Discovery of Biologically-Optimized Polymyxin Derivatives Facilitated by Peptide Scanning and *in situ* Screening Chemistry

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ABSTRACT

Peptides can be converted to highly active compounds by introducing appropriate substituents on the suitable amino acid residue. Although modifiable residues in peptides can be systematically identified by peptide scanning methodologies, there is no practical method for optimization at the "scanned" position. With the purpose of using derivatives not only for scanning, but also as a starting point for further chemical functionalization, we herein report the 'scanning and direct derivatization' strategy through chemoselective acylation of embedded threonine residues by a serine/threonine ligation (STL) with the help of *in situ* screening chemistry. We have applied this strategy to the optimization of the polymyxin antibiotics, which were selected as a model system to highlight the power of the rapid derivatization of active scanning derivatives. Using this approach, we explored the structure-activity relationships of the polymyxins and successfully prepared derivatives with activity against polymyxin-resistant bacteria and those with *Pseudomonas aeruginosa* selective antibacterial activity. This strategy opens up efficient structural exploration and further optimization of peptide sequences.

1. Introduction

Peptides offer advantages of both small-molecule therapeutics and biologics such as proteins and antibodies, making them an attractive modality for drug discovery.[1-3] Like biologics, peptides have a larger surface area and multiple functional groups that contribute to their binding affinity arising from well-ordered interactions and are amenable to chemical synthesis. These attractive features prompted us to investigate peptides against some challenging targets such as protein-protein interaction (PPI), leading to the development of novel therapeutics targeting cancer, infectious diseases, diabetes, and others.[4-12] Structural modification of peptides is particularly helpful to enhance their biological activities and improve membrane permeability and metabolic stability, which are crucial for drug development.[1,13-16] For example, micafungin, a FDA approved antifungal medicine, was developed through the optimization of echinocandin B, which is a cyclic peptide natural product (Figure 1a).[15,17] Liraglutide, a top-selling anti-diabetic medication drug, is a synthetic derivative of a human glucagon-like peptide-1 (7-37).[16,18] To optimize physicochemical and/or pharmacological properties of the parent peptide, both the substituent placed on the peptide and its position are important. The position should be carefully determined as the newly introduced moiety should not alter the functions of the original peptides and their interactions with target molecules.

However, because the interaction surfaces between peptides and their target molecules are dynamic and broad, even with modern docking or molecular dynamics (MD) simulations, it is a challenging task to predict suitable substituents and modification sites to improve their activities.[19]

As for the determination of the modification site on peptides, a sophisticated methodology has been developed: peptide scanning.[20-36] In this methodology, the systematic substitution of each amino acid in the peptide sequence for a certain scanning unit is used to investigate the importance of the corresponding amino acid residue. Alanine scanning is the most frequently used and classical method among peptide scanning, where the contribution of each amino acid side chain for the activity of the parent peptide can be systematically identified (Figure 1b).[20-34] This adroit approach was followed by the development of other amino acid scanning. For example, by D-scanning, the importance of the orientation of each amino acid side chain can be investigated by systematic incorporation of the corresponding D-amino acid for each amino acid residue.[32-35] *N*-Alkyl amino acid scanning can be helpful to investigate the importance of each amide proton as a hydrogen bond donor.[36] While peptide scanning has enabled the rapid search for potentially modifiable sites in peptides, only a few cases of activity enhancement by structural optimization based on this information have been achieved.[30]

This is because these methods are specialized for identifying the modifiable amino acid residue and elucidating the structural features of the parent peptide, and obtaining potent derivatives requires the arduous process of re-designing and synthesizing several candidates from scratch. For example, in the case of alanine scanning, amino acid residues that decrease or retain activity after alanine modification could be identified, but the derivatives which contain various substituents on the identified site could not be directly prepared due to the chemical inertness of the methyl group of the alanine side chain (Figure 1b). In this respect, peptide scanning methodology has room for improvement, especially to obtain highly active derivatives.

In consideration of the problems described above regarding the synthesis of derivatives using peptide scanning, we have devised ‘scanning and direct derivatization’ strategy, that exploits scanning derivatives with a suitably designed side chain instead of the methyl group used in alanine scanning (Figure 1c, left). By making it possible to introduce various functional moieties directly to the screening derivatives in a chemoselective manner utilizing a reactive site in the side chain, the scanning derivatives could be used not only for scanning amino acid residues but also as a starting point for further chemical derivatization. The strategy is composed of two stages: scanning and direct derivatization. Specifically, in the first step, exploration of the amino acid residues

that could be modified is performed by synthesis and biological activity evaluation of a series of scanning derivatives. In the second step, those scanning derivatives which retain biological activity are subjected to the direct derivatization stage, where various substituents are directly introduced into the reactive site pre-installed in the scanning derivatives to obtain derivatives with the desired properties. As a whole, it is possible to elucidate amino acid residues that can be modified, as well as suitable substituents required for high affinity.

This 'scanning and direct derivatization' strategy should, in principle, allow the synthesis of several hundreds of derivatives, thereby enabling the efficient discovery of highly active derivatives. However, purification of every derivative individually by reverse-phase high-performance liquid chromatography (RP-HPLC), which is generally applied in peptide chemistry, could significantly impair its efficiency.[37-39] To this end, we planned to combine *in situ* high-throughput screening chemistry, in which purification of individual derivatives could be omitted and the reaction products are directly used for the biological evaluations (Figure 1c, right).[40-53] Although this method was originally developed for small molecules, it has been recently applied to peptide derivatives. For example, Wong *et al.* reported the synthesis of dozens of peptide derivatives by amination and evaluation for their HIV protease inhibitory activity.[40] Recently, Heinis *et al.* reported

the synthesis of macrocycles and their screening for thrombin inhibitory activity.[52,53] Combining our strategy with *in situ* high-throughput screening chemistry, it would be possible to elucidate amino acid residues which could be modified, and discover the optimized substituents introduced to achieve desired properties seamlessly. As a specific embodiment, we applied this strategy to polymyxin antibiotics, which is a congener of colistin, an antibacterial drug of last resort.[54-56] It allowed for the synthesis and evaluation of 648 series of polymyxin derivatives to discover structurally-optimized polymyxin derivatives.

2. Results

2.1 Molecular design for the 'scanning and direct derivatization' strategy

Scanning derivatives and accessories used in the 'scanning and direct derivatization' strategy should fulfill the following three requirements. First, the scanning unit pre-installed in the unprotected scanning derivatives must be available as a reactive group in the direct derivatization stage. Second, despite its reactivity, the scanning unit should not inhibit the biological evaluation at the scanning stage. Third, facile access to synthesize the functional group accessories is desirable. Serine/threonine ligation (STL) is a two step transformation to obtain amide compounds from 1,2-aminoalcohols such as

N-terminal serine and threonine, and *C*-terminal salicylaldehyde (SAL) ester via chemoselective oxazolidine formation, followed by acidolysis of the aminal intermediate (Figure 2a).[57,58] Li and co-workers have developed its application to the total synthesis of proteins, natural products such as daptomycin and teixobactin, and the synthesis of diverse peptide structural architectures, which gave rise to expectations of chemoselective structural modification of the scanning derivatives.[59-62] *N*-Terminal serine and threonine are native amino acid residues and would not be expected to interfere with the biological evaluation during the scanning stage. Furthermore, a salicylaldehyde ester can be synthesized in a single step from a corresponding carboxylic acid, one of the most abundant substances, which allows for the synthesis of a variety of derivatives at the direct derivatization stage. These features prompt us to use a threonine as a reactive site on the scanning unit: When investigating the amino acid side chain of the peptide, threonyldiaminobutyric acid (TDA) is used (Figure 2b). This originally devised amino acid residue has an *N*-terminal threonine attached to the peptide backbone, which serves as a reactive site for STL. Compared to the commonly used scanning unit alanine, the amino acid sidechain is larger, which may be suitable for scanning the permissibility of introducing further substituents. We also use *N*-terminal threonine for investigating the possibility of introducing the substituents in the peptide main chain. With such scanning

units, in the scanning stage, synthesis of peptides bearing a TDA or *N*-terminal threonine, and following biological evaluation efficiently explore the convertible position at the next direct derivatization stage. In the direct derivatization stage, various substituents could be introduced with salicylaldehyde ester accessories by STL, and the crude products are then directly subjected to the biological activity evaluation to obtain highly active derivatives.

The emergence of antimicrobial resistance (AMR) is one of the urgent global threats to our health and welfare, and polymyxin antibiotics composed of polymyxin B_1 (**1**) and polymyxin B_2 (**2**) are used as drugs of last resort for treatment of serious infections caused by gram-negative bacteria (Figure 2c).[54-56,63-65] Polymyxin resistance through loss of function mutations to lipopolysaccharide (LPS) have been reported while the emergence of plasmid-mediated colistin (polymyxin E) resistance (*mcr*) was first described in 2015. [66,67] The latter, in particular, poses a serious threat because it can be easily spread to other bacteria by horizontal transmission. To prevent further outbreaks of infection caused by drug-resistant gram-negative bacteria, the development of effective antibacterial agents is urgently needed.[68-70] Several groups including ours have been trying to find polymyxin derivatives that exhibit antibacterial activity against drug-resistant gram-negative bacteria, but their development has not been fruitful because even slight structural modifications significantly reduce activity.[71-77] The antibacterial

spectrum of antibiotics is also important in clinical viewpoint, since a selection of antibiotics with proper antibacterial spectrum is recommended taking into account of the trade-off criteria: a clinical severity and the emergence of drug resistance.[70,78] Therefore, a development of antibiotics with a variety of antibacterial spectrum is needed. Despite a common recognition of these challenges, a robust method has not been developed to address them. Thus, there is no choice to discover derivatives overcoming drug resistance or showing the desired antibacterial spectrum through iterative synthesis and antibacterial activity evaluation of diverse derivatives. Focusing on these challenges in the development of antibacterial derivatives, we aimed to explore two types of polymyxin derivatives using a 'scanning and direct derivatization' strategy. Namely, derivatives that are effective against polymyxin-resistant bacteria and those that have a narrow and broad antibacterial spectrum. Structurally, polymyxin B is composed of two types of peptide fragments: a linear and a cyclic peptide, making it an ideal proving ground to examine our strategy.

