6 h. After being cooled to room temperature, the solvent was evaporated under reduced pressure and diluted with ethyl acetate and washed with 1M HCl. At last the solvents were removed. The products were purified using flash chromatography using mixtures of DCM and MeOH as eluent. The isolated yields were 50-80 %.

Synthesis of compound 15 The synthetic procedure complied with the synthesis of compound **9.**

General procedure to synthesize compound 16-24 The synthetic procedure of compound **16-24** complied with the synthesis of compound **9**.

General procedure to synthesize compound 25-34 The synthetic procedure of compound **25-34** complied with the synthesis of compound **9**. Fmoc group was removed under 33% diethylamine in MeCN for 15 min and the solvent was evaporated. At last, deprotection was conducted with TFA/H_2O/TIPS (*v/v/v* = 95:2.5:2.5) for 1 h.

Synthesis of compound 35 Compound **15** (19.2 mg, 0.026 mmol) was dissolved in the DCM. $Pd(PPh_3)_4$ (6 mg, 0.005 mmol), 1,3-Dimethylbarbituric acid (8 mg, 0.052 mmol) was added and the mixture was stirred at RT for 1 hour. Then the solvent was evaporated. At last, deprotection was conducted with TFA/H_2O/TIPS (*v/v/v* = 95:2.5:2.5) for 1h.

Compound **9**,**16-35** were purified by semiprep HPLC, all compounds synthesized showed a purity >95% by HPLC (See SI for chromatograms).

^{1}H NMR for Compound **24** (400 MHz, MeOH-d4) δ (ppm) 7.65-7.597 (m, 4H), 7.44 (t, *J* = 7.84 Hz, 2H), 7.38-7.32 (m, 3H), 5.60-5.50 (m, 1H), 4.53-4.47 (m, 1H), 4.43-4.25 (m, 4H), 4.21-4.06 (m, 5H), 3.96-3.89 (m, 2H), 3.89-3.75 (m, 2H), 3.30-3.17 (m, 4H), 2.32 (t, *J* = 7.44 Hz, 2H), 2.185-2.05 (m, 1H), 2.05-1.921 (m, 2H), 1.92-1.75 (m, 4H), 1.71-1.55 (m, 4H), 1.55-1.45 (m, 5H), 1.32-1.10 (m, 8H), 0.97-0.70 (m, 23H).

^{1}H NMR for compound **25** (400 MHz, MeOH-d4) δ (ppm) 7.40-7.22 (m, 5H), 5.58-5.52 (m, 1H), 4.50 (t, *J* = 8.60 Hz, 1H), 4.45-4.35 (m, 2H), 4.35-4.28 (m, 2H), 4.28-4.18 (m, 2H), 4.18-4.08 (m, 2H), 4.05 (d, *J* = 7.24 Hz, 1H), 3.98-3.90 (m, 2H), 3.86-3.72 (m, 2H), 4.28-3.18 (m, 1H), 3.18-3.05 (m, 1H), 2.94 (t, *J* = 7.66 Hz, 1H), 2.68 (s, 3H), 2.32 (t, *J* = 7.64 Hz, 2H), 2.15-2.08 (m, 1H), 2.03-1.93 (m, 2H), 1.90-1.78 (m, 4H), 1.78-1.68 (m, 2H), 1.68-1.60 (m, 2H), 1.60-1.40 (m, 6H), 1.38-1.10 (m, 9H), 1.10-0.80 (m, 19H), 0.80-0.77 (m, 6H).

^{1}H NMR for compound **26** (400 MHz, MeOH-d4) δ (ppm) 7.64-7.59 (m, 4H), 7.44 (t, *J* = 7.36 Hz, 2H), 7.38-7.35 (m, 3H), 5.55-5.54 (m, 1H), 4.52 (t, *J* = 8.60 Hz, 1H), 4.43-4.31 (m, 5H), 4.24-4.11 (m, 4H), 4.19-4.09 (m, 2H), 3.93-3.67 (m, 4H), 3.24-3.17 (m, 4H), 2.32 (t, *J* = 8.16 Hz, 2H), 2.18-2.02 (m, 1H), 2.02-1.91 (m, 2H), 1.91-1.71 (m, 4H), 1.69-1.58 (m, 4H), 1.56-1.48 (m, 5H), 1.28-1.12 (m, 8H), 0.97-0.82 (m, 25H).

