‘Exploration and Expansion’ in Peptide Sequence: Discovery of Structurally-Optimized Polymyxin Derivatives Facilitated by Peptide Scanning and *in situ* Screening Chemistry

Rintaro Kaguchi,[1] Akira Katsuyama,[1,2,3] Toyotaka Sato,[4,5,6] Satoshi Takahashi, [7,8] Motohiro Horiuchi,[4,5,6] Shin-ichi Yokota,[9] and Satoshi Ichikawa[1,2,3*]

[1]Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan
[2]Center for Research and Education on Drug Discovery, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan
[3]Global Station for Biosurfaces and Drug Discovery, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Sapporo, Japan
[4]Laboratory of Veterinary Hygiene, School/Faculty of Veterinary Medicine, Hokkaido University, Kita-18, Nishi-9, Kita-ku, Sapporo 060-0818, Japan
[5]Graduate School of Infectious Diseases, Hokkaido University, Sapporo 060-0818, Japan
[6]One Health Research Center, Hokkaido University, Sapporo 060-0818, Japan
[7]Department of Infection Control and Laboratory Medicine, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo, 060-8543, Japan
[8]Division of Laboratory Medicine, Sapporo Medical University Hospital, South-1, West-16, Chuo-ku, Sapporo, 060-8543, Japan
[9]Department of Microbiology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo, 060-8556, Japan

Corresponding Authors: To whom correspondence should be addressed. Tel: (+81) 11-706-3229. Fax: (+81) 11-706-4980. ichikawa@pharm.hokudai.ac.jp

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 'exploration and expansion' 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 expansion 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 *P. 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 (Fig. 1a).[15,17] Liraglutide, a top-selling anti-diabetic medication drug, is a synthetic derivative of a human glucagon-like peptide-1.[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 (Fig. 1b).[20-33] 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.[33-35] *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.[31]

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 (Fig. 1b). In this respect, peptide scanning methodology has room for improvement, especially to obtain highly active derivatives.

We devise a strategy, named 'exploration and expansion', exploiting scanning derivatives with a suitably designed side chain instead of the methyl group used in alanine scanning (Fig. 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. 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 ‘expansion’ stage, where various accessories 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 ‘exploration and expansion’ 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 (Fig. 1c, right).[40-53] Although this method was originally developed in 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 converted, 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, a last resort of antibacterial drug.[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 'exploration and expansion' strategy

Scanning derivatives and accessories used in the 'exploration and expansion' 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 expansion stage. Second, despite its reactivity, the scanning unit should not inhibit the biological evaluation at the exploration stage. Third, facile access to synthesize the functional group accessories is desirable. Serine/threonine ligation (STL) is a two steps transformation to obtain amide compounds from 1,2-aminoalcohol such as *N*-terminal serine and threonine, and *C*-terminal salicylaldehyde (SAL) ester via chemoselective oxazolidine formation, followed by acidolysis of the aminal intermediate (Fig. 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 exploration 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 expansion 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 (Fig. 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 exploration stage, synthesis of peptides bearing a TDA or *N*-terminal threonine, and following biological evaluation efficiently explore the convertible position at the next expansion stage. In the expansion 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 known as the last resort for treatment of serious infections caused by gram-negative bacteria (Fig. 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 an 'exploration and expansion' 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, the 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 expansion 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 (Fig. 3a).[74,79] After preparing 20 mM buffer solutions [pyridine/AcOH = 1/1 (mol/mol)] of salicylaldehyde 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** (Fig. 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 (Fig. 3a, see also Supplementary 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 without calibration (Fig. 3c): The addition of 40 μL of DMSO to this crude 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 Exploration 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 (Fig. 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. **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 Supplementary Information).[80]

As an exploration 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 attributes 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 expansion 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 50 or 25 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 expansion 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 an expansion 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 Expansion stage

Case 1: Discover 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 expansion 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 (Fig. 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. The resulting library was prepared as a 5 mM stock solution in DMSO, based on the limiting amount of salicylaldehyde. The purity of all samples was assessed by LC-MS (Fig. 6a).

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 expansion 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 (Fig. 6b). 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 expansion showed

antibacterial activity at 3 μM, the same concentration as polymyxin B. More than 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: 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 3.13, 1.56, and 3.13 μM, respectively, which is equipotent or two-fold stronger activity than that of polymyxin B (Fig. 6c). Next, the 324 compounds obtained by the expansion 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 Supplementary 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 (Fig. 6c).

Case 2: Discover 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 expansion 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 expansion from **AM02** had a narrow antibacterial spectrum effective against only one strain, consistent with the antibacterial activity of **AM02** (Fig. 7a). 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 64-fold weaker antibacterial activity than that of polymyxin B (Fig. 7b). 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 eight-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. 6a), displayed four times stronger antibacterial activity than polymyxin B against *E. coli* SME98/PORTpmrB34 strain. 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

We herein developed an 'exploration and expansion' 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. By combining *in situ* screening chemistry, hundreds of derivatives could be synthesized and evaluated seamlessly. We applied this strategy to explore 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 expansion stage. These results indicate that acquisition of even slight biological activity in the exploration stage is sufficient since activity can be significantly improved during the expansion 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 exploration 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 Supplementary 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 optical substituents efficiently. In the case of the discovery of derivatives with the various antibacterial spectrum, the unique antibacterial spectrum of the scanning derivatives **AM02** and **AM10** were used as a starting point to discover **9**, **10**, **11**, and **12**.

To the best of our knowledge, the STL used in our research has not been applied to *in situ* screening chemistry so far. It was revealed that LC-MS analysis showed that 71.6% (464 derivatives) over the 648 derivatives (case 1 + case 2) had a UV purity of >50%. This high conversion and wide substrate scope of STL demonstrate that this reaction can provide a large number of derivatives with a high purity sufficient for our 'exploration and expansion' strategy. 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 'exploration and expansion' 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 exploration stage was successfully unleashed in the expansion 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 5 μM [= 6.45 μg/mL for **AM04**, **AM06**, and **AM10** (MWs 1290.58); = 6.39 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 Supplementary Information).[85] 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**: 3.13 μM (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 contrary to Sakura's report that replacing D-Phe[6] to D-alanine did not affect antibacterial activity against *E. coli* IFO 12734.[85] 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.[85] 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.[86] However, TDA scanning revealed that **AM10** exhibited antibacterial activity at 1.56 μM (2.02 μg/mL, MW 1290.58) against the strain. These deviations would 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. 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. 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 reliable modification site; on the contrary, in the case of our TDA scanning, **AM10** retained antibacterial activity against the bacteria, indicating that the possibility of Dab^8 modification remains. The hypothesis was demonstrated by the results showing that dansylated derivative **13** and biotinylated derivative **14** retained their antibacterial activity. 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.

Peptides are one of the most accessible modalities to target such as protein-protein interactions and multi-body interactions between lipid chains on lipid rafts, which are difficult to target with small molecules, and the development of highly potent derivatives and chemical probes are earnestly needed to discover novel therapeutics and understand the 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 ‘exploration and expansion’ 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 is considered widely applicable as a general strategy for amino acid scanning and further optimization of peptide sequences.

Methods

Experimental procedures for the synthesis of compounds, the construction of the polymyxin derivatives, and *in situ* screening is disclosed in Supplementary Information.

Data availability

The data supporting the findings of this study are available from the corresponding author upon request. Experimental procedures are disclosed in Supplementary Information.

References

1. Wang, L., Wang, N., Zhang, W., Cheng, X., Yan, Z., Shao, G., Wang, X., Wang, R. & Fu, C. Therapeutic peptides: Current applications and future directions. *Sig. Transduct. Target Ther.* **7**, 48 (2022). https://doi.org/10.1038/s41392-022-00904-4
2. Muttenthaler, M., King, G. F., Adams, D. J. & Alewood, P. F. Trends in peptide drug discovery. *Nat. Rev. Drug Discov.* **20**, 309-325 (2021). https://doi.org/10.1038/s41573-020-00135-8

3. Henninot, A., Collins, J. C. & Nuss, J. M. The current state of peptide drug discovery: Back to the future? *J. Med. Chem.* **61**, 1382-1414 (2018). https://doi.org/10.1021/acs.jmedchem.7b00318

4. Bhat, A., Roberts, L. R., & Dwyer, J. J. Lead discovery and optimization strategies for peptide macrocycles. *Eur. J. Med. Chem.* **94**, 471-479 (2015). https://doi.org/10.1016/j.ejmech.2014.07.083

5. Tsomaia, N. Peptide therapeutics: targeting the undruggable space. *Eur. J. Med. Chem.* 94, 459-470 (2015). https://doi.org/10.1016/j.ejmech.2015.01.014

6. Fosgerau, K. & Hoffman, T. Peptide therapeutics: current status and future directions. *Drug Discov. Today* **20**, 122-128 (2015). https://doi.org/10.1016/j.drudis.2014.10.003

7. Kaspar, A. A. & Reichert, J. M. Future directions for peptide therapeutics development. *Drug Discov. Today* **18**, 807-817 (2013). https://doi.org/10.1016/j.drudis.2013.05.011

8. Craik, D. J., Fairlie, D. P. Liras, S. & Price, D. The future of peptide-based drugs. *Chem. Biol. Drug Des.* **81**, 136-147 (2013). https://doi.org/10.1111/cbdd.12055

9. Lu, H., Zhou, Q., He, J., Jiang, Z., Peng, C., Tong, R., & Shi, J. Recent advances in the development of protein–protein interactions modulators: mechanisms and clinical

trials. *Sig Transduct Target Ther* **5**, 213 (2020). https://doi.org/10.1038/s41392-020-00315-3