2.2 Investigation of an *in situ* screening chemistry with serine/threonine ligation

In general, a reliability of *in situ* screening is perturbed by multiple factors such as reaction yield and byproducts.[46] Especially at a very small scale, the progress of the

reactions sometimes provides unexpected results. The unprotected side chain of peptide as well as the conformation of the peptide affect the intrinsic reactivity at the reaction center impacting the reliability of the reaction. In the case of STL, the chemoselectivity against several functional groups, especially amino groups, in the peptide sequence and yields should be high in the direct derivatization stage. We examined polymyxin derivative **3**, which was developed as an antibacterial agent against *Pseudomonas aeruginosa*, as a synthetic target to investigate the robustness of our nanomole-scale synthesis and *in situ* screening sequence (Figure 3a).[74,79] After preparing 20 mM buffer solutions [pyridine/AcOH = 1/1 (mol/mol)] of salicylaldehyde ester named **SL002** and aminoalcohol named **AM02**, **SL002** (1 equiv., 10 μL, 200 nmol) and a slight excess amount of **AM02** (1.1 equiv., 11 μL, 220 nmol) were added into the buffer (19 μL). After 3 hours of reaction at room temperature, the solvent was removed, and the product was analyzed by LC-MS. The LC-MS analysis suggested that aminoalcohol **AM02** had almost been completely consumed and a single UV peak was detected, showing *m*/*z* corresponding to the oxazolidine **4** (Figure 3b). Next, the product was treated with 50% aqueous TFA solution (50 μL) for 12 h. The solvent was removed under reduced pressure again, and the product was treated with 2% aqueous DMSO (150 μL) at 76 °C for 12 h. After removing the solvent, LC-MS analysis of this crude product, designated

AM02SL002, showed only a single abundant UV peak whose *m*/*z* was consistent with the target product. To confirm that the compound giving this main peak is identical to the compound obtained by *N*-octanoylation of the threonine residue, authentic compound **3** was synthesized by a solid-phase protocol (Figure 3a, see also Supporting Information). Co-elution of the crude **AM02SL002** and **3** confirm that the main component of **AM02SL002** is identical to **3**, which should be obtained by STL. The three-step yield of this conversion was found to be 70% (140 nmol) based on an external standard. To investigate the applicability of the crude product for *in situ* screening, **AM02SL002** was directly subjected to antibacterial activity evaluation (Figure 3c): The addition of 40 μL of DMSO to this crude product mixture can be considered as a stock solution of 5 mM in DMSO, assuming all the above-mentioned three steps have quantitatively proceeded. We found that **AM02SL002** and its pure form **3** showed comparable antibacterial activity against *Escherichia coli* (ATCC 25922, SME98) and *P. aeruginosa*, indicating that low abundant byproducts from STL and reaction yield did not affect *in situ* screening. In a series of experiments, only 39 nmol of crude STL product was consumed for structural determination by LC-MS (15 nmol) and antibacterial evaluation (24 nmol), which means that only 60 μg of **AM02** was required. Thus, with a few milligrams of scanning derivatives, dozens of functionalized crude derivatives can be obtained by derivatization

with STL.

2.3 Scanning stage: Antibacterial activities of 12 series of scanning derivatives against nine bacterial strains

Having established the synthesis using STL and *in situ* investigation of antibacterial activity, we designed a series of scanning derivatives named **AM01**-**AM12** for the discovery of superior antibacterial agents, including **AM02** used in the initial experiment (Figure 4). Scanning derivatives **AM01**-**AM03** are designed to modulate the length of the linear peptide moiety; **AM04**-**AM12** are scanning derivatives with TDA replaced for all amino acid residues except for Dab^4 whose side chain is embedded in the macrocycle. Scanning derivative **AM08** has a TDA unit composed of Thr-D-Dab sequence, considering that polymyxin B has a D-amino acid at this scanning position. The Fmoc solid-phase peptide synthesis of these scanning derivatives was carried out in a parallel manner according to our previously reported method with some modifications.[71] Tailor-made cyclization conditions were identified for each scanning derivative by high-throughput examination using a 96-well microplate (see Supporting Information).[80]

As a scanning stage, the scanning derivatives are subjected to an antibacterial activity assay against nine bacterial strains to identify the amino acid residues that retain

activity (Table 1). The 'ESKAPE' pathogens, where the emergence of bacterial resistance is now a clinical problem, are used in this study. In addition, two clinically threatening polymyxin-resistant strains were also evaluated. The SME98/PORTpmrB34 strain, in which a mutation attributable to the deletion of amino acid residues in PmrB (Δ27-45) was introduced to the *pmrB* gene in the chromosomal DNA of the SME98 strain, and the SME98/plncl2_*mcr-1* strain, in which a plasmid containing *mcr-1* was introduced.[81,82] In both strains, the phosphate moiety of lipopolysaccharide which is a target of polymyxin B is modified by a phosphoethanolamine transferase, resulting in resistance to these antibiotics.[83] Considering the direct derivatization stage which involves *in situ* parallel syntheses, the activity was evaluated at molarity (μM).

In terms of the discovery of the derivatives effective against polymyxin-resistant *E. coli*, we found that six scanning derivatives (**AM04**, **AM05**, **AM06**, **AM10**, **AM11**, and **AM12**) showed antibacterial activity against *E. coli* SME98/plncl2_*mcr-1* strain at 25–50 μM (Table 1). Scanning derivatives **AM04**, **AM06**, **AM10**, and **AM12** also showed antibacterial activity against *E. coli* SME98/PORTpmrB34 strain at similar concentrations. In contrast, other scanning derivatives **AM07**, **AM08**, and **AM09** were devoid of antibacterial activity against our panel of pathogens after introducing TDA units. Based on our hypothesis that derivatization of active scanning derivatives may lead to

compounds exhibiting improved antibacterial activity against the corresponding polymyxin-resistant *E. coli*, we selected **AM05**, **AM10**, and **AM12** for further derivatization (case 1 in section 2.4). Aside from the derivatives effective against polymyxin-resistant *E. coli.*, we also found several scanning derivatives possessed a unique antibacterial spectrum of activity. Namely, **AM02** displayed selective antibacterial activity against *P. aeruginosa* among the nine bacterial species. On the other hand, **AM04**, **AM06**, and **AM10** exhibited a broader antibacterial spectrum with antibacterial activity against *E. coli* including polymyxin-resistant bacteria, as well as *P. aeruginosa* and *Acinetobacter baumannii*. Based on these results, we decided to perform the derivatization on scanning derivatives **AM02** to discover narrow-spectrum derivatives and **AM04**, **AM06**, and **AM10** to discover broad-spectrum derivatives (case 2 in section 2.4).

2.4 Direct derivatization stage

Case 1: Development of derivatives that show antibacterial activity against polymyxin-resistant *Escherichia coli*

First, we pursued the discovery of derivatives effective against polymyxin-resistant *E. coli* with the direct derivatization stage. For the diversification starting from

the scanning derivatives, fifty-four salicylaldehyde ester accessories **SL001**-**SL054** were prepared, including **SL002** used in the initial experiment (Figure 5). These could be synthesized in parallel by a condensation reaction of salicylaldehyde to the corresponding carboxylic acid. Saturated or unsaturated alkyl carboxylic acids (**SL001**-**SL012**, **SL052**, and **SL053**), as well as (hetero)aryl carboxylic acids (**SL013**-**SL039**) with a variety of substituents, amino acids (**SL040**-**SL051**), and dicarboxylic acid (**SL054**) are converted to the corresponding salicylaldehyde esters. Salicylaldehyde esters **SL037** and **SL038** have a dimethyl acetal moiety, and deprotection proceeds simultaneously in the acidolysis step of STL, producing aldehydes and volatile methanol. In the case of **SL044** and **SL047**, the Boc groups would be removed, and in **SL048** and **SL049**, the ^{t}Bu group would be removed as well under the conditions. In general, salicylaldehyde esters with lysine or glutamic acid at the *C*-terminal cannot be synthesized due to their instability, but in this study, these ligations were made possible by using a protecting group that leaves no-byproduct during the deprotection.[84]

The aforementioned three scanning derivatives (**AM05**, **AM10**, and **AM12**) and fifty-four SAL esters (**SL001**-**SL054**) were subjected to the STL. As a control, **AM07**, **AM08**, and **AM09**, all of which showed no antibacterial activity against polymyxin-resistant bacteria, were also subjected to synthesize 324 (6 × 54) polymyxin derivatives.

The crude materials obtained from the STL of AMpp and SLqqq (where p and q are arbitrary numbers) are hereinafter referred to as AMppSLqqq. The scale and conditions of the reaction were the same as in the initial experiment (section 2.2), and the library was constructed in a parallel manner using a 96-well microplate as a reaction vessel. Namely, a buffer solution of scanning derivatives and salicylaldehyde esters were mixed in each well, which was then subjected to lyophilization, acid treatment, and solubilization in DMSO to prepare a 5 mM DMSO solution of each STL product (Figure 6). The purity of all samples was assessed by LC-MS (Figure 7a). Based on the UV purity from the LC chromatograms, 73.5% (238 derivatives) of the total samples were found to have UV purity greater than 50%. Only 1.5% (5 derivatives) of the total samples had UV purity of less than 5%, and all of these were a combination of adamantylsalicylaldehyde ester (**SL010**) and scanning derivatives. In all cases where the UV purity was less than 5%, the main peak was identified as the corresponding unreacted scanning derivatives (**AM05**, **AM07**, **AM08**, **AM09**, and **AM12**), suggesting that the oxazolidine formation step in STL did not proceed because of the prominent bulkiness of the adamantyl group of **SL010**. The observed remarkably high conversion and wide substance scope of STL except for **SL010** demonstrate that this reaction can provide a large number of derivatives at sufficient purity in the direct derivatization stage.