^{1}H NMR for compound **35** (400 MHz, MeOH-d4) δ (ppm) 7.64-7.59 (m, 4H), 7.44 (t, *J* = 7.36 Hz, 2H), 7.40-7.30 (m, 3H), 4.65-4.55 (m, 2H), 4.55-4.48 (m, 1H), 4.45-4.20 (m, 6H), 4.13-4.03 (m, 2H), 4.03-3.75 (m, 5H), 3.18 (d, *J* = 7.84 Hz, 2H), 2.92 (t, *J* = 7.32 Hz, 2H), 2.33 (t, *J* = 7.36 Hz, 2H), 2.20-2.05 (m, 2H), 2.05-1.60 (m, 10H), 1.60-1.35 (m, 8H), 1.28-1.10 (m, 8H), 1.00-0.85 (m, 16H), 0.79 (d, *J* = 6.84 Hz, 7H), 0.78-0.71 (m, 3H).

Minimum Inhibitory Concentration[13] (**MIC**) MIC was determined by broth microdilution according to CLSI guidelines. The test medium for most species was cation adjusted Mueller-Hinton Broth (MHB). The physical state of all analogues was amorphous, in the form of trifluoroacetates. Analogues were prepared to 10 mg/mL in DMSO, then they were diluted with MHB. The same test medium was supplemented with 3% lysed horse blood (Cleveland Scientific, Bath, OH) for growing Streptococci. All test media were supplemented with 0.002% polysorbate 80 to prevent drug binding to plastic surfaces, and cell concentration was adjusted to approximately 5×10^5 cells per ml. After 20 h of incubation at 37ºC, the MIC was defined as the lowest concentration of antibiotic with no visible growth. The assay was done three times to confirm results.

Antagonization assays[13] Compound **26**, compound **35**, or teixobactin, each at 4x or 8x the broth MIC concentration, were mixed with lipid II or undecaprenyl-phosphate (C55-P) in MHII broth (with 0.05% tween-80). Lipid II and C55-P (purified by Tanja Schneider) were added in 2x, or 0.5x molar excess to the test compound. After incubation at rt for 30 min, compound and lipid mixtures were transferred to a round-bottom 96-well plate and exponential *S. aureus* ATCC 29213 added to a final OD600 of 0.001. Plates were incubated overnight at 37 °C, and then examined by eye and by spectrophotometer plate reader to determine growth of *S. aureus*. The assay was done three times for compound **35** and two times for compound **26** to confirm results.

Lipid thin layer chromatography[13] In vitro complex formation of compounds with lipid II or C_{55}-P was analyzed as described. In 50 mM Tris/HCl pH 7.5, 1 nmol of lipid II or C_{55}-P was incubated with 1, 2, 3, or 4 nmol of test compound (**26**, **35** and teixobactin) for 30 min at rt. An equal volume of pyridinium acetate (6 M pH 4.2)/butanol (1:2 v/v) was added, vortexed for 30 s, and centrifuged at high speed for 30 s. The organic phase was collected, and the extraction repeated.

The pooled organic fraction was washed with butanol-saturated water, and then loaded onto a silica TLC plate (Sigma, 02599). TLC was run with a mobile phase of chloroform/methanol/water/ammonia (88:48:10:1 v/v/v/v) and stained with phosphomolybdic acid. The assay was done two times to confirm results.

ITC UDP-MurNAc-L-Ala-D-Glu-meso-Dap-D-Ala-D-Ala (6, Park's nucleotide) was isolated from Bacillus subtilis according to the method of Holtje [36]. The titration was performed using MicroCal ITC200 system (GE Healthcare) at 25 °C. Lipid II analogue was made to 0.375 mM solution with co-solvent (DMSO:H_2O/85:15), compound **26** was prepared with the same co-solvent to 0.025 mM solution. The lipid II analogue was titrated into compound **26** (0.025 mM). The resultant ITC curves were processed using the Origin 7.0 software. The assay was done two times to confirm results.

In vivo study[13]. All animal experiments were done under the guidelines of Laboratory Animal Research Center, Tsinghua University and used an approved animal protocol (16-RY2, PI, Yu Rao). Mice source：Beijing Vital River Laboratory Animal Technology Co., Ltd.. Age: 6-8 weeks. Sex: female. Species: C57BL/6JCnc. Strain of animals: C57BL/6J, The Jackson laboratory (https://www.jax.org/strain/000664). The physical state of compound **26** was amorphous. It was used in the form of trifluoroacetates. Compound **26** and vancomycin were dissolved in a mixture solvent (PBS:Cremophor-EL:DMSO/94:5:1).

1) Compound **26** was tested against *Streptococcus pneumoniae* D39 in a mouse septicemia protection assay. C57BL/6J female mice (6-8 week) were infected with 0.2 mL of bacterial suspension (1×10^4 c.f.u. per mouse, 6 mice per group) via intraperitoneal injection, a concentration that achieves at least 90% mortality within 48 h after infection. At one hour post-infection, female

mice (6 female per group) were treated with compound **26** at single intravenous doses of 5, 2 and 1 mg per kg. Infection control female mice (6 per group) were dosed with vehicle or vancomycin (5 mg per kg). 48 h after infection, female mice in negative group died. The survival rate of all other animals was 100%. The probability was determined by non-parametric log-rank test. It was analyzed by GraphPad Prism 5.01.