10. Wójcik, P. & Berlicki, Ł. Peptide-based inhibitors of protein-protein interactions. *Bioorg. Med. Chem. Lett.* **26**, 707-713 (2016). https://doi.org/10.1016/j.bmcl.2015.12.084
11. Wang, H., Dawber, R. S., Zhang, P., Walko, M., Wilson, A. J. & Wang, X. Peptide-based inhibitors of protein-protein interactions: Biophysical, structural and cellular consequences of introducing a constraint. *Chem. Sci.* **12**, 5977-5993 (2021). https://doi.org/10.1039/D1SC00165E
12. Viller, E. A., Beglov, D., Chennamadhavuni, S., Porco, J. A., Kozakov, D., Vajda, S. & Whitty, A. How proteins bind macrocycles. *Nat. Chem. Biol.* **10**, 723-734 (2014). https://doi.org/10.1038/nchembio.1584
13. Vinogradov, A. A., Yin, Y. & Suga, H. Macrocyclic peptides as drug candidates: recent progress and remaining challenges. *J. Am. Chem. Soc.* 141, 4167-4181 (2019). https://doi.org/10.1021/jacs.8b13178
14. Boto, A., González, C. C., Hernández, D., Romero-Estudillo, I. & Saavedra, C. J. Site-selective modification of peptide backbones. *Org. Chem. Front.* **8**, 6720-6759 (2021). https://doi.org/10.1039/D1QO00892G

15. Tomishima, M., Ohki, H., Yamada, A., Takasugi, H., Maki, K., Tawara, S., & Tanaka, H. J. FK463, a novel water-soluble echinocandin lipopeptide: synthesis and antifungal activity. *J. Antibiot.* **52**, 674-676 (1999). https://doi.org/10.7164/antibiotics.52.674

16. Knudsen, L. B., Nielsen, P. F., Huusfeldt, P. O., Johansen, N. L., Madsen, K., Pedersen, F. Z., Thøgersen, H., Wilken, M. & Agersø, H. Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J. Med. Chem.* **43**, 1664-1669 (2000). https://doi.org/10.1021/jm9909645

17. Nyfeler, R. & Keller-Schierlein, W. Stoffwechselprodukte von Mikroorganismen 143. Mitteilung. Echinocandin B, ein neuartiges polypeptid-antibiotium aus *Aspergillus nidulans var. echinulatus*: Isolierung und bausteine. *Helv. Chim. Acta.* **57**, 2459-2477 (1974). https://doi.org/10.1002/hlca.19740570818

18. Mojsov, S., Heinrich, G., Wilson, I. B., Ravazzola, M., Orci, L. & Habener, J. F. Preproglucagon gene expression in pancreas and intestine diversifies at the level of post-translational processing. *J. Biol. Chem.* **261**, 11880-11889 (1986). https://doi.org/10.1016/S0021-9258(18)67324-7

19. Kapla, J., Rodríguez-Espigares, I., Ballante, F., Selent, J. & Carlsson, J. Can molecular dynamics simulations improve the structural accuracy and virtual screening

performance of GPCR models? *PLoS Comput. Biol.* **17**, e1008936 (2021). https://doi.org/10.1371/journal.pcbi.1008936

20. Jamieson, A. G., Boutand, N., Sabatino, D. & Lubell, W. D. Peptide scanning for studying structure-activity relationships in drug discovery. *Chem. Biol. Drug Des.* **81**, 148-165 (2013). https://doi.org/10.1111/cbdd.12042
21. Vigneaud, V. D., Denning, G. S., Drabarek, S. & Chan, W. Y. The synthesis and pharmacological study of 4-decarboxamido-oxytocin (4-α-aminobutyric acid-oxytocin) and 5-decarboxamido-oxytocin (5-alanine-oxytocin). *J. Biol. Chem.* **239**, 472-478 (1964). https://doi.org/10.1016/S0021-9258(18)51704-X
22. Hodges, R. S. & Merrifield, R. B. Synthetic study of the effect of tyrosine at position 120 of ribonuclease. *Int. J. Pept. Protein Res.* **6**, 397-405 (1974). https://doi.org/10.1111/j.1399-3011.1974.tb02401.x
23. Cunningham, B. C. & Wells, J. A. High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. *Science* **244**, 1081-1085 (1989). https://doi.org/10.1126/science.2471267
24. Peeters, T. L., Macielag, M. J., Depoortere, I., Konteatis, Z. D., Florance, J. R., Lessor, R. A. & Galdes, A. D-amino acid and alanine scans of the bioactive portion of porcine

motilin. *Peptides*, **13**, 1103-1107 (1992). https://doi.org/10.1016/0196-9781(92)90014-T

25. Beck-Sickinger, A. G., Wieland, H. A., Wittneben, H., Willim, K.-D., Rudolf, K. & Jung, G. Complete L-alanine scan of neuropeptide Y reveals ligands binding to Y1 and Y2 receptors with distinguished conformations. *Eur. J. Biochem.* **225**, 947-958 (1994). https://doi.org/10.1111/j.1432-1033.1994.0947b.x
26. Massova, I. & Kollman, P. A. Computational alanine scanning to probe protein−protein interactions: A novel approach to evaluate binding free energies. *J. Am. Chem. Soc.* **121**, 8133-8143 (1999). https://doi.org/10.1021/ja990935j
27. Johnson, H. J., Boys, S. K., Makda, A., Carragher, N. O. & Hulme, A. N. Naturally inspired peptide leads: Alanine scanning reveals an actin-targeting thiazole analogue of bisebromoamide. *ChemBioChem* **17**, 1621-1627 (2016). https://doi.org/10.1002/cbic.201600257
28. Chen, K. H., Le, S. P., Han, X., Frias, J. M. & Nowick, J. S. Alanine scan reveals modifiable residues in teixobactin. *ChemCommun* **53**, 11357-11359 (2017). https://doi.org/10.1039/C7CC03415F
29. Niida, A., Sasaki, S., Yonemori, K., Sameshima, T., Yaguchi, M., Asami, T., Sakamoto, K. & Kamaura, M. Investigation of the structural requirements of K-

Ras(G12D) selective inhibitory peptide KRpep-2d using alanine scans and cysteine bridging. *Bioorg. Med. Chem. Lett.* **27**, 2757-2761 (2017). https://doi.org/10.1016/j.bmcl.2017.04.063

30. Cochrane, S. A., Findlay, B., Vederas, J. C. & Ratemi, E. S. Key residues in octyl-tridecaptin A1 analogues linked to stable secondary structures in the membrane. *ChemBioChem* **15**, 1295-1299 (2014). https://doi.org/10.1002/cbic.201402024
31. Kavianinia, I., Stubbing, L. A., Abbattista, M. R., Harris, P. W. R., Smaill, J. B., Patterson, A. V. & Brimble, M. A. Alanine scan-guided synthesis and biological evaluation of analogues of culicinin D, a potent anticancer peptaibol. *Bioorg. Med. Chem. Lett.* **30**, 127135 (2020). https://doi.org/10.1016/j.bmcl.2020.127135
32. Proniewicz, E., Burnat, G., Domin, H., Małuch, I., Makowska, M. & Prahl, A. Application of alanine scanning to determination of amino acids essential for peptide adsorption at the solid/solution interface and binding to the receptor: Surface-enhanced Raman/infrared spectroscopy versus bioactivity assays. *J. Med. Chem.* **64**, 8410-8422 (2021). https://doi.org/10.1021/acs.jmedchem.1c00397
33. Ramalingam, K., Eaton, S. R., Cody, W. L., Lu, G. H., Panek, R., Waite, L. A., Decker, S. J., Keiser, J. A. & Doherty, A. M. Structure-activity studies of phosphorylated peptide inhibitors of the association of phosphatidylinositol 3-kinase with PDGF-β

receptor. *Bioorg. Med. Chem.* **3**, 1263-1272 (1995). https://doi.org/10.1016/0968-0896(95)00112-T

34. Grieco, P., Balse, P. M., Weinberg, D., MacNeil, T. & Hruby, V. J. D-amino acid scan of γ-melanocyte-stimulating hormone: Importance of Trp8 on human MC3 receptor selectivity. *J. Med. Chem.* **43**, 4998-5002 (2000). https://doi.org/10.1021/jm000211e

35. Roth, A. L., Marzola, E., Rizzi, A., Arduin, M., Trapella, C., Corti, C., Vergura, R., Martinelli, P., Salvadori, S., Regoli, D., Corsi, M., Cavanni, P., Caló, G. & Guerrini, R. Structure-activity studies on neuropeptide S: Identification of the amino acid residues crucial for receptor activation. *J. Biol. Chem.* **281**, 20809-20816 (2006). https://doi.org/10.1074/jbc.M601846200

36. Miller, S. C. & Scanlan, T. S. Site-selective *N*-methylation of peptides on solid support. *J. Am. Chem. Soc.* **119**, 2301-2302 (1997). https://doi.org/10.1021/ja9635443

37. Kulkarni, S. S., Sayurs, J., Premdjee, B. & Payne, R. J. Rapid and efficient protein synthesis through expansion of the native chemical ligation concept. *Nat. Rev. Chem.* **2**, 0122 (2018). https://doi.org/10.1038/s41570-018-0122

38. Isidro-Llobet, A., Kenworthy, M. N., Mukherjee, S., Kopach, M. E., Wegner, K., Gallou, F., Smith, A. G. & Roschangar, F. Sustainability challenges in peptide synthesis and purification: From R&D to production. *J. Org. Chem.* **84**, 4615-4628 (2019). https://doi.org/10.1021/acs.joc.8b03001

39. Loibl, S. F., Harpaz, Z., Zitterbart, R. & Seitz, O. Total chemical synthesis of proteins without HPLC purification. *Chem. Sci.* **7**, 6753-6759 (2016). https://doi.org/10.1039/C6SC01883A

40. Brik, A., Lin, Y.-C., Elder, J. & Wong, C.-H. A quick diversity-oriented amide-forming reaction to optimize P-Subsite residues of HIV protease inhibitors. *Chem. Biol.* **9**, 891-896 (2002). https://doi.org/10.1016/S1074-5521(02)00184-9

41. Wu, C.-Y., Chang, C.-F., Chen, J. S.-Y., Wong, C.-H. & Lin, C.-H. Rapid diversity-oriented synthesis in microtiter plates for in situ screening: Discovery of potent and selective α-fucosidase inhibitors. *Angew. Chem. Int. Ed.* **42**, 4661-4664 (2003). https://doi.org/10.1002/anie.200351823