The 324 derivatives were screened for antibacterial activity against polymyxin-resistant *E. coli*. Each derivative was evaluated at 12, 3, and 1 μM, based on the amount of salicylaldehydes (Figure 7b). When evaluating against *E. coli* SME98/plncl2_*mcr-1* strain, polymyxin B showed antibacterial activity at 3 μM. As a reference, the scanning derivatives (**AM05**, **AM07**, **AM08**, **AM09**, **AM10**, and **AM12**) and salicylaldehyde esters (**SL001**-**SL054**) were subjected to STL conditions without ligation partners, and the resulting crude products did not show antibacterial activity at highest concentration evaluated (12 μM). By contrast, 15 derivatives obtained by the derivatization showed antibacterial activity at 3 μM, the same concentration as polymyxin B. More than or equal to two combinations from each of the three scanning derivatives (**AM05**, **AM10**, **AM12**) showed antibacterial activity below 3 μM, in particular, nine combinations were found from **AM05**. In terms of the structure-activity relationship of salicylaldehyde esters, we found that those with hydrophobic functional groups, such as lauroyl (in **SL003**), 4-octylbenzoyl (in **SL018**), biphenyl (in **SL020**, **SL021**), and 4-*tert*-butylphenyl (in **SL039**) groups, tend to give active compounds. Based on the screening results, the hit compounds were synthesized by a standard solid-phase manner to conduct antibacterial assay with purified compounds: Antibacterial assay of authentic **5**, **6**, and **7**, the main components of **AM05SL018**, **AM05SL020**, **AM05SL039**, revealed that they exhibit antibacterial

activity against *E. coli* SME98/plncl2_*mcr-1* strain at 1.56–3.13, 1.56, and 1.56–3.13 μM, respectively, which is 1- to 4-fold stronger activity than that of polymyxin B (Figure 7c). Next, the 324 compounds obtained by the direct derivatization stage were evaluated for antibacterial activity against the *E. coli* SME98/PORTpmrB34 strain, and nine derivatives were found to exhibit activity at 3 μM, the same concentration as polymyxin B (see Supporting Information). Most of these derivatives also showed antibacterial activity against *E. coli* SME98/plncl2_*mcr-1* strain at 3 μM, but **AM10SL021** was the sole one among 324 compounds that showed antibacterial activity only against SME98/PORTpmrB34 strain at 3 μM. Authentic **8** of the main component of **AM10SL021** showed antibacterial activity comparable to that of polymyxins (Figure 7c).

Case 2: Development of derivatives that show a narrow or broad antibacterial spectrum

Based on the successful acquisition of derivatives effective against polymyxin-resistant bacteria, we further pursued the discovery of narrow- and broad-spectrum derivatives utilizing the direct derivatization stage. The aforementioned four scanning derivatives (**AM02**, **AM04**, **AM06**, and **AM10**), which showed a narrow or broad antibacterial spectrum, were ligated with 54 salicylaldehyde esters (**SL001**-**SL054**). As a

control, scanning derivatives (**AM01**, **AM03** and **AM11**) were also subjected to afford 378 (7 × 54) derivatives, which were evaluated for antibacterial activity against nine strains of bacteria. To discuss the antibacterial spectrum semi-quantitatively, we defined it based on how many bacterial species out of nine showed antibacterial activity below 3 μM. It was found that 23 out of the 54 derivatives obtained by the derivatization from **AM02** had a narrow antibacterial spectrum effective against only one strain, consistent with the antibacterial activity of **AM02** (Figure 8a). In addition, 10 of them showed antibacterial activity only against *P. aeruginosa* at 3 μM, and no antibacterial activity against the other eight bacterial strains at 12 μM, the highest concentration in this screening. As expected, authentic **9**, the main component of **AM02SL042**, exhibited antibacterial activity against *P. aeruginosa* at 3.13 μM, while it showed no antibacterial activity against other bacteria below 10 μM, especially against *E. coli* ATCC 25922, displaying 128- to 32-fold weaker antibacterial activity than that of polymyxin B (Figure 8b). In addition, **10**, and **11**, which are the main component of **AM02SL041** and **AM02SL044**, possessed strong antibacterial activity against *P. aeruginosa*. It should be noted that although compound **10** and **11** were active against polymyxin-susceptible *E. coli*, the activity was at least four-fold weaker than that against *P. aeruginosa*. Contrary to **9**-**11**, compound **12**, the main component of **AM10SL018**, which showed antibacterial

activity against nine strains (Fig. 8a), displayed four times stronger antibacterial activity than polymyxin B against *E. coli* SME98/PORTpmrB34 strain (Figure 8b). Moreover, it showed antibacterial activity against two polymyxin-susceptible *E. coli* strains, *P. aeruginosa*, *A. baumannii*, and even against gram-positive bacteria which is generally less susceptible to polymyxin B. As a whole, we have succeeded in developing derivatives with a selective antibacterial activity or an expanded antibacterial spectrum of activity.

3. Discussion

The combination of a 'scanning and direct derivatization' strategy and *in situ* screening chemistry enables seamless synthesis and evaluation of hundreds of derivatives. The strength of this strategy is well demonstrated by the discovery of two types of polymyxin derivatives: one effective against polymyxin-resistant bacteria and the other that has either an ultra-narrow or broader antibacterial spectrum of activity. In the case of the former study, scanning derivatives **AM05** and **AM10**, corresponding to **5**, **6**, **7**, and **8**, showed antibacterial activity at least four-fold weaker than that of polymyxins, however, the activity of the scanning derivatives could be successfully improved in the direct derivatization stage. These results indicate that acquisition of even slight biological activity in the scanning stage is sufficient since activity can be significantly improved

during the direct derivatization stage. In contrast, among the 162 derivatives obtained from the control scanning derivatives (**AM07**, **AM08**, and **AM09**), only two derivatives showed antibacterial activity at 3 μM, the same concentration as polymyxin B. These results are consistent with the scanning results, indicating that the scanning stage works well to identify the modifiable amino acid residue. It is of interest to understand the mode of **6** against polymyxin-resistant bacteria. Molecular dynamics simulations of **6** indicate that the installation of a biphenyl moiety would play two important roles: strengthen the affinity to the membrane and accelerate the membrane penetration (for details, see Supporting Information). The logical design of molecules with a desired dynamics are not trivial; our strategy worked well even for acquiring such molecules because it allows the scanning of the peptide and identification of optimal substituents efficiently. The benefit of our strategy was also highlighted in the case 2 study. In this case, we obtained a derivative showing superior antibacterial activity to polymyxin against some strains. For example, compound **12** exhibits 64- to 32-fold and at least 16-fold stronger activity against *S. aureus* and *Enterobacter cloacae*, respectively. Although the origin of the activity enhancement remains unclear, the result demonstrates the usefulness of our strategy to improve the biological activity of a parent peptide. Moreover, the unique antibacterial spectrum of the scanning derivatives **AM02** and **AM10** were augmented to

discover **9**, **10**, **11**, and **12**. It is reported that antibacterial susceptibility would be varied by the component and composition of the bacterial membrane.[85,86] In our case, these deviations could determine the antibacterial spectrum of activity.

To the best of our knowledge, the STL used in our research has not been applied to *in situ* screening chemistry so far. The LC-MS analysis revealed that 71.6% (464 derivatives) of the 648 derivatives (case 1 + case 2) had a UV purity of >50%. This conversion rates of STL and purity of crude products are considered sufficient to identify hit compounds, since the activity of the crude STL product and those of purified products **5**, **6**, **7**, and **8** were comparable, albeit with some variation in values of MICs (case 1). The high conversion rate, wide substrate scope, and sufficient purity of a crude product of STL enabled the synthesis and biological activity evaluation of >600 derivatives, including hundreds of control derivatives. It is also important to note that although 324 derivatives were screened against two series of polymyxin-resistant bacteria, only 16 derivatives showed antibacterial activity comparable or superior to polymyxin B. This highlights the significance of using the 'scanning and direct derivatization' strategy to efficiently develop active compounds, as it is a daunting process to synthesize derivatives one by one for achieving compounds active against low hit-rate targets.

In the two studies described above, the potential biological activity of the

scanning derivatives found in the scanning stage was successfully exploited in the direct derivatization stage. This suggests that the aminoalcohol moiety of the scanning derivatives itself had a capability of accepting modifications to improve their activity. Of the 12 scanning derivatives, the scanning with **AM04**-**AM12** can be regarded as an augmented scanning technique with TDA instead of those with alanine. Therefore, we compared the antibacterial activity of **AM04**-**AM12** with those of reported alanine scanning to unveil the characteristic of the 'TDA scanning'. As shown in Table 1, scanning derivatives **AM04**, **AM06**, **AM09**, and **AM10** showed antibacterial activities against two polymyxin-sensitive *E. coli* (ATCC 25922 and SME98) with minimum inhibitory concentration (MIC) values of below 10 μM [= 12.9 μg/mL for **AM04**, **AM06**, and **AM10** (MWs 1290.58); = 12.8 μg/mL for **AM09** (MW 1277.54)]. Alanine scanning against *E. coli* IFO12734 was reported by Sakura *et al.* and indicated that changing the amino acid residues Dab^{1}, Dab^{3}, D-Phe^{6}, Leu^{7}, and Thr^{10} to alanine did not significantly impair the activity (see also Supporting Information).[87] These results show, for example, that in both cases where Dab^{1} was changed to alanine or TDA, the antibacterial activities are similar to that of polymyxin B [**AM04**: 1.56−3.13 μM (2.02−4.03 μg/mL, MW 1290.58) against *E. coli* ATCC 25922; **[Ala^{1}]-PMB_3**: 2 μg/mL against *E. coli* IFO12734]. Similarly, alanine scanning and TDA scanning showed consistent results in terms of

maintaining original activity when Dab[3] and Leu[7] were converted. By contrast, the substitution of some amino acid residues showed differences in antibacterial activity depending on whether they were converted to alanine or TDA. For example, **AM08**, where the D-Phe[6] is replaced with TDA, showed no antibacterial activity against two strains of polymyxin-susceptible *E. coli* even at 50 μM (62.2 μg/mL, MW 1243.52), and the result was distinct from Sakura's report that replacing D-Phe[6] to D-alanine did not affect antibacterial activity against *E. coli* IFO 12734.[87] Against *P. aeruginosa* ATCC 27853 strain, only **AM04**, **AM06**, and **AM10** showed comparable antibacterial activity with polymyxin B. However, in the alanine scanning conducted by Sakura's group, all of the amino acid residue (Dab[1]-Dab[3], Dab[5]-Thr[10]) could be converted to alanine without significant loss of antibacterial activity.[87] In some cases, TDA scanning gave stronger scanning derivatives. For example, Li *et al.* reported that **[Ala[8]]-PMB3** showed antibacterial activity at 16 μg/mL against the *A. baumannii* ATCC 19606 strain.[88] However, TDA scanning revealed that **AM10** exhibited antibacterial activity at 1.56−3.13 μM (2.02−4.03 μg/mL, MW 1290.58) against the strain. These deviations arise from the essential difference between alanine scanning and TDA scanning. When alanine scanning is applied to the native amino acid residues except for glycine, the amino acid's side chains become sterically smaller, and the polar functional groups are completely removed.