2) C57BL/6J female mice were infected with 0.2 mL of *Streptococcus pneumoniae* D39 suspension (5×10^4 c.f.u. per mouse, 4 mice per group) via intraperitoneal injection, at one hour post-infection, female mice (4 per group) were treated with compound **26** at single intravenous doses of 5 and 1 mg per kg. Infection control female mice (4 per group) were dosed with vehicle or vancomycin (5 mg per kg). For all animals, blood colony-forming units (c.f.u.) were determined at 12 h, 18 h, 22 h post-infection. The probability was determined by non-parametric log-rank test. It was analyzed by GraphPad Prism 5.01.

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Author Contributions

Yu Rao and Yu Zong designed the project. Yu Zong, Xiuyun Sun, and Hongying Gao synthesized compounds and performed MIC, ITC, and in vivo studies. Yu Zong, Xiuyun Sun, Hongying Gao and Yu Rao drafted the manuscript. Kirsten Meyer, Kim Lewis performed lipid II antagonization and TLC assays, provided teixobactin, and approved manuscript. ‡These authors contributed equally.

Funding Sources

This work was supported by National Natural Science Foundation of China (# 81573277, 81622042, 81773567), National Major Scientific and Technological Special Project for "Significant New Drugs Development" (# SQ2017ZX095003) and Tsinghua University Initiative Scientific Research Program.

Notes

Any additional relevant notes should be placed here.

ACKNOWLEDGMENT

We thank Professor Jingren Zhang and Jingwen Li from Tsinghua university for providing pathogenic microorganisms. We thank Yang Wang from Tsinghua university to help us to conduct test of blood colony-forming units (c.f.u.). We thank Tanja Schneider from University of Bonn for providing purified lipid II and undecaprenyl-phosphate.

ABBREVIATIONS

DMAP, 4-Dimethylaminopyridine; EDCI, 1-Ethyl-3-(3-dimethyl aminopropyl) carbodiimide; HATU, O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate; HOAt, 1-Hydroxy-7-azabenzotriazole; HPLC High-performance liquid chromatography; SPPS, solid phase peptide synthesis; TFA, Trifluoroacetic acid; TFE, 2,2,2-Trifluoroethanol; THF, Tetrahydrofuran; TIPS, Triisopropylsilane.

Supporting Information The Supporting Information is available free of charge on the ACS Publications website at DOI:

Supplementary data for Lipid thin layer chromatography.

Supplementary data for blood colony-forming units (c.f.u.) in vivo study.

Characterization data for synthetic intermediates and analogues, HPLC, HRMS, LRMS and NMR.

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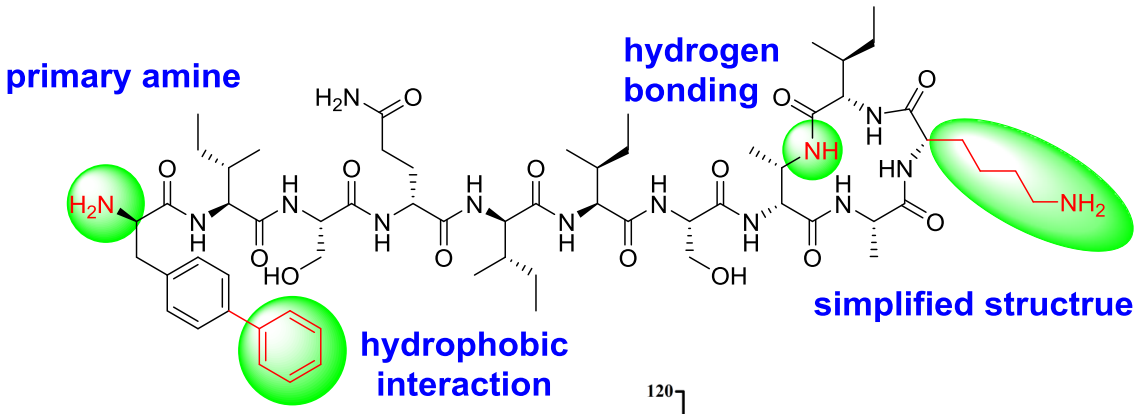

MIC (μg/ml):

S. aureus (MRSA)	0.25
Enterococcus faecalis (VRE)	0.5
Streptococcus pneumoniae	< 0.032
Streptococcus pyogenes	< 0.25