42. Chang, C.-F., Ho, C.-W. Wu, C.-Y., Chao, T.-A., Wong, C.-H. & Lin, C.-H. Discovery of picomolar slow tight-binding inhibitors of α-fucosidase. *Chem. Biol.* **11**, 1301-1306 (2004). https://doi.org/10.1016/j.chembiol.2004.07.009

43. Brik, A., Wu, C.-Y., Best, M. D. & Wong, C.-H. Tetrabutylammonium fluoride-assisted rapid N^9-alkylation on purine ring: Application to combinatorial reaction in microtiter plates for the discovery of potent sulfotransferase inhibitors in situ. *Bioorg. Med. Chem.* **13**, 4622-4626 (2005). https://doi.org/10.1016/j.bmc.2005.02.066

44. Wu, C.-Y., Brik, A., Wang, S.-K., Chen, Y.-H. & Wong, C.-H. Tetrabutylammonium fluoride-mediated rapid alkylation reaction in microtiter plates for the discovery of enzyme inhibitors in situ. *ChemBioChem* **6**, 2176-2180 (2005). https://doi.org/10.1002/cbic.200500295

45. Numa, M. M. D., Lee, L. V., Hsu, C.-C., Bower, K. E. & Wong, C.-H. Identification of novel anthrax lethal factor inhibitors generated by combinatorial Pictet-Spengler reaction followed by screening in situ. *ChemBioChem* **6**, 1002-1006 (2005). https://doi.org/10.1002/cbic.200500009

46. Brik, A., Wu, C.-Y. & Wong, C.-H. Microtiter plate based chemistry and in situ screening: A useful approach for rapid inhibitor discovery. *Org. Biomol. Chem.* **4**, 1446-1457 (2006). https://doi.org/10.1039/B600055J

47. van der Zouwen, A. J., Lohse, J., Wieske, L. H. E., Hohmann, K. F., van der Vlag, R. & Witte, M. D. An in situ combinatorial methodology to synthesize and screen

chemical probes. *Chem. Commun.* **55**, 2050-2053 (2019). https://doi.org/10.1039/C8CC06991C

48. Immel, J. R., Chilamari, M. & Bloom, S. Combining flavin photocatalysis with parallel synthesis: A general platform to optimize peptides with non-proteinogenic amino acids. *Chem. Sci.* **12**, 10083-10091 (2021). https://doi.org/10.1039/D1SC02562G
49. Gao, K., Shaabani, S., Xu, R., Zarganes-Tzitzikas, T., Gao, L., Ahmadianmoghaddam, M., Groves, M. R. & Dömling, A. Nanoscale, automated, high throughput synthesis and screening for the accelerated discovery of protein modifiers. *RSC Med. Chem.* **12**, 809-818 (2021). https://doi.org/10.1039/D1MD00087J
50. Elías-Rodríguez, P., Pingitore, V., Carmona, A. T., Moreno-Vargas, A. J., Ide, D., Miyawaki, S., Kato, A., Álvarez, E. & Robina, I. Discovery of a potent α-galactosidase inhibitor by in situ analysis of a library of pyrrolizidine–(thio)urea hybrid molecules generated via click chemistry. *J. Org. Chem.* **83**, 8863-8873 (2018). https://doi.org/10.1021/acs.joc.8b01073
51. Pingitore, V., Martínez-Bailén, M., Carmona, A. T., Mészáros, Z., Kulik, N., Slámová, K., Křen, V., Bojarová, P., Robina, I. & Moreno-Vargas, A. J. Discovery of human hexosaminidase inhibitors by in situ screening of a library of mono- and divalent

pyrrolidine iminosugars. *Bioorg. Chem.* **120**, 105650 (2022). https://doi.org/10.1016/j.bioorg.2022.105650

52. Kale, S. S., Bergeron-Brlek, M., Wu, Y., Kumar, M. G., Pham, M. V., Bortoli, J., Vesin, J., Kong, X.-D., Machado, J. F., Deyle, K., Gonschorek, P., Turcatti, G., Cendron, L., Angelini, A. & Heinis, C. Thiol-to-amine cyclization reaction enables screening of large libraries of macrocyclic compounds and the generation of sub-kilodalton ligands. *Sci Adv.* **5**, eaaw2851 (2019). https://doi.org/10.1126/sciadv.aaw2851

53. Mothukuri, G. K., Kale, S. S., Stenbratt, C. L., Zorzi, A., Vesin, J., Chapalay, J. B., Deyle, K., Turcatti, G., Cendron, L., Angelini, A. & Heinis, C. Macrocycle synthesis strategy based on step-wise "adding and reacting" three components enables screening of large combinatorial libraries. *Chem. Sci.* **11**, 7858-7863 (2020). https://doi.org/10.1039/D0SC01944E

54. Anisworth, G. C., Brown, A. M. & Brownlee, G. 'Aerosporin', an Antibiotic Produced by Bacillus aerosporus Greer. *Nature* **160**, 263 (1947). https://doi.org/10.1038/160263a0

55. Benedict, R. G. & Langlykke, A. F. Antibiotic activity of Bacillus polymyxa. *J. Bacteriol.* **54**, 24 (1947). https://doi.org/10.1128/jb.54.1.1-30.1947

56. Stansly, P. G., Shepherd, R. G. & White, H. J. Polymyxin: A new chemotherapeutic agent. *Bull. Johns Hopkins Hosp.* **81**, 43-54 (1947).

57. Li, X., Lam, H. Y., Zhang, Y. & Chan, K. Salicylaldehyde ester-induced chemoselective peptide ligations: Enabling generation of natural peptidic linkages at the serine/threonine sites. *Org. Lett.* **12**, 1724-1727 (2010). https://doi.org/10.1021/ol1003109

58. Liu, H. & Li, X. Serine/threonine ligation: Origin, mechanistic aspects, and applications. *Acc. Chem. Res.* **51**, 1643-1655 (2018). https://doi.org/10.1021/acs.accounts.8b00151

59. Zhang, Y., Xu, C., Lam, H. Y., Lee, C. L. & Li, X. Protein chemical synthesis by serine and threonine ligation. *Proc. Nat. Acad. Sci.* **110**, 6657-6662 (2013). https://doi.org/10.1073/pnas.1221012110

60. Lam, H. Y., Zhang, Y., Liu, H., Xu, J., Wong, C. T. T., Xu, C. & Li, X. Total synthesis of daptomycin by cyclization via a chemoselective serine ligation. *J. Am. Chem. Soc.* **135**, 6272-6279 (2013). https://doi.org/10.1021/ja4012468

61. Jin, K., Sam, I. H., Po, K. H. L., Lin, D., Zadeh, E. H. G., Chen, S., Yuan, Y. & Li, X. Total synthesis of teixobactin. *Nat. Commun.* **7**, 12394 (2016). https://doi.org/10.1038/ncomms12394

62. Cheung, C. H. P., Xu, J., Lee, C. H., Zhang, Y., Wei, R., Bierer, D., Huang, X. & Li, X. Construction of diverse peptide structural architectures via chemoselective peptide ligation. *Chem. Sci.* **12**, 7091-7097 (2021). https://doi.org/10.1039/D1SC01174J

63. Murray, C. J. L., Ikuta, K. S., Sharara, F., Swetschinski, L., Aguilar, G. R., Gray, A., Han, C., Bisignano, C., Rao, P., Wool, E., Johnson, S. C., Browne, A. J., Chipeta, M. G., Fell, F., Hackett, S., Haines-Wood house, G., Hamadani, B. H. K., Kumaran, E. A. P., McManigal, B., Agarwal, R., Akech, S., Albertson, S., Amuasi, J., Andrews, J., Aravkin, A., Ashley, E., Bailey, F., Baker, S., Basnyat, B., Bekker, A., Bender, R., Bethou, A., Bielicki, J., Boonkasidecha, S., Bukosia, J., Carvalheiro, C., Castañeda-Orjuela, C., Chansamouth, V., Chaurasia, S., Chiurchiù, S., Chowdhury, F., Chowdhury, F., Cook, A. J., Cooper, B., Cressey, T. R., Criollo-Mora, E., Cunningham, M., Darboe, S., Day, N. P. J., Luca, M. D., Dokova, K., Dramowski, A., Dunachie, S. J., Eckmanns, T., Eibach, D., Emami, A., Feasey, N., Fisher-Pearson, N., Forrest, K., Garrett, D., Gastmeier, P., Giref, A. G., Greer, R. C., Gupta, V., Haller, S., Haselbeck, A., Hay, S, I., Holm, M., Hopkins, S., Iregbu, K. C., Jacobs, J., Jarovsky, D., Javanmardi, F., Khorana, M., Kissoon, N., Kobeissi, E., Kostyanev, T., Krapp, F., Krumkamp, R., Kumar, A., Kyu, H. H., Lim, C., Limmathurotsakul, D., Loftus, M. J., Lunn, M., Ma, J., Mturi, N., Munera-Huertas, T., Musicha, P., Mussi-

Pinhata, M. M., Nakamura, T., Nanavati, R., Nangia, S., Newton, P., Ngoun, C., Novotney, A., Nwakanma, D., Obiero, C. W., Olivas-Martinez, A., Olliaro, P., Ooko, E., Ortiz-Brizuela, E., Peleg, A. Y., Perrone, C., Plakkal, N., Ponce-de-Leon, A., Raad, M., Ramdin, T., Riddell, A., Roberts, T., Robotham, J. V., Roca, A., Rudd, K. E., Russell, N., Schnall, J., Scott, J, A. G., Shivamallappa, M., Sifuentes-Osornio, J., Steenkeste, N., Stewardson, A. J., Stoeva, T., Tasak, N., Thaiprakong, A., Thwaites, G., Turner, C., Turner, P., van Doorn, H. R., Velaphi, S., Vongpradith, A., Vu, H., Walsh, T., Waner, S., Wangrangsimakul, T., Wozniak, T., Zheng, P., Sartorius, B., Lopez, A. D., Stergachis, A., Moore, C., Dolecek, C. & Naghavi, M. Global burden of bacterial antimicrobial resistance in 2019: A systematic analysis. *Lancet* **399**, 629-655 (2022). https://doi.org/10.1016/S0140-6736(21)02724-0