Therefore, alanine scanning could examine whether the loss of the side chain has any adverse effect on biological activity (Figure 9a). On the other hand, in TDA scanning, the side chain of the TDA unit is larger than those of all-natural amino acids, and aminoalcohol moieties derived from threonine are also introduced. Therefore, TDA scanning could investigate the influence of the introduction of large substituents and polar functional groups at the amino acid residue (Figure 9a). The deviation between TDA scanning and alanine scanning was also outstanding in the interpretation of the structural modification on Dab^8. Substitution of Dab^8 for alanine has been reported to diminish the antibacterial activity against *A. baumannii*, hence the Dab^8 has not been considered as a viable modification site. On the contrary, in the case of our TDA scanning, **AM10** retained antibacterial activity against the bacteria, indicating the possibility of Dab^8 modification. The hypothesis was demonstrated by the results showing that dansylated derivative **13** and biotinylated derivative **14** retained their antibacterial activity (Figure 9b). These derivatives could be used for elucidating the detailed antibacterial mechanism of **8** and **12**. Thus, TDA scanning can be considered as a new option for the identification of modifiable amino acid residues, especially for improving activity. This method works well, particularly when identifying positions for introducing large and/or polar affinity tags like biotin and fluorophores, and provides the opportunity to modify even amino acid

residues deemed ‘essential’ by the result of alanine scanning. Furthermore, by combining this scanning with *in situ* screening, rapid structural optimization could be achieved.

4. Conclusions

In summary, we have developed a ‘scanning and direct derivatization’ strategy as a new option for facilitating peptide-based medicinal chemistry to improve biological activity. With this strategy, it is possible to elucidate amino acid residues that can be modified, as well as suitable substituents required for the improvement of their activity. Peptides have higher potentials to modulate protein-protein interactions and multi-body interactions between lipid chains on lipid rafts, which are difficult to target with small molecules. The development of highly potent derivatives and chemical probes are earnestly needed utilize this class of molecule as novel therapeutics and for the understanding of biological event. In contrast, peptides often show significant loss of biological activity with slight structural modification, and the time-consuming and trial-and-error structural optimization process is often problematic. The ‘scanning and direct derivatization’ strategy developed here can be applied not only to natural linear and cyclic peptides, but also to non-natural peptide sequences developed *in-house*. It may be considered widely applicable as a general strategy for amino acid scanning and further

optimization of peptide sequences.

Figure 1. Structural modification of peptide for the development of highly active derivatives. a Representative drug developments through the amino acid modification of parent peptides. **b** Overview of alanine scanning. Systematic search for potentially modifiable amino acid residue provides the information for the modification of peptides to improve their activity. **c** Overview of 'scanning and direct derivatization' strategy. A suitable scanning unit enables identification of modifiable amino acid residues, as well as subsequent installation of substituents to improve the activity. Combined with an *in situ* screening chemistry, several hundreds of syntheses and evaluation of derivatives can be performed in the direct derivatization stage.

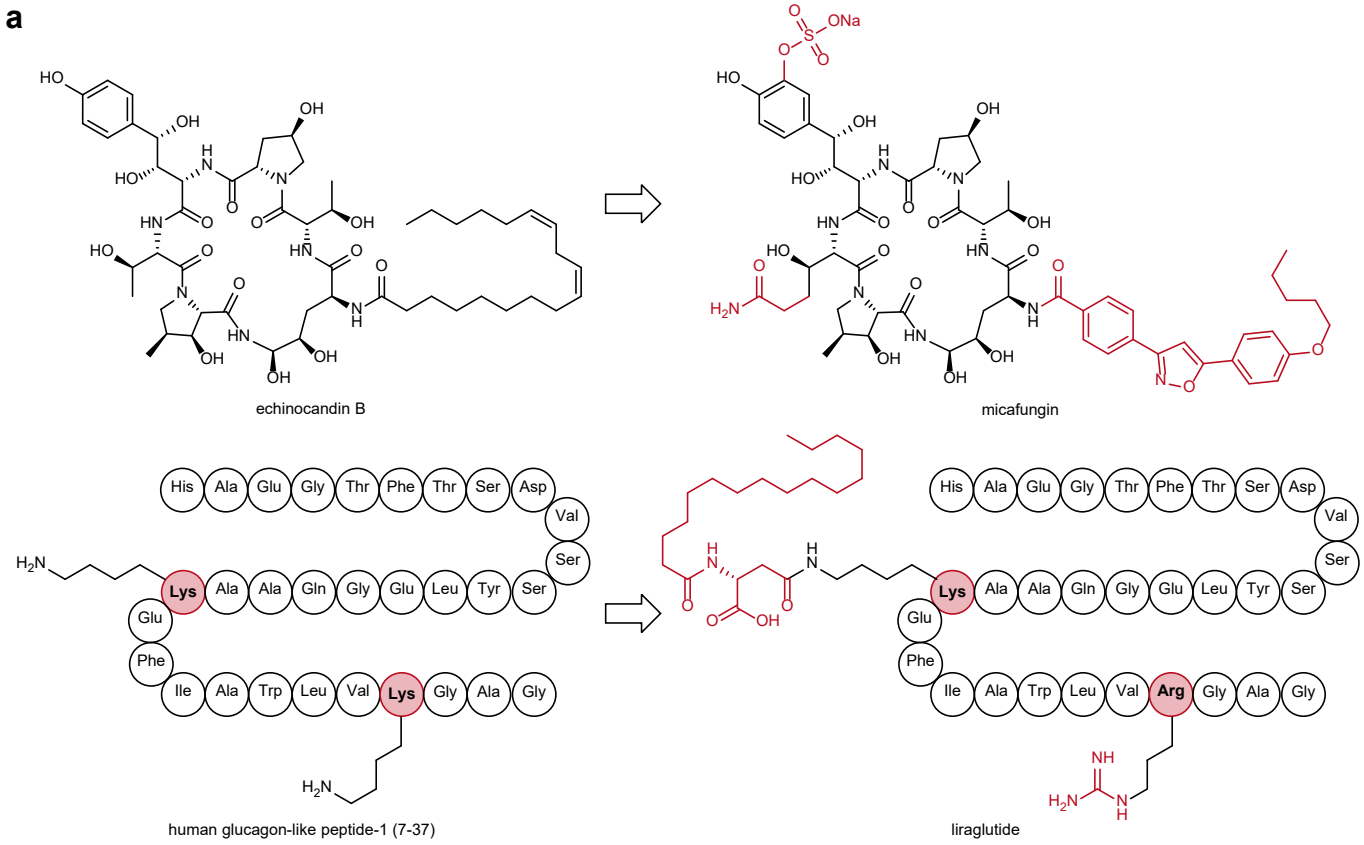

a
echinocandin B
micafungin
human glucagon-like peptide-1 (7-37)
liraglutide
b
alanine scanning
activity: −
activity: +
activity: +
additional function
activity: ++
c
scanning
activity: −
activity: +
derivatization
step 2
activity: +
additional function
activity: ++
'scanning and direct derivatization' strategy
✓ chemoselective functionalization on the scanning derivatives
✓ straightforward access for the structural diversification
in situ screening chemistry
✓ omitted purification process
✓ facile access to the desired properties

Figure 2. ‘Scanning and direct derivatization’ strategy applied to the polymyxin antibiotics. a General scheme of a serine/threonine ligation (STL) developed by Li. **b** ‘Scanning and direct derivatization’ strategy using STL. The scanning unit, which possesses an aminoalcohol moiety, could be used for the identification of modifiable amino acid residues and derivatization at the position by a chemoselective acylation with STL. **c** Chemical structure of polymyxin antibiotics.

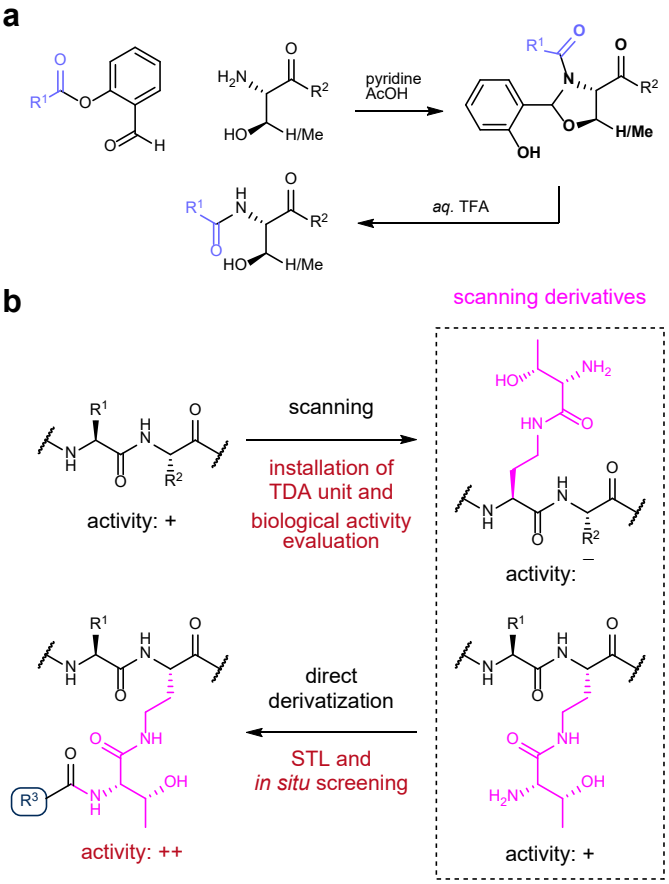
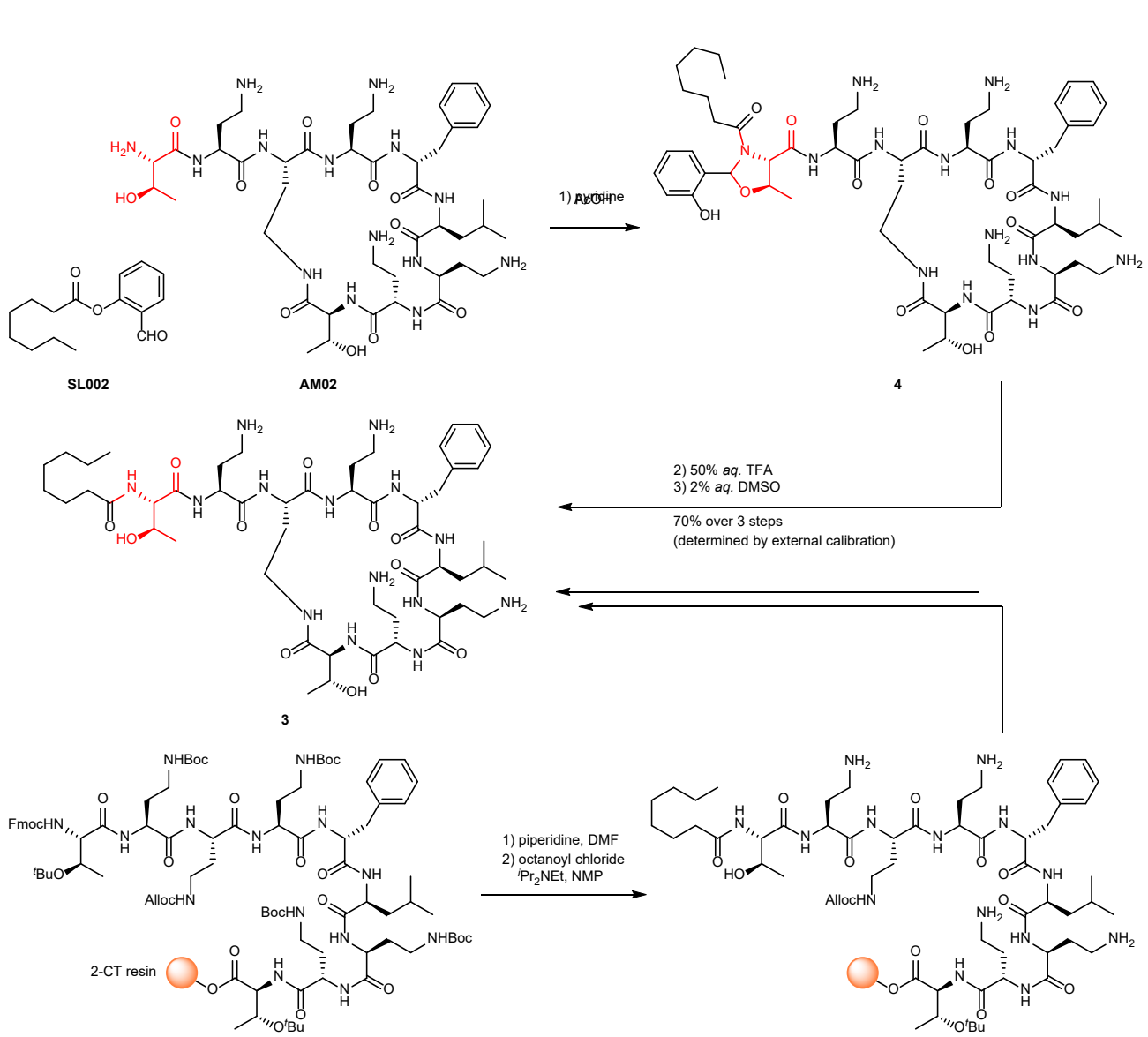
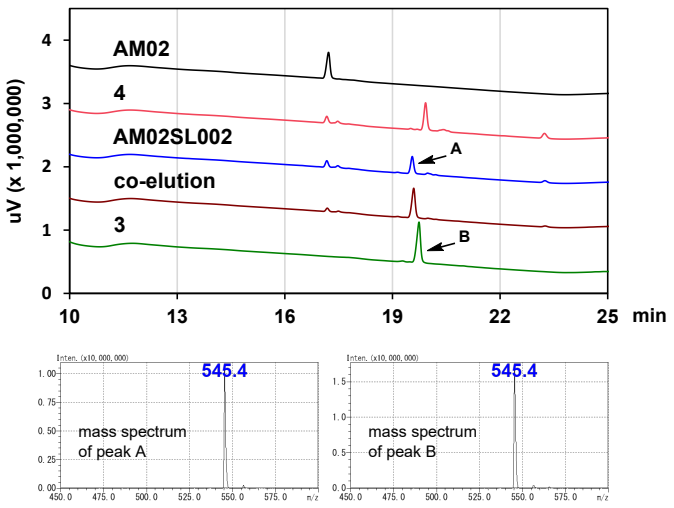

a
pyridine
AcOH
aq. TFA
H/Me
b
scanning derivatives
scanning
installation of
TDA unit and
biological activity
evaluation
activity: +
activity:
direct
derivatization
STL and
in situ screening
activity: ++
activity: +
c
L-Dab1
L-Dab3
L-Dab5
D-Phe6
L-Leu7
L-Thr2
L-Dab4
L-Dab8
L-Thr10
L-Dab9
polymyxin B1 (1): R1 =
polymyxin B2 (2): R1 =

Figure 3. Initial investigation of the *in situ* screening using STL. a Reaction scheme of the initial experiment. **b** Analytical LC-MS chromatogram of the reactions. Co-elution of **AM02SL002** and authentic **3** shows these are identical compounds. Mass spectrums at these peaks gave same the *m*/*z* values (observed: 545.4 and 545.4, respectively; calculated: 545.34 as $[M+2H]^{2+}$). See also Supporting Information. **c** The results of *in situ* screening. Crude ligation product **AM02SL002** and authentic **3** showed comparable antibacterial activity. The minimum inhibitory concentrations (MICs) were determined by performing the same experiment at least three times.

a



b

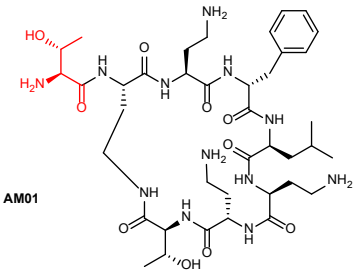


c

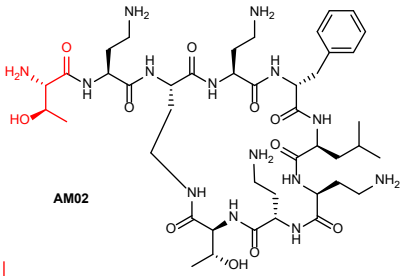
compounds	antibacterial activity MIC (μM)			
	E. coli ATCC 25922	*E. coli* SME98	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853
AM02	>12	>12	>12	12–>12
SL002	>12	>12	>12	>12
AM02SL002	0.38–0.75	0.38–0.75	>12	1.5–3
3	0.38–0.75	0.38–0.75	12–>12	1.5
colistin	0.19–0.38	0.09	0.38	0.38
polymyxin B	0.19–0.38	0.19	0.38	0.38

weak activity — potent activity

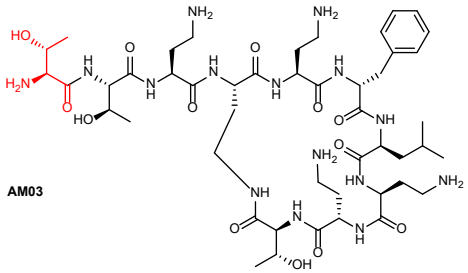
Figure 4. Design of the scanning derivatives. Scanning derivatives **AM01**-**AM03** are designed to modulate the length of the linear peptide moiety. **AM04**-**AM12** have a TDA, which can be used for scanning a permissibility of introducing substituents on the side chain of the peptide.