64. Privalsky, T. M., Soohoo, A. M., Wang, J., Walsh, C. T., Wright, G. D., Gordon, E. M., Gray, N. S. & Khosla, C. Prospects for antibacterial discovery and development. *J. Am. Chem. Soc.* **143**, 21127-21142 (2021). https://doi.org/10.1021/jacs.1c10200

65. Roberts, K. D., Azad, M. A. K., Wang, J., Horne, A. S., Thompson, P. E., Nation, R. L. & Li, J. Antimicrobial activity and toxicity of the major lipopeptide components of polymyxin B and colistin: Last-line antibiotics against multidrug-resistant Gram-

negative bacteria. *ACS Infect. Dis.* **1**, 568-575 (2015). https://doi.org/10.1021/acsinfecdis.5b00085

66. Khondker, A. & Rheinstädter, M. C. How do bacterial membranes resist polymyxin antibiotics? *Commun. Biol.* **3**, 77 (2020). https://doi.org/10.1038/s42003-020-0803-x

67. Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H. & Shen, J. Emergence of plasmid-mediated colistin resistance mechanism MCR-1animal and human being in Chena: A microbiological and molecular biological study. *Lancet Infect. Dis.* **16**, 161-168 (2016). https://doi.org/10.1016/S1473-3099(15)00424-7

68. Chellat, M. F., Raguž, L. & Riedl, R. Targeting antibacterial resistance. *Angew. Chem. Int. Ed.* **55**, 6600-6626 (2016). https://doi.org/10.1002/anie.201506818

69. Agnello, S., Brand, M., Chellat, M. F., Gazzola, S. & Riedl, R. A structural view on medicinal chemistry strategies against drug resistance. *Angew. Chem. Int. Ed.* **58**, 3300-3345. (2019). https://doi.org/10.1002/anie.201802416

70. Baym, M., Stone, L. K. & Kishony, R. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* **351**, aad3292 (2016). https://doi.org/10.1126/science.aad3292

71. Kinoshita, Y., Yakushiji, F., Matsui, H., Hanaki, H. & Ichikawa, S. Study of the structure-activity relationship of polymyxin analogues. *Bioorg. Med. Chem. Lett.* **28**, 2713-2716 (2018). https://doi.org/10.1016/j.bmcl.2018.03.028

72. Velkov, T., Thompson, P. E., Nation, R. L. & Li, J. Structure-activity relationships of polymyxin antibiotics. *J. Med. Chem.* **53**, 1898-1916 (2010). https://doi.org/10.1021/jm900999h

73. Magee, T. V., Brown, M. F., Starr, J. T., Ackley, D. C., Abramite, J. A., Aubrecht, J., Butler, A., Crandon, J. L., Dib-Hajj, F., Flanagan, M. E., Granskog, K., Hardink, J. R., Huband, M. D., Irvine, R., Kuhn, M., Leach, K. L., Li, B., Lin, J., Luke, D. R., MacVane, S. H., Miller, A. A., McCurdy, S., McKim, J. M., Nicolau, D. P., Nguyen, T. T., Noe, M. C., O'Donnell, J. P., Seibel, S. B., Shen, Y., Stepan, A. F., Tomaras, A. P., Wilga, P. C., Zhang, L., Xu, J. & Chen, J. M. Discovery of Dap-3 polymyxin analogues for the treatment of multidrug-resistant Gram-negative nosocomial infections. *J. Med. Chem.* **56**, 5079-5093 (2013). https://doi.org/10.1021/jm400416u

74. Gallardo-Godoy, A., Muldoon, C., Becker, B., Elliott, A. G., Lash, L. H., Huang, J. X., Butler, M. S., Pelingon, R., Kavanagh, A. M., Ramu, S., Phetsang, W., Blaskovich, M. A. & and Cooper, M. A. Activity and predicted nephrotoxicity of synthetic

antibiotics based on polymyxin B. *J. Med. Chem.* **59**, 1068-1077 (2016). https://doi.org/10.1021/acs.jmedchem.5b01593

75. Cui, A-L., Hu, X.-X., Chen, Y., Jin, J., Yi, H., Wang, X.-K., He, Q.-Y., You, X.-F. & Li, Z.-R. Design, synthesis, and bioactivity of cyclic lipopeptide antibiotics with varied polarity, hydrophobicity, and positive charge distribution. *ACS Infect. Dis.* **6**, 1796-1806 (2020). https://doi.org/10.1021/acsinfecdis.0c00056

76. Li, J., Guan, D., Chen, F., Shi, W., Lan, L. & Huang, W. Total and semisyntheses of polymyxin analogues with 2-Thr or 10-Thr modifications to decipher the structure–activity relationship and improve the antibacterial activity. *J. Med. Chem.* **64**, 5746-5765 (2021). https://doi.org/10.1021/acs.jmedchem.0c02217

77. Roberts, K. D., Zhu, Y., Azad, M. A. K., Han, M.-L., Wang, J., Wang, L., Yu, H. H., Horne, A. S., Pinson, J.-A., Rudd, D., Voelcker, N. H., Patil, N. A., Zhao, J., Jiang, X., Lu, J., Chen, K., Lomovskaya, O., Hecker, S. J., Thompson, P. E., Nation, R. L., Dudley, M. N., Griffith, D. C., Velkov, T. & Li, J. A synthetic lipopeptide targeting top-priority multidrug-resistant Gram-negative pathogens. *Nat. Commun.* **13**, 1625 (2022). https://doi.org/10.1038/s41467-022-29234-3

78. Evans, L., Rhodes, A., Alhazzani, W., Antonell, M., Coopersmith, C. M., French, C., Machado, F. R., Micintyre, L., Ostermann, M., Prescott, H. C., Schorr, C., Simpson,

S., Wiersinga, W. J., Alshamsi, F., Angus, D. C., Arabi, Y., Azevedo, L., Beale, R., Beilman, G., Belley-Cote, E., Burry, L., Cecconi, M., Centofanti, J., Yataco, A. C., Waele, J. D. & Dellinger, R. P. Surviving sepsis campaign: International guidelines for management of sepsis and septic shock 2021. *Clit. Care Med.* **49**, e1063-e1143 (2021). https://doi.org/10.1097/CCM.0000000000005337

79. O'Dowd, H., Kim, B., Margolis, P., Wang, W., Wu, C., Lopez, S. L. & Blais, J. Preparation of tetra-Boc-protected polymyxin B nonapeptide. *Tetrahedron Lett.* **48**, 2003-2005 (2007). https://doi.org/10.1016/j.tetlet.2007.01.071
80. Mennen, S. M., Alhambra, C., Allen, C. L., Barberis, M., Berritt, S., Brandt, T. A., Campbell, A. D., Castañón, J., Cherney, A. H., Christensen, M., Damon, D. B., de Diego, J. E., García-Cerrada, S., García-Losada, P., Haro, R., Janey, J., Leitch, D. C., Li, L., Liu, F., Lobben, P. C., MacMillan, D. W. C., Magano, J., McInurff, E., Monfette, S., Post, R. J., Schultz, D., Sitter, B. J., Stevens, J. M., Strambeanu, I. I., Twilton, J., Wang, K. & Zajac, M. A. The evolution of high-throughput experimentation in pharmaceutical development and perspectives on the future. *Org. Process Res. Dev.* **23**, 1213-1242 (2019). https://doi.org/10.1021/acs.oprd.9b00140
81. Sato, T., Shiraishi, T., Hiyama, Y., Honda, H., Shinagawa, M., Usui, M., Kuronuma, K., Masumori, N., Takahashi, S., Tamura, Y. & Yokota, S.-I. Contribution of novel

amino acid alterations in PmrA or PmrB to colistin resistance in *mcr*-negative *Escherichia coli* clinical isolates, including major multidrug-resistant lineages O25b:H4-ST131-*H*30Rx and non-x. *Antimicrob. Agents Chemother.* **62**, e00864-18 (2018). https://doi.org/10.1128/AAC.00864-18

82. Sato, T., Fukuda, A., Usui, M., Shinagawa, M., Shiraishi, T., Tamura, Y., Takahashi, S. & Yokota, S.-I. Isolation of a *mcr-1*-harbouring *Escherichia coli* isolate from a human clinical setting in Sapporo, Japan. *J. Grob. Antimicrob. Resist.* **13**, 20 (2018). https://doi.org/10.1016/j.jgar.2018.02.010
83. Needham, B. D. & Trent, M. S. Fortifying the barrier: The impact of lipid A remodelling on bacterial pathogenesis. *Nat. Rev. Microbiol.* **11**, 467-481 (2013). https://doi.org/10.1038/nrmicro3047
84. Wong, C. T. T., Li, T., Lam, H. Y., Zhang, Y. & Li, X. Realizing serine/threonine ligation: Scope and limitations and mechanistic implication thereof. *Front. Chem.* **2**, 28 (2014). https://doi.org/10.3389/fchem.2014.00028
85. Kanazawa, K., Sato, Y., Ohki, K., Okimura, K., Uchida, Y., Shindo, M. & Sakura, N. Contribution of each amino acid residue in polymyxin B_3 to antimicrobial and lipopolysaccharide binding activity. *Chem. Pharm. Bull.* **57**, 240-244 (2009). https://doi.org/10.1248/cpb.57.240

86. Jiang, X., Patil, N. A., Azad, M. A. K., Wickremasinghe, H., Yu, H., Zhao, J., Zhang, X., Li, M., Gong, B., Wan, L., Ma, W., Thompson, P. E., Yang, K., Yuan, B., Schreiber, F., Wang, L., Velkov, T., Roberts, K. D. & Li, J. A novel chemical biology and computational approach to expedite the discovery of new-generation polymyxins against life-threatening *Acinetobacter baumannii*. *Chem. Sci.* **12**, 12211-12220 (2021). https://doi.org/10.1039/D1SC03460J

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).