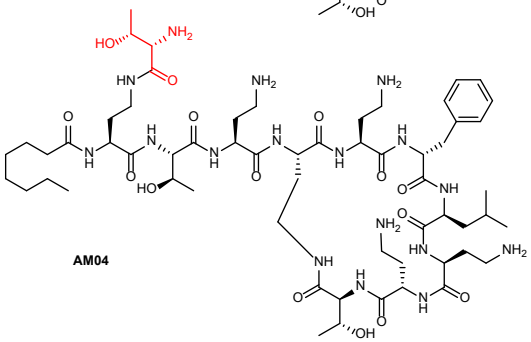
AM01



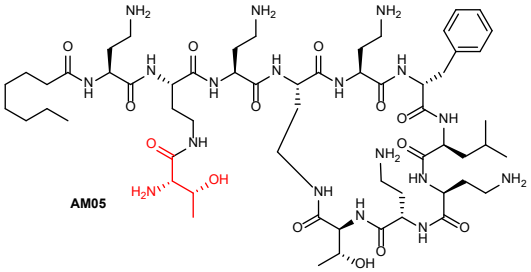
AM02



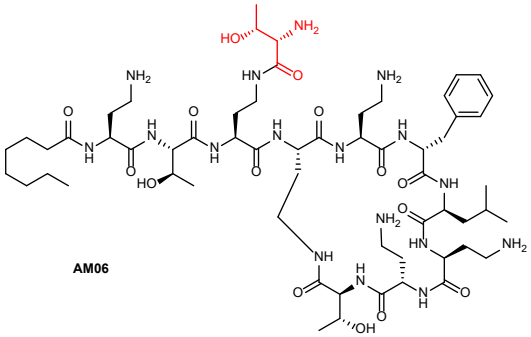
AM03



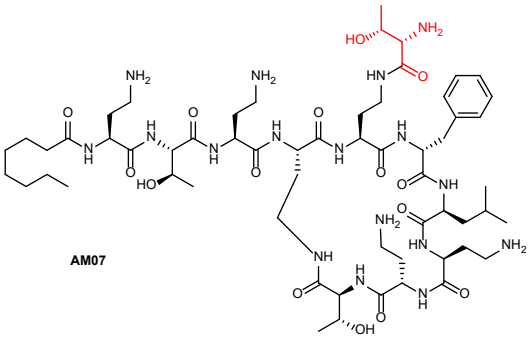
AM04



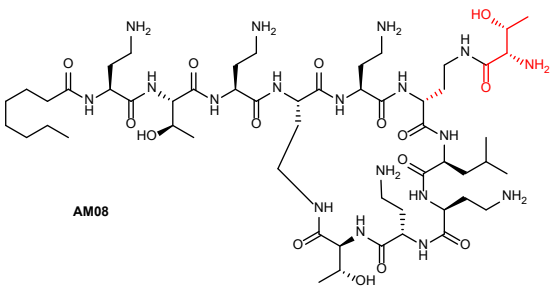
AM05



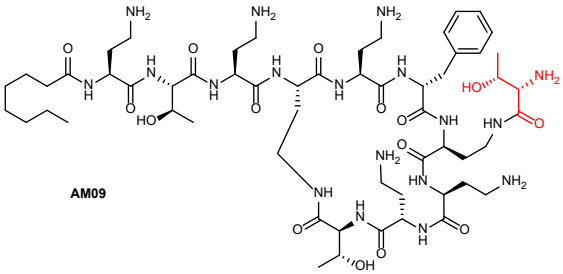
AM06



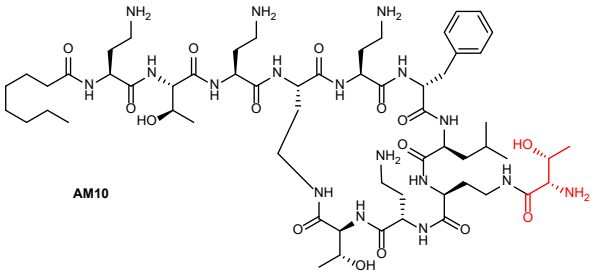
AM07



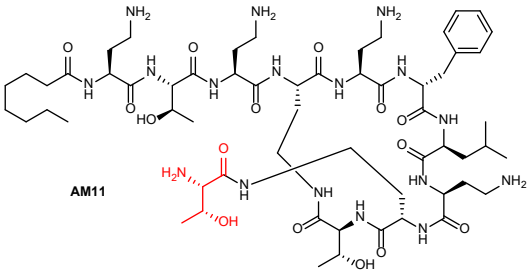
AM08



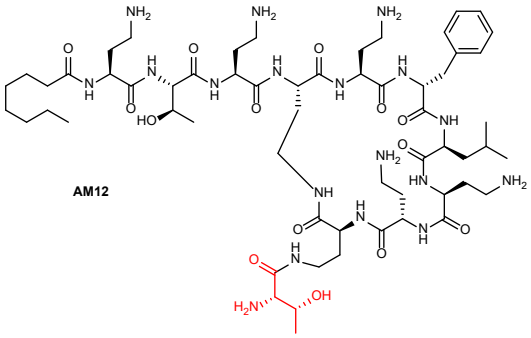
AM09



AM10



AM11



AM12

Table 1. Scanning stage of polymyxin antibiotics: Antibacterial activity of scanning derivatives. The minimum inhibitory concentrations (MICs) were determined by performing the same experiment at least three times. During the test of **AM02** against *P. aeruginosa* ATCC 27853, skipped well was observed (growth inhibition was seen at 3.13–6.25 μM, but regrew at 12.5 μM); during the test of **AM03** against *P. aeruginosa* ATCC 27853, skipped well was observed (growth inhibition was seen at 3.13–12.5 μM, but regrew at ≥ 25 μM). See also Supporting Information.

compounds	antibacterial activity MIC (μM)								
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853	*E. cloacae* ATCC 13047	*E. faecium* ATCC 35667	*E. coli* SME98	*E. coli* SME98 /pIncI2_*mcr-1*	*E. coli* SME98 /PORTpmrB34
AM01	>50	>50	>50	>50	>50	>50	>50	>50	>50
AM02	>50	>50	>50	25, 3.13	>50	>50	>50	>50	>50
AM03	>50	>50	>50	>50, 3.13	>50	>50	>50	>50	>50
AM04	1.56–3.13	>50	12.5–25	1.56–3.13	>50	>50	1.56	25–50	50–>50
AM05	50–>50	>50	>50	50	>50	>50	50	50	>50
AM06	0.39–0.78	>50	3.13	1.56–3.13	>50	>50	0.20–0.39	25–50	50
AM07	12.5–25	>50	>50	50	>50	>50	12.5–25	>50	>50
AM08	>50	>50	>50	>50	>50	>50	>50	>50	>50
AM09	3.13	>50	>50	>50	>50	>50	3.13–6.25	>50	>50
AM10	0.39–0.78	>50	1.56–3.13	1.56	>50	>50	0.39	25	25–50
AM11	25	>50	25–50	50–>50	>50	>50	12.5	50	>50
AM12	12.5	>50	>50	12.5	>50	>50	12.5	25–50	50
colistin	0.20–0.39	>50	0.39–0.78	0.78	>50	>50	0.05–0.10	3.13–6.25	6.25–12.5
polymyxin B	0.20–0.39	50	0.20–0.39	0.39–0.78	>50	>50	0.20–0.39	3.13–6.25	6.25

Figure 5. Salicylaldehyde esters used in this study. During the ligation, dimethylacetal moieties (**SL037** and **SL038**), Boc groups (**SL041**, **SL043**, **SL044**, **SL045**, **SL047**, **SL049**, and **SL051**), and ^{t}Bu groups (**SL048** and **SL049**) are deprotected to afford aldehyde, amine, and carboxylic acid, respectively.

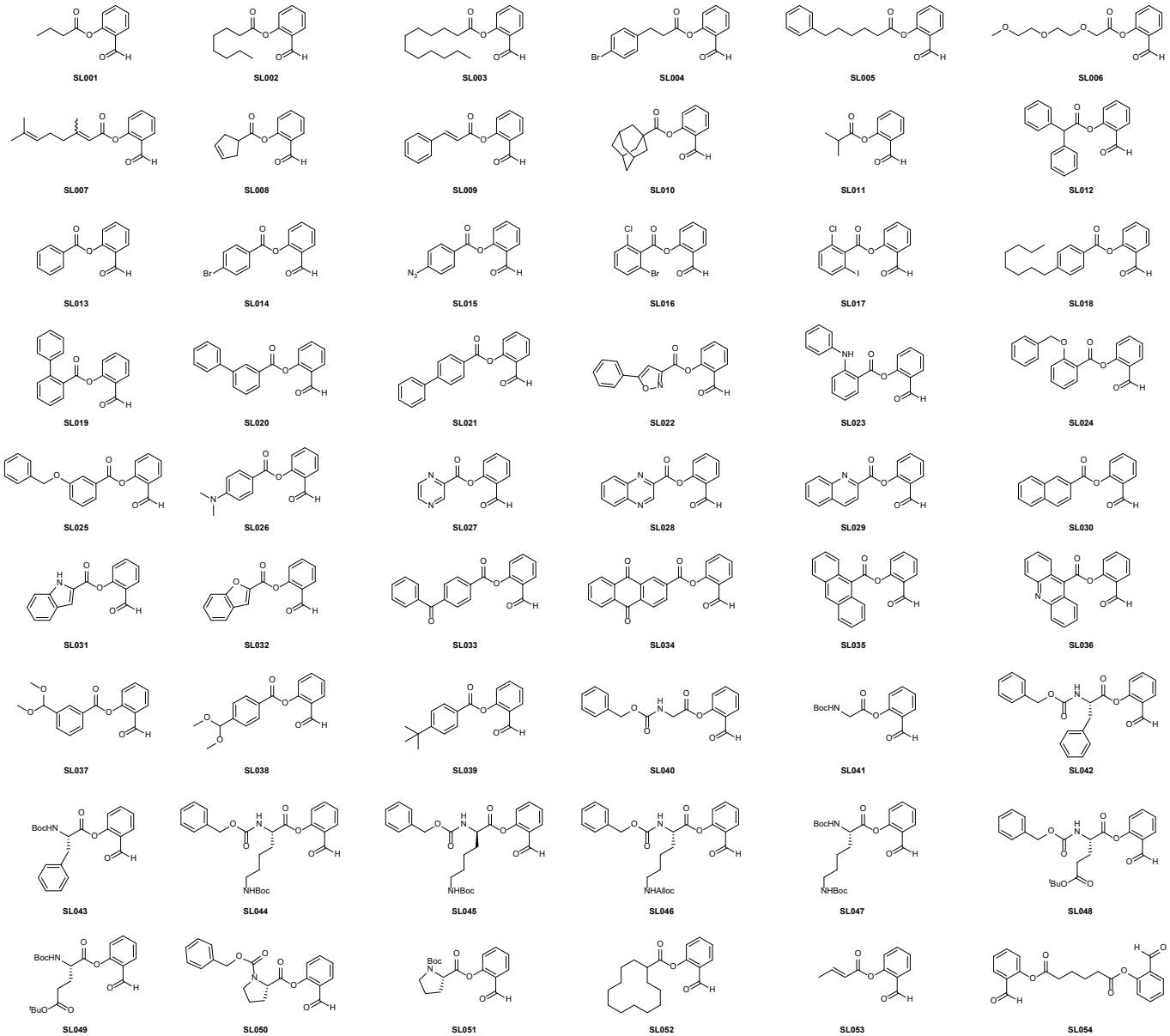


Figure 6. Overall workflow for the preparation of derivatives on 96-well microplate. Each solution of scanning derivatives or SAL esters in pyridine/AcOH buffer was mixed on the 96-well microplate, which was subjected to the following sequence (including removal of solvents and acidolysis) to prepare STL products. The resulting crude product was diluted with DMSO to prepare a 5 mM stock solution, assuming all reactions are proceeded quantitatively. See also Supporting Information for details.

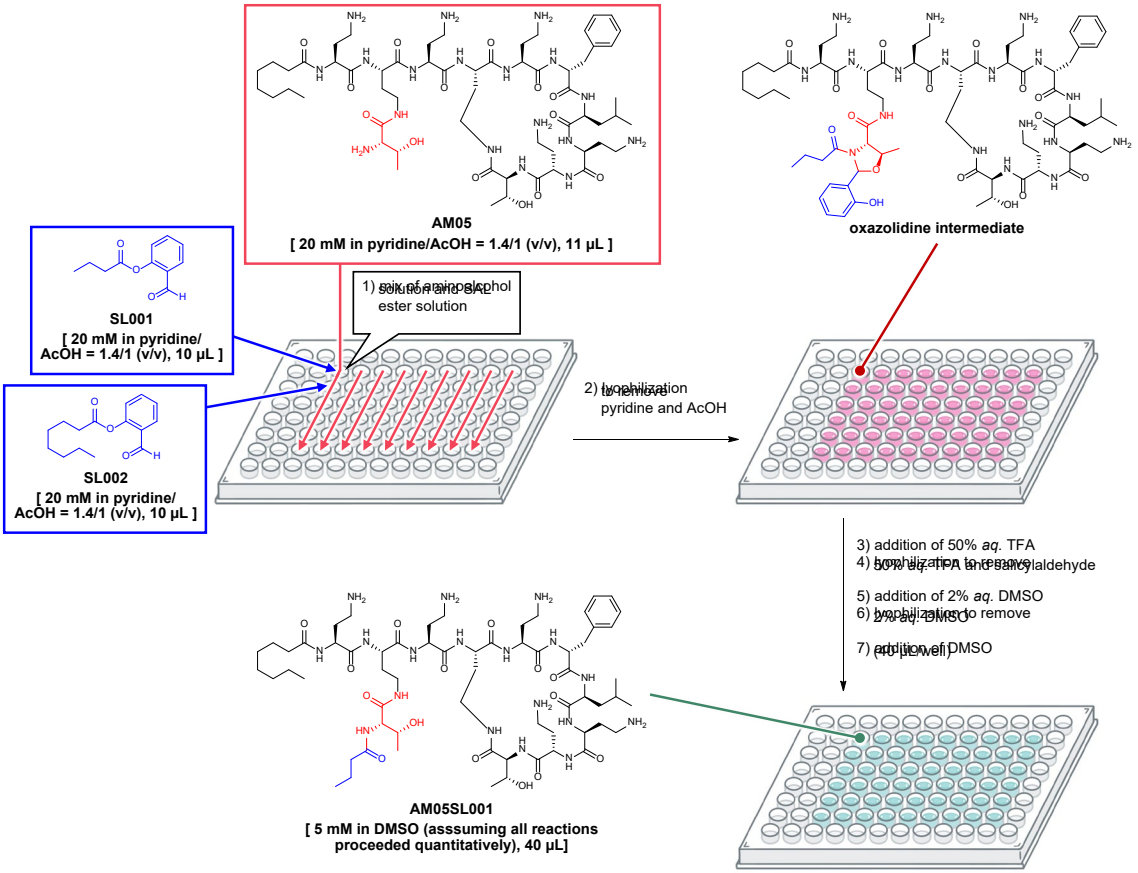
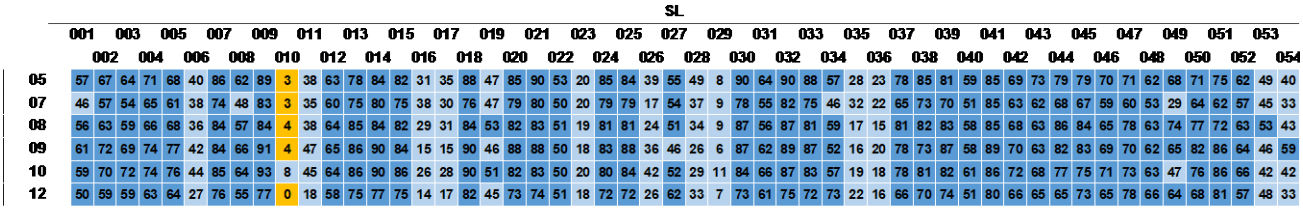
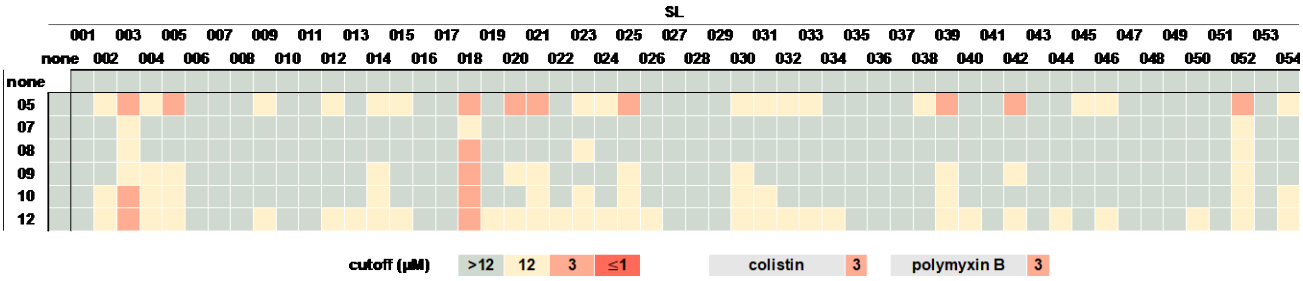

AM05
[20 mM in pyridine/AcOH = 1.4/1 (v/v), 11 µL]
oxazolidine intermediate
SL001
[20 mM in pyridine/
AcOH = 1.4/1 (v/v), 10 µL]
SL002
[20 mM in pyridine/
AcOH = 1.4/1 (v/v), 10 µL]
1) mix of aminoalcohol
ester solution
2) lyophilization
pyridine and AcOH
3) addition of 50% aq. TFA
5) addition of 2% aq. DMSO
6) lyophilization to remove
7) addition of DMSO
AM05SL001
[5 mM in DMSO (asssuming all reactions
proceeded quantitatively), 40 µL]

Figure 7. Direct derivatization stage to discover derivatives that show antibacterial activity against polymyxin-resistant *E. coli*. **a** Analysis of purity of the 324 series of polymyxin derivatives by the LC-MS. The purity of each compound was defined as follows: First, the integrals of all peaks during the gradient elution on UV chromatograms (210 nm) were calculated. A peak showing *m*/*z* corresponding to the ligation product was then identified by mass chromatogram, and the ratio of the integral out of all integrals was defined as purity. See also Supporting Information. **b** Antibacterial activity screening of 324 series of polymyxin derivatives against *E. coli* SME98/plncl2_*mcr-1*. **c** Synthesis and antibacterial activity evaluation of hit compounds. The minimum inhibitory concentrations (MICs) were determined by performing the same experiment at least three times.

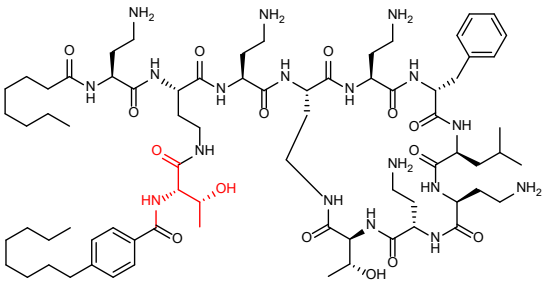
a



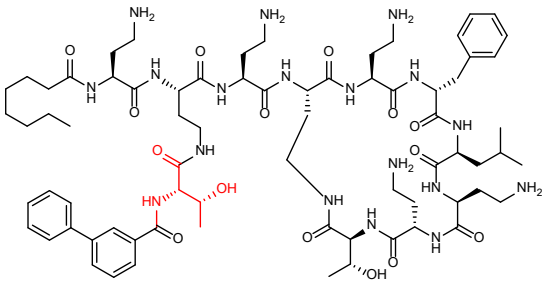
b



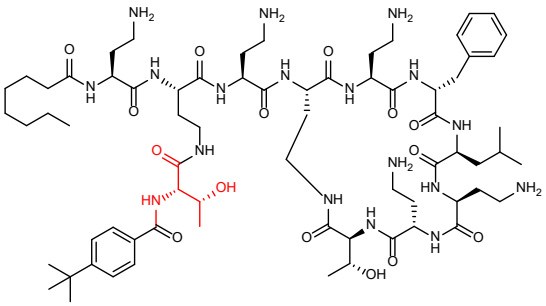
c



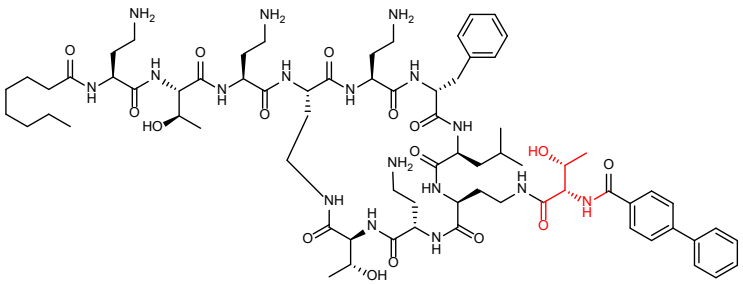
5 (main component of AM05SL018)



6 (main component of AM05SL020)



7 (main component of AM05SL039)



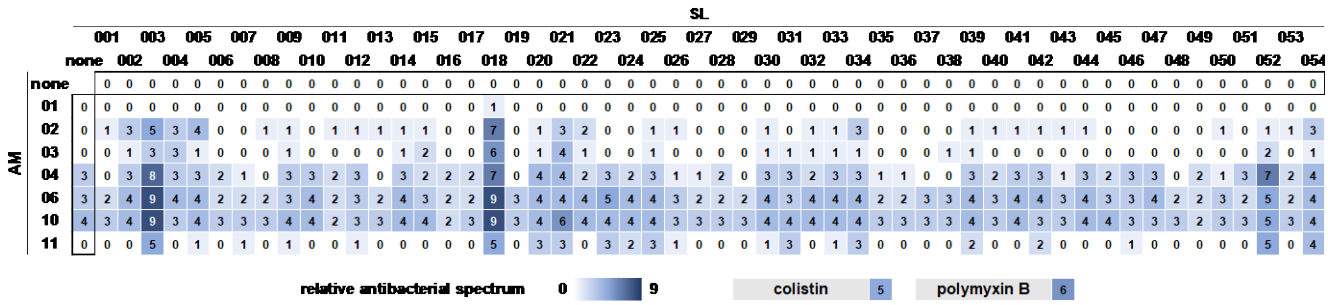
8 (main component of AM10SL021)

compounds	antibacterial activity MIC (μM)		compounds	antibacterial activity MIC (μM)	
	E. coli SME98 /pIncI2_*mcr-1*	*E. coli* SME98 /PORTpmrB34		*E. coli* SME98 /pIncI2_*mcr-1*	*E. coli* SME98 /PORTpmrB34
AM05	50	>50	7	1.56–3.13	6.25
AM10	25	25–50	8	6.25–12.5	6.25–12.5
5	1.56–3.13	3.13–6.25	colistin	3.13–6.25	6.25–12.5
6	1.56	3.13–6.25	polymyxin B	3.13–6.25	6.25

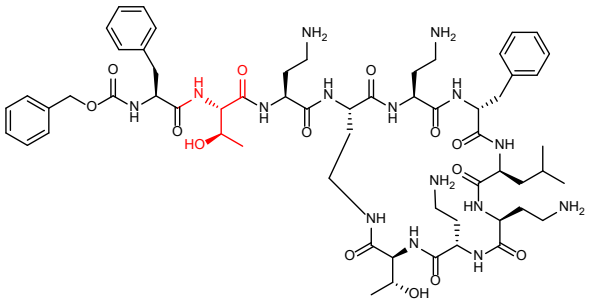
weak activity — potent activity

Figure 8. Direct derivatization stage to discover derivatives that show narrow or broad antibacterial spectrum. a Antibacterial activity screening of 378 series of polymyxin derivatives against nine strains of bacteria. Relative antibacterial spectrum was defined based on how many bacterial species out of nine showed antibacterial activity below 3 μM. **b** Synthesis and antibacterial activity evaluation of hit compounds. The minimum inhibitory concentrations (MICs) were determined by performing the same experiment at least three times. During the test of compound **12** against *E. faecium* ATCC 35667, skipped well was observed (growth inhibition was seen at 1.56–12.5 μM, but regrew at 25 μM). See also Supporting Information.