Author Contributions

S.I. and R.K. conceived the concept of ‘exploration and expansion’ strategy and jointly launched the idea for combining with *in situ* screening methodology utilising serine/threonine ligation. S.I. directed and oversaw the project. A.K. and R.K. jointly designed experiments including procedures for the library construction on the microplate and high-throughput examination (HTE) disclosed in Supplementary Information. R.K. synthesized and characterized the compound, conducted HTE, and constructed the library. R.K. performed LC-MS measurements and these data analyses with A.K. providing guidance of interpretation of data. R.K. performed antibacterial activity evaluations, screening, and these data analyses with T.S. providing guidance of experimental procedure and interpretation of data. A.K. and R.K. jointly performed MD simulations. A.K. launched the idea for preparing probe derivative **13** and **14** to interpret the scanning. S.T. prepared polymyxin-resistant bacteria. T.S., S.Y., and M.H. provided helpful discussion on antibacterial activity evaluations. R.K. wrote the original draft manuscript with a contribution of A.K., which was reviewed, edited, and revised by S.I.; R.K., A.K., and S.I. prepared the final version of manuscript with input and contributions from all authors.

Corresponding Author

Satoshi Ichikawa - Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan; Center for Research and Education on Drug Discovery, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan; Global Station for Biosurfaces and Drug Discovery, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Sapporo, Japan

Rintaro Kaguchi - Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan

Akira Katsuyama - Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan; Center for Research and Education on Drug Discovery, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Japan; Global Station for Biosurfaces and Drug Discovery, Global Institution for Collaborative Research and Education (GI-CoRE), Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060-0812, Sapporo, Japan

Toyotaka Sato - Laboratory of Veterinary Hygiene, School/Faculty of Veterinary Medicine, Hokkaido University, Kita-18, Nishi-9, Kita-ku, Sapporo 060-0818, Japan; Graduate School of Infectious Diseases, Hokkaido University, Sapporo 060-0818, Japan; One Health Research Center, Hokkaido University, Sapporo 060-0818, Japan

Satoshi Takahashi - Department of Infection Control and Laboratory Medicine, Sapporo Medical University School of Medicine, South-1, West-16, Chuo-ku, Sapporo, 060-8543, Japan; Division of Laboratory Medicine, Sapporo Medical University Hospital, South-1, West-16, Chuo-ku, Sapporo, 060-8543, Japan

Motohiro Horiuchi - Laboratory of Veterinary Hygiene, School/Faculty of Veterinary Medicine, Hokkaido University, Kita-18, Nishi-9, Kita-ku, Sapporo 060-0818, Japan; Graduate School of Infectious Diseases, Hokkaido University, Sapporo 060-0818, Japan; One Health Research Center, Hokkaido University, Sapporo 060-0818, Japan

Shin-ichi Yokota - Department of Microbiology, Sapporo Medical University School of Medicine, South-1, West-17, Chuo-ku, Sapporo, 060-8556, Japan

Ethics declarations

The authors declare no competing interests.

Fig. 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 'exploration and expansion' 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 a *in situ* screening chemistry, several hundreds of syntheses and evaluation of derivatives can be performed in the expansion stage.

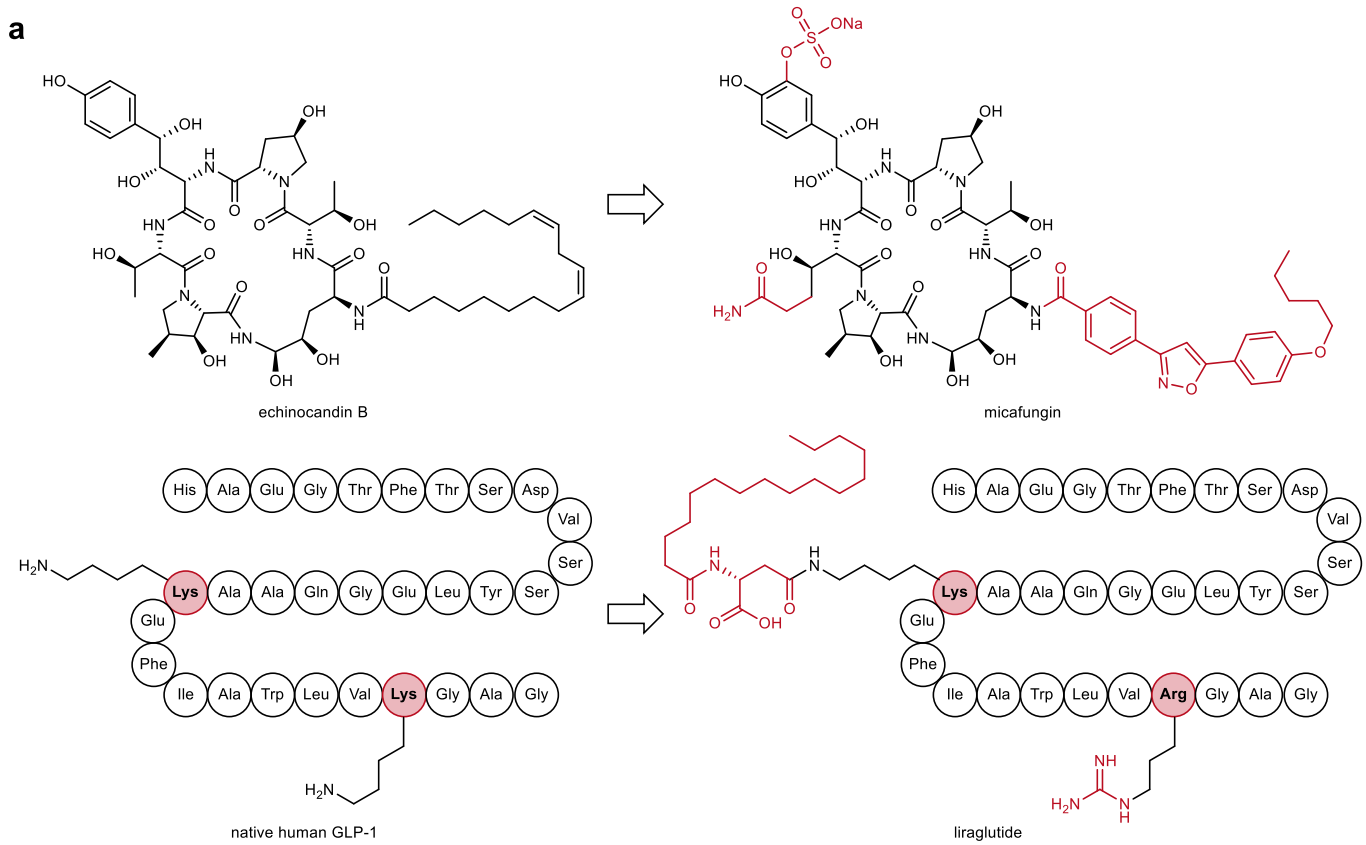

a
echinocandin B
micafungin
native human GLP-1
liraglutide
b
alanine scanning
step 1
activity: −
activity: +
point diversification
step 2
activity: + additional function
activity: ++
c
exploration
step 1
activity: −
activity: +
expansion
step 2
"exploration and expansion" strategy
✓ chemoselective functionalization on the scanning derivatives
✓ straightforward access for the structural diversification
activity: + additional function
activity: ++
in situ screening chemistry
✓ omitted purification process
✓ facile access to the desired properties

Fig. 2 'Exploration and expansion' strategy applied to the polymyxin antibiotics. a General scheme of a serine/threonine ligation (STL) developed by Li. **b** 'Exploration and expansion' 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.