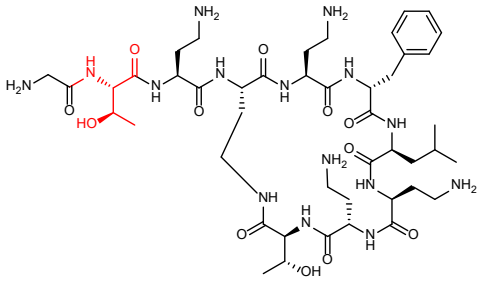
a



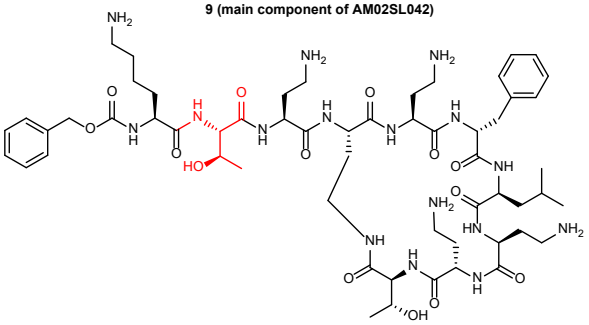
b



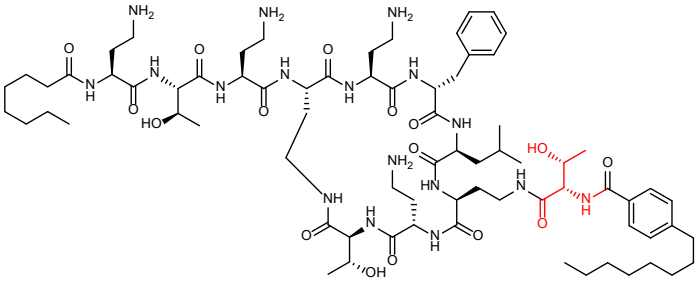
9 (main component of AM02SL042)



10 (main component of AM02SL041)



11 (main component of AM02SL044)



12 (main component of AM10SL018)

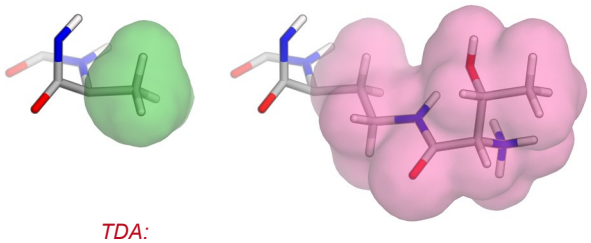
compounds	antibacterial activity MIC (μM)								
	E. coli ATCC 25922	*S. aureus* ATCC 25923	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853	*E. cloacae* ATCC 13047	*E. faecium* ATCC 35667	*E. coli* SME98	*E. coli* SME98 /pIncI2_*mcr-1*	*E. coli* SME98 /PORTpmrB34
AM02	>50	>50	>50	25, 3.13	>50	>50	>50	>50	>50
AM10	0.39–0.78	>50	1.56–3.13	1.56	>50	>50	0.39	25	25–50
9	12.5–25	>50	>50	3.13	>50	>50	12.5	25–50	50
10	12.5–25	>50	50–>50	1.56–3.13	>50	>50	25–50	>50	>50
11	3.13–6.25	>50	50	0.78	>50	>50	6.25	50	50
12	1.56–3.13	0.78–1.56	0.78–1.56	1.56–3.13	3.13	50, 1.56	0.78–1.56	1.56–3.13	1.56
colistin	0.20–0.39	>50	0.39–0.78	0.78	>50	>50	0.05–0.10	3.13–6.25	6.25–12.5
polymyxin B	0.20–0.39	50	0.20–0.39	0.39–0.78	>50	>50	0.20–0.39	3.13–6.25	6.25

weak activity — potent activity

Figure 9. The characteristic of TDA residue on the peptide scanning. a Comparison between alanine and TDA residues. TDA contains polar functional groups in a bulky side chain. **b** Structures and antibacterial activities of probe compounds of polymyxin antibiotics. The design of probe compounds was guided by a TDA scanning. The minimum inhibitory concentrations (MICs) were determined by performing the same experiment at least three times.

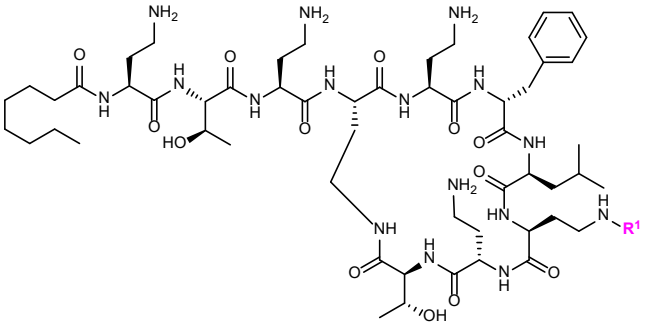
a

alanine threonyldiaminobutyric acid (TDA)

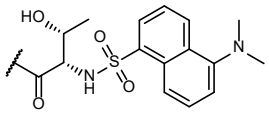


TDA:
suitable for scanning the permissibility of introducing large and/or polar substituents

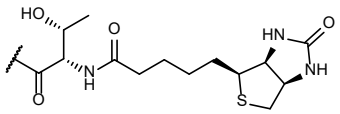
b



13: R^1 =



14: R^1 =



compounds	antibacterial activity MIC (μM)			
	E. coli ATCC 25922	*E. coli* SME98	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853
AM10	0.39–0.78	0.39	1.56–3.13	1.56
13	1.56	1.56	1.56	6.25
14	12.5	6.25–12.5	12.5	25

weak activity — potent activity

ASSOCIATED CONTENT

Supporting Information (1): experimental procedures, characterization data, and detail of the molecular dynamics simulation (PDF). Supporting Information (2): NMR spectra and high-resolution mass spectra for all compounds (PDF). Supporting Information (3): LC-MS chromatograms (PDF). Supporting Information (4): raw data of OD600 (PDF).

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The authors declare no competing interests.

ACKNOWLEDGEMENT

We thank Yuma Terasawa (Faculty of Pharmaceutical Sciences, Hokkaido University, Japan) for helpful discussion on the research concept. We thank Kazuki Takashina (Faculty of Pharmaceutical Sciences, Hokkaido University, Japan) for preparing synthetic plusbacin A_3. We also thank Prof. Dr. Toshiyuki Wakimoto (Faculty of Pharmaceutical Sciences, Hokkaido University, Japan) for helpful discussion on the structural analysis of the library. We further thank Prof. Dr. Courtney C. Aldrich

(Department of Medicinal Chemistry, University of Minnesota, United States) for helpful discussion on the manuscript. Finally, we thank all of the former and current members of the Ichikawa group for fruitful discussion on the design of the experiment.

This research was supported by JSPS KAKENHI Grant-in-Aid for Scientific Research (B) (SI, Grant Number 22H02738 and JP19H03345), Grant-in-Aid for Scientific Research on Innovative Areas 'Frontier Research on Chemical Communications' (SI, Grant Number JP20H04757), Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from Japan Agency for Medical Research and Development (AMED) (SI, Grant Number JP22ama121039, JP18ae0101047h0001, and JP19ae0101047h0002), JSPS KAKENHI Grant-in-Aid for Early-Career Scientist (AK, Grant Number JP22K15241 and JP19K16308), JSPS KAKENHI (TS, Grant Number JP21H03622), JST START Program (TS, Grant Number ST211004JO) , AMED (TS, Grant Number JP21ak0101118h9903), JST SPRING (Grant Number JPMJSP2119), North Tech Foundation, Takeda Science Foundation and was partly supported by Hokkaido University, Global Facility Center (GFC), Pharma Science Open Unit (PSOU) funded by MEXT under "Support Program for Implementation of New Equipment Sharing System", JST for their support through the DX (digital

transformation) Doctoral Fellowship (RK), G-7 Foundation for their support through the G-7 Scholarship (RK), and Tsukushi Fellowship and Research Foundation for their support through the Tsukushi Scholarship (RK).

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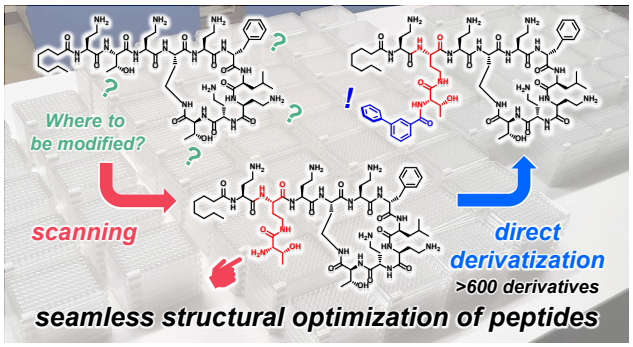
TOC Graphic


Where to
be modified?
scanning
direct
derivatization
>600 derivatives
seamless structural optimization of peptides