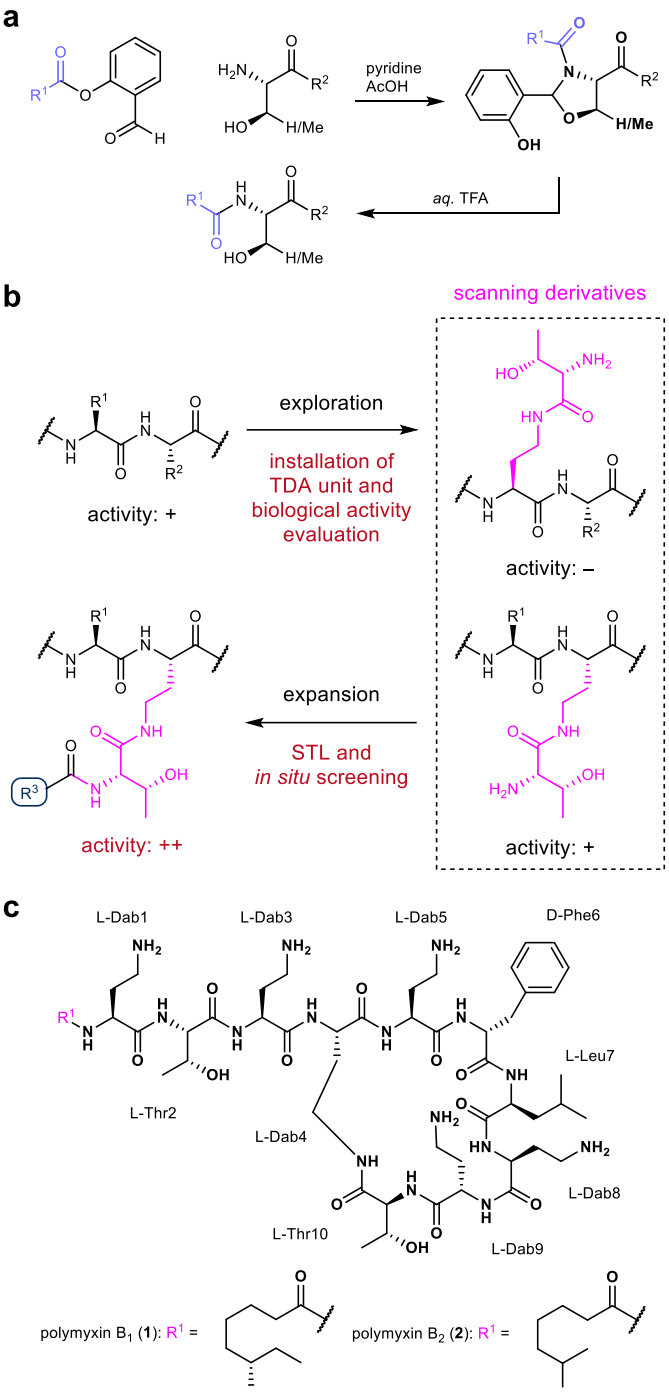
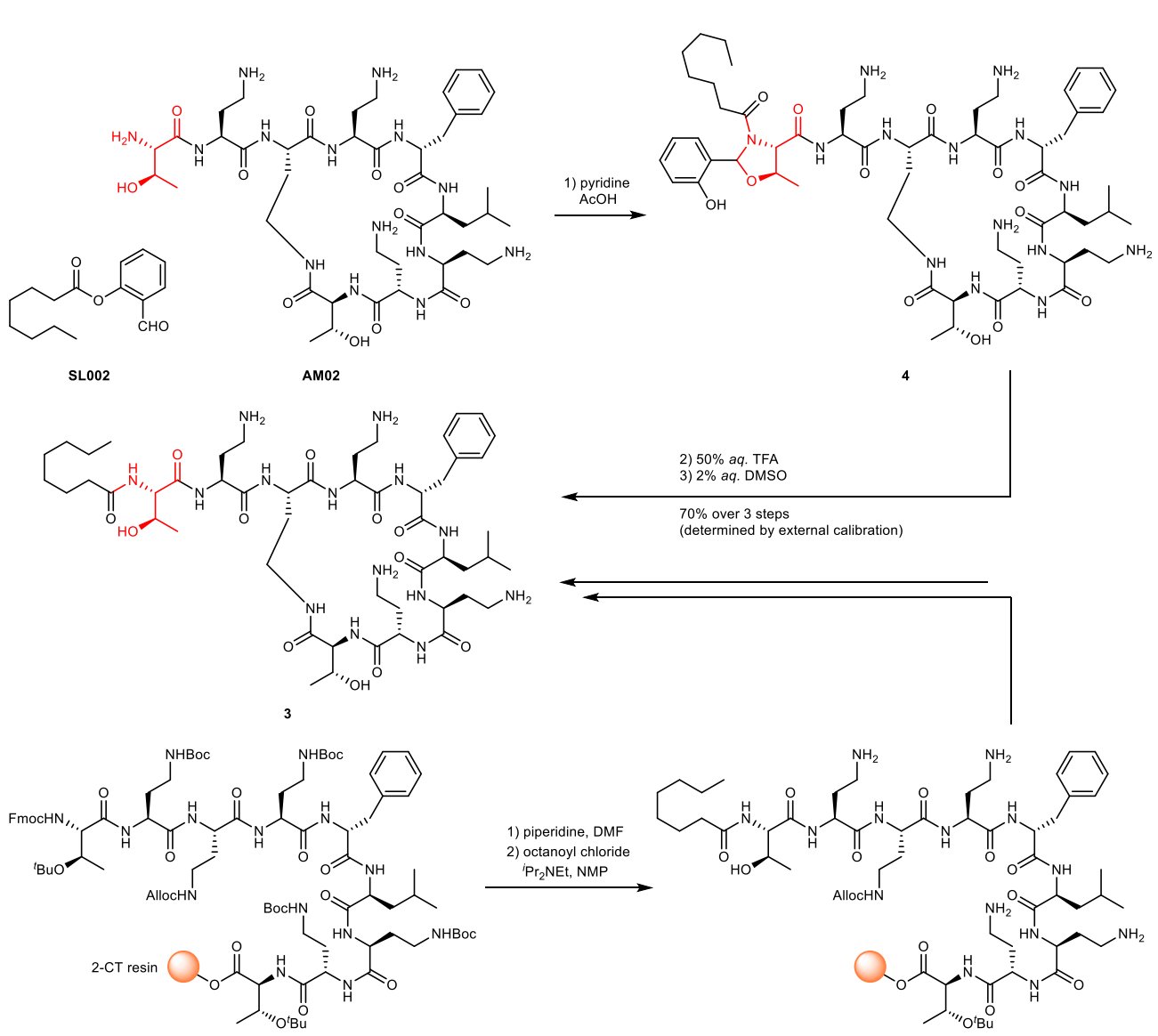
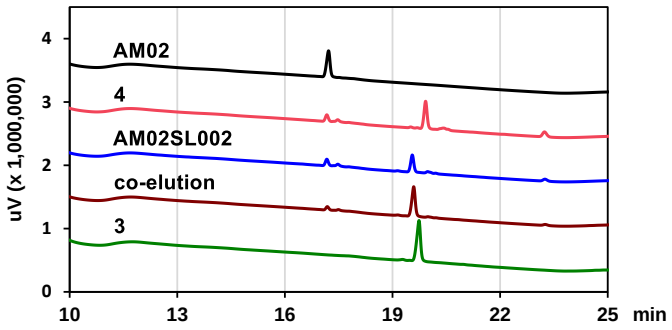

a
pyridine
AcOH
aq. TFA
H/Me
b
scanning derivatives
exploration
installation of
TDA unit and
biological activity
evaluation
activity: +
activity: –
expansion
STL and
in situ screening
activity: ++
activity: +
c
L-Dab1
L-Dab3
L-Dab5
D-Phe6
L-Leu7
L-Thr2
L-Dab4
L-Dab8
L-Thr10
L-Dab9
polymyxin B1 (1): R1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

Fig. 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. **c** The results of *in situ* screening. Ligation product **AM02SL002** and authentic **3** showed comparable antibacterial activity.

a



b

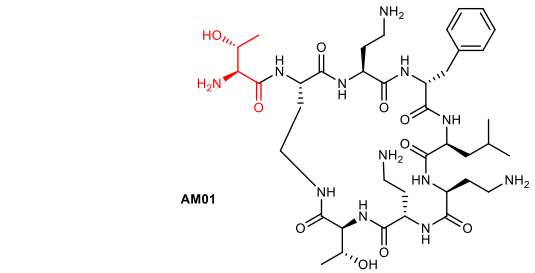


c

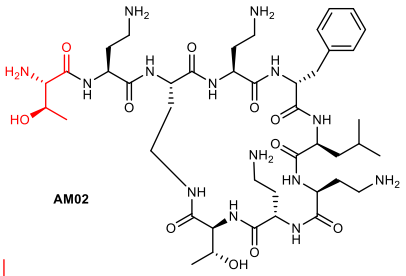
compounds	antibacterial activity MIC (μM)			
	E. coli ATCC 25922	*E. coli* SME98	*A. baumannii* ATCC 19606	*P. aeruginosa* ATCC 27853
AM02	>12	>12	>12	>12
SL002	>12	>12	>12	>12
AM02SL002	0.75	0.75	>12	1.5
3	0.75	0.38	>12	1.5
colistin	0.19	0.09	0.38	0.38
polymyxin B	0.38	0.19	0.38	0.38

weak activity — potent activity

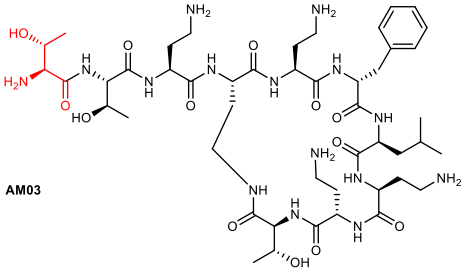
Fig. 4 Design of the scanning derivatives. Scanning derivatives **AM01**-**AM03** are designed to modulate the length of the linear peptide moiety. **AM04**-**AM12** have a TDA, which can be used for scanning a permissibility of introducing substituents on the side chain of the peptide.



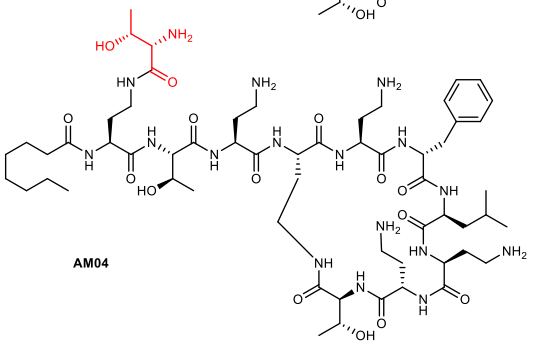
AM01



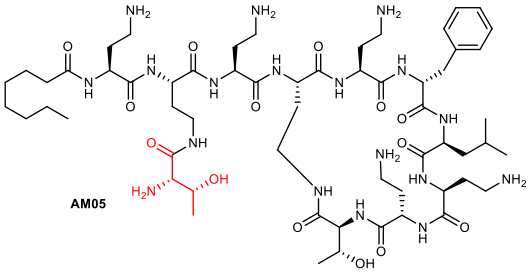
AM02



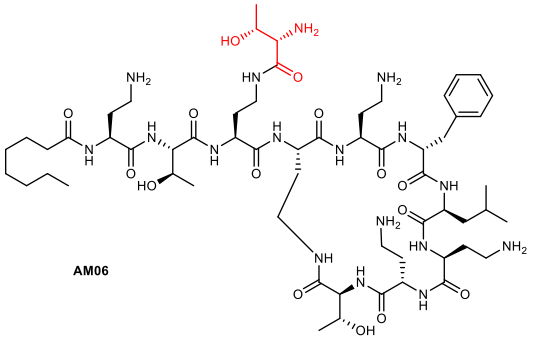
AM03



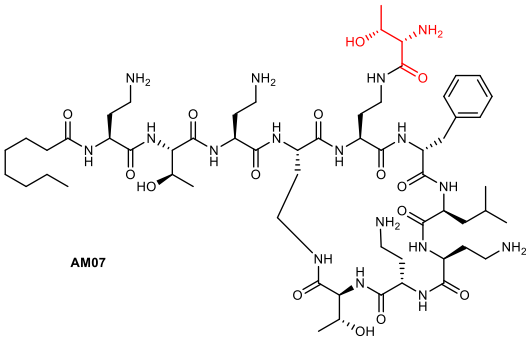
AM04



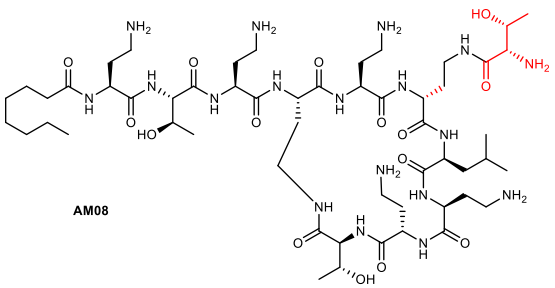
AM05



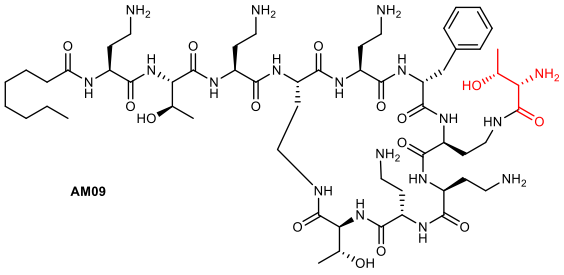
AM06



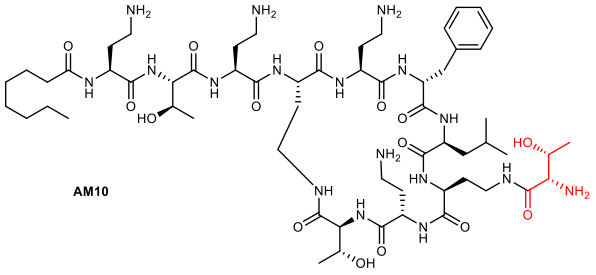
AM07



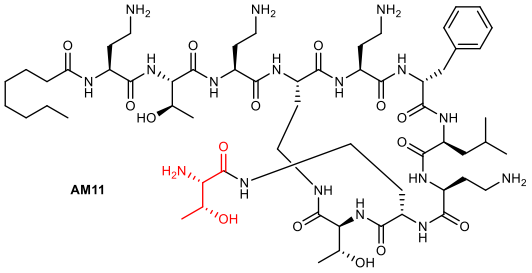
AM08



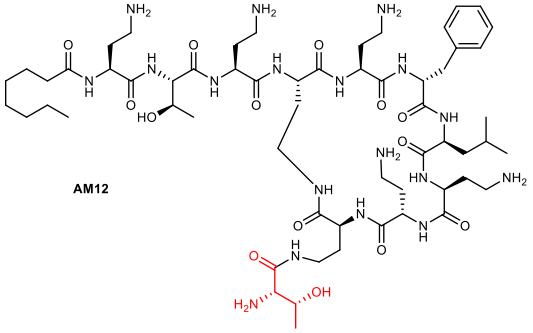
AM09



AM10



AM11



AM12

Table 1 Exploration 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. The most frequently observed values are shown in this figure. 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 supplementary 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	3.13	>50	12.5	3.13	>50	>50	1.56	50	50
AM05	50	>50	>50	50	>50	>50	50	50	>50
AM06	0.78	>50	3.13	3.13	>50	>50	0.39	25	50
AM07	25	>50	>50	50	>50	>50	25	>50	>50
AM08	>50	>50	>50	>50	>50	>50	>50	>50	>50
AM09	3.13	>50	>50	>50	>50	>50	3.13	>50	>50
AM10	0.39	>50	1.56	1.56	>50	>50	0.39	25	25
AM11	25	>50	50	50	>50	>50	12.5	50	>50
AM12	12.5	>50	>50	12.5	>50	>50	12.5	25	50
colistin	0.39	>50	0.78	0.78	>50	>50	0.05	6.25	6.25
polymyxin B	0.39	50	0.39	0.78	>50	>50	0.20	3.13	6.25

Fig. 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.

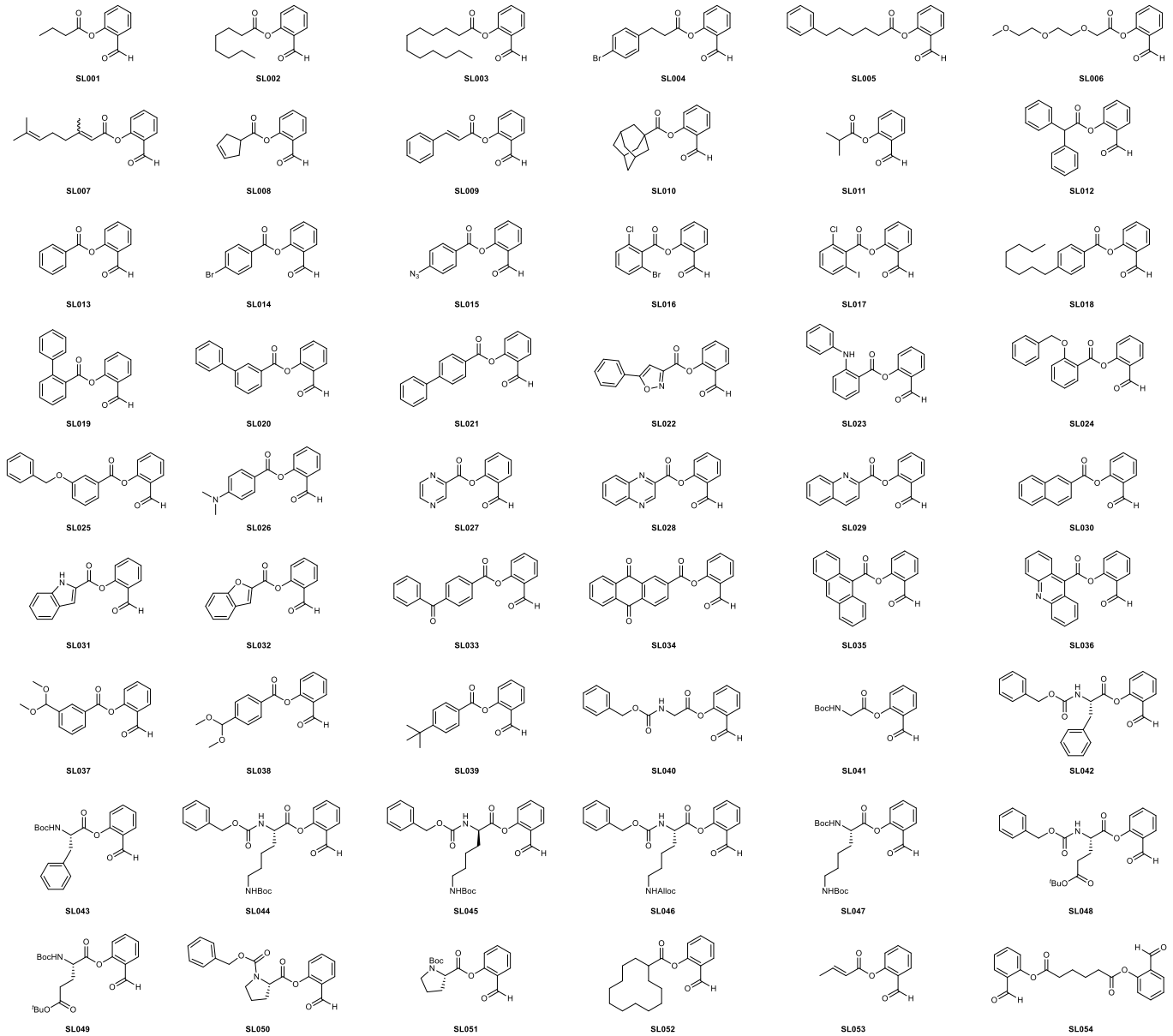
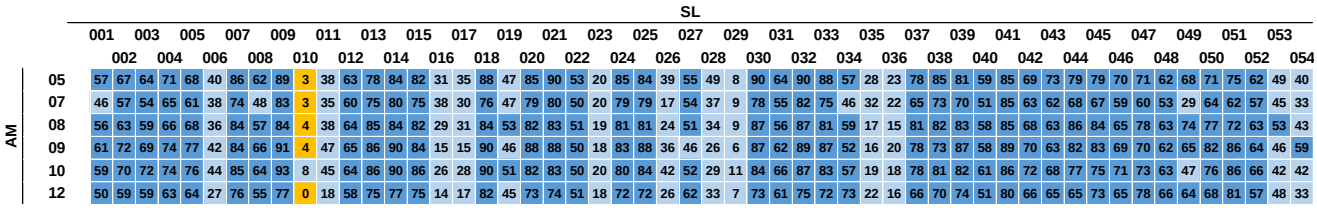
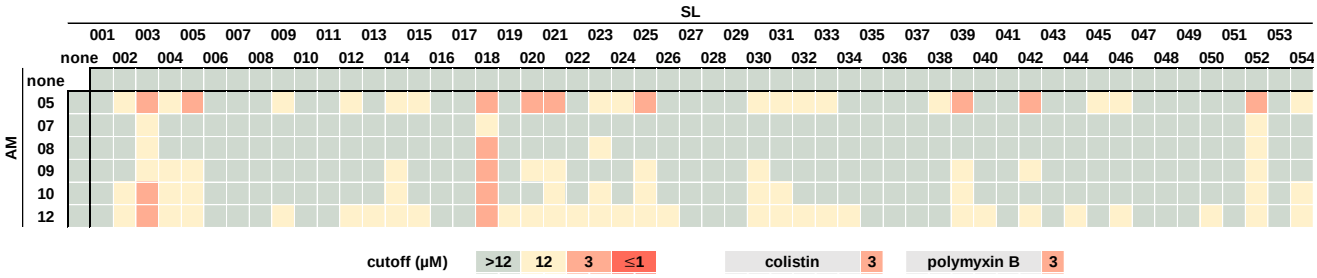


Fig. 6 Expansion stage to discover derivatives that show antibacterial activity against polymyxin-resistant *E. coli*. **a** Analysis of purity of the 324 series of polymyxin derivatives by the LC-MS. **b** Antibacterial activity screening of 324 series of polymyxin derivatives against *E. coli* SME98/plncl2_*mcr-1*. **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. The most frequently observed values are shown in this figure.

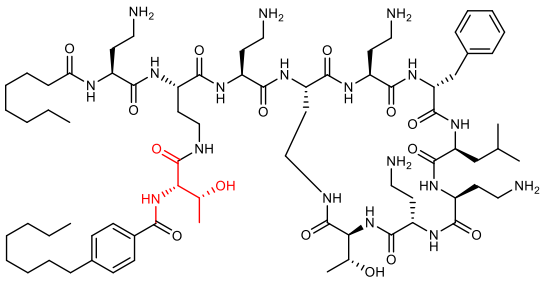
a



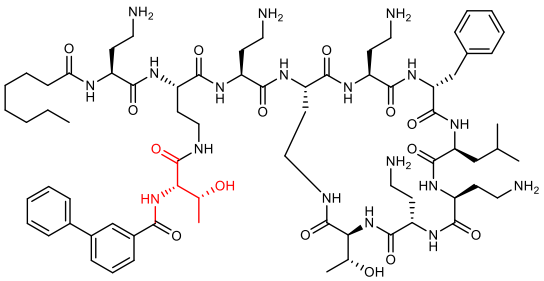
b



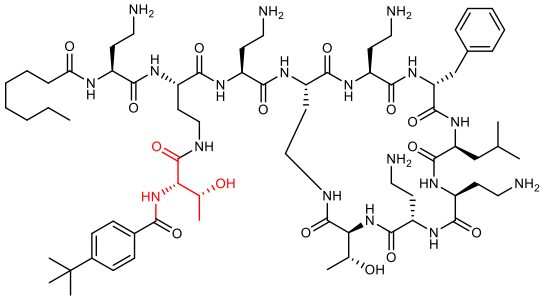
c



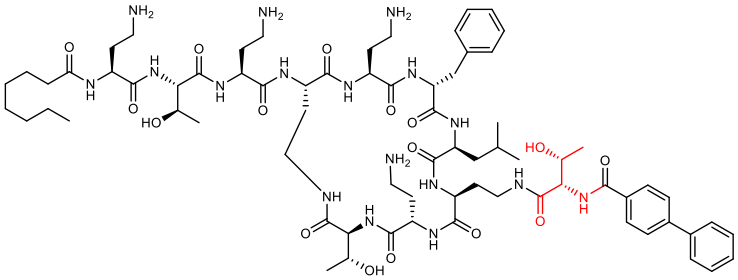
5 (main component of AM05SL018)



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	3.13	6.25
AM10	25	25	8	6.25	6.25
5	3.13	3.13	colistin	6.25	6.25
6	1.56	3.13	polymyxin B	3.13	6.25

weak activity — potent activity

Fig. 7 Expansion 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. The most frequently observed values are shown in this figure. 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 Supplementary Information.

a

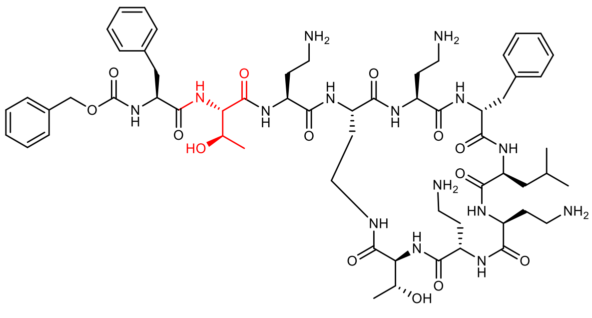
SL

AM	none	001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019	020	021	022	023	024	025	026	027
none		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
02	0	1	3	5	3	4	0	0	1	1	0	1	1	1	1	1	0	0	7	0	1	3	2	0	0	1	1	0
03	0	0	1	3	3	1	0	0	0	1	0	0	0	0	1	2	0	0	6	0	1	4	1	0	0	1	0	0
04	3	0	3	8	3	3	2	1	0	3	3	2	3	0	3	2	2	2	7	0	4	4	2	3	2	3	1	1
06	3	2	4	9	4	4	2	2	2	3	4	2	3	2	4	3	2	2	9	3	4	4	4	5	4	4	3	2
10	4	3	4	9	3	4	3	3	3	4	4	2	3	3	4	4	2	3	9	3	4	6	4	4	4	4	3	3
11	0	0	0	5	0	1	0	1	0	1	0	0	1	0	0	0	0	0	5	0	3	3	0	3	2	3	1	0

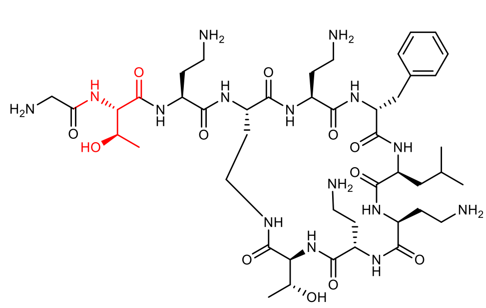
AM	028	029	030	031	032	033	034	035	036	037	038	039	040	041	042	043	044	045	046	047	048	049	050	051	052	053	054
none	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
02	0	0	1	0	1	1	3	0	0	0	0	1	1	1	1	1	1	0	0	0	0	0	1	0	1	1	3
03	0	0	1	1	1	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1
04	2	0	3	3	2	3	3	1	1	0	0	3	2	3	3	1	3	2	3	3	0	2	1	3	7	2	4
06	2	2	4	3	4	4	4	2	2	3	3	4	3	4	4	3	4	3	3	4	2	2	3	2	5	2	4
10	3	3	4	4	4	4	4	3	3	3	3	4	3	4	3	3	4	4	3	3	3	2	3	3	5	3	4
11	0	0	1	3	0	1	3	0	0	0	0	2	0	0	2	0	0	0	1	0	0	0	0	0	5	0	4

relative antibacterial spectrum 0 – 9 colistin 5 polymyxin B 6

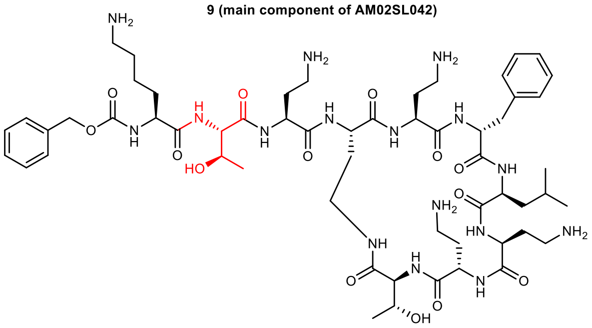
b



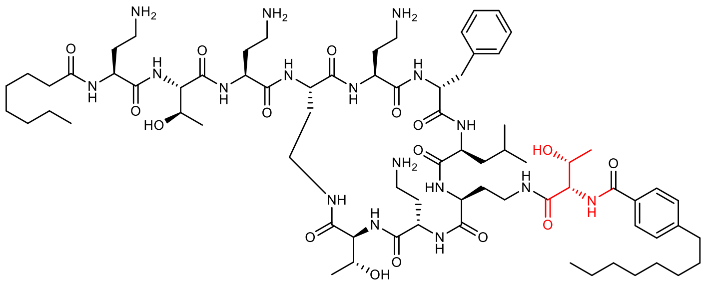
9 (main component of AM02SL042)



10 (main component of AM02SL041)



11 (main component of AM02SL044)



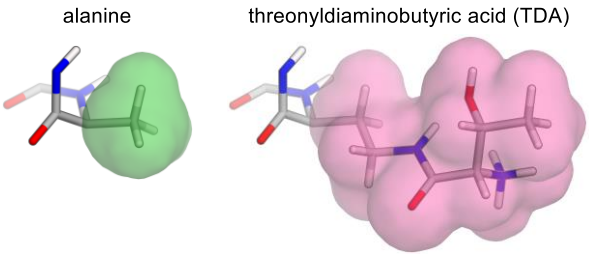
12 (main component of AM10SL018)

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	>50	1.56	1.56	>50	>50	0.39	25	25
9	25	>50	>50	3.13	>50	>50	12.5	25	50
10	12.5	>50	>50	1.56	>50	>50	25	>50	>50
11	6.25	>50	50	0.78	>50	>50	6.25	50	50
12	3.13	1.56	1.56	3.13	3.13	50, 1.56	1.56	3.13	1.56
colistin	0.39	>50	0.78	0.78	>50	>50	0.05	6.25	6.25
polymyxin B	0.39	50	0.39	0.78	>50	>50	0.20	3.13	6.25

weak activity – potent activity

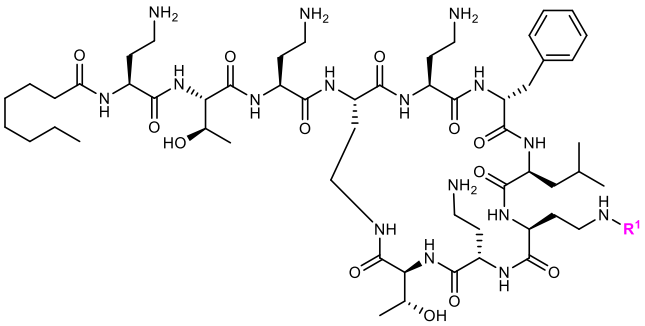
Fig. 8. The characteristic of TDA residue on the peptide scanning. a Comparison between alanine and TDA residues. TDA contains polar functional groups in a bulky side chain. **b** Structures and antibacterial activities of probe compounds of polymyxin antibiotics. The design of probe compounds was guided by a TDA scanning. The minimum inhibitory concentrations (MICs) were determined by performing the same experiment at least three times. The most frequently observed values are shown in this figure.

a

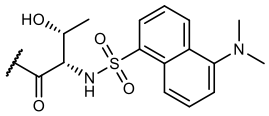


TDA:
suitable for scanning the permissibility of
introducing large and/or polar substituents

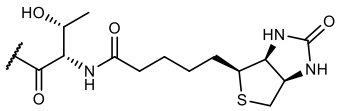
b



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	0.39	1.56	1.56
13	1.56	1.56	1.56	6.25
14	12.5	6.25	12.5	25

weak activity — potent activity