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The Synthesis and Bioactivity Investigation of the Individual Components of Cyclic Lipopeptide Antibiotics

A-Long Cui[‡], Xin-Xin Hu[‡], Yan Gao, Jie Jin, Hong Yi, Xiu-Kun Wang, Tong-Ying Nie, Yang Chen, Qi-Yang He, Hui-Fang Guo, Jian-Dong Jiang, Xue-Fu You, Zhuo-Rong Li**

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KEYWORDS: individual polymyxin component, synthesize, bioactivity evaluation, SAR and toxicity

ABSTRACT: In this paper, twenty-six natural polymyxin components and a new derivative S_2 were synthesized, and their differences in efficacy and toxicity have been investigated. Almost all of the synthesized components showed strong activity against both susceptible and resistant strains of *E. coli*, *K. pneumoniae*, *P. aeruginosa,* and *A. baumannii*. The toxicities were obviously different between the components. Only some of the components were tested for toxicity *in vivo*. Compounds E_2, E_2-Val, A_2, M_2, D_2, and S_2 showed obviously lower renal cytotoxicity and acute toxicity than polymyxins B and E. The *in vivo* nephrotoxicity of E_2, M_2, and S_2 were similar to that of polymyxin E. Compound S_2, with four positive charges, was especially interesting as it possessed both increased efficacy and decreased toxicity. The SAR

and toxicity studies indicated that further structural modification could concentrate on polymyxin S. The results also indicated that S_2 could be a new drug candidate.

INTRODUCTION

In February 2017, the World Health Organization published its first list of antibiotic-resistant critical pathogens and the "critical group" including carbapenem-resistant *A. baumannii*, *P. aeruginosa* and various *Enterobacteriaceae* (such as *E. coli*, *K. pneumoniae*, etc.). These bacteria are dangerous and can cause deadly bloodstream infections and pneumonia. Polymyxins were first discovered in 1947 and subsequently were used to treat infections caused by Gram-negative bacteria.[1-3] Unfortunately, their initial application in the 1970's was limited because of the occurrence of serious side effects, especially nephrotoxicity. The incidence of acute kidney injury induced by polymyxins occurred in up to 40-60% of patients.[4-7] Due to the lack of new antibiotics, polymyxins are now being employed as a last-resort therapy against infections caused by multidrug-resistant Gram-negative bacteria, especially *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*.[8] Therefore, further studies are urgently needed to better balance their efficacy and nephrotoxicity in clinical application.[9] Some recent studies have already demonstrated that improved dosing regimens are possible.[10-12]

Polymyxins are cyclic lipopeptide antibiotics manufactured via fermentation from different strains of *Paenibacillus polymyxa*, and the obtained lipopeptides are chemically unresolved mixtures that containing multiple structurally similar constituents. There are more than eleven distinct types of polymyxins that have been identified to date from the different fermentation products, including polymyxins A, B, C, D, E, F, K, M, P, S, and T.[13-14]

Currently, polymyxin B sulfate and colistimethate sodium (prodrug of colistin) are clinically available for the treatment of infections caused by Gram-negative superbugs. The clinically used polymyxins are cationic antimicrobial lipopeptides that possess a cyclic heptapeptide backbone with three positive charges, a linear tripeptide segment with two positive charges, and a hydrophobic fatty acyl chain. They have a common primary sequence, the differences being at the fatty acyl group and the amino acids occupying positions 6 and 7. A nomenclature for the polymyxins employs a subscript number for different acyl groups and different letters for different amino acids composition. For polymyxin B, the major components are polymyxins B_1 and B_2, whereas for polymyxin E (colistin), they are polymyxins E_1 (colistin A) and E_2 (colistin B) (Table 1).[8] Orwa et al isolated and characterized the individual components of commercial polymyxins B and E.[15–16] Polymyxins B_1, B_2, B_3, B_4, B_5, B_6 and B_1-Ile were isolated and characterized from polymyxin B, but the stereochemistry of the fatty acyl chain of B_6 was unidentified. Polymyxins E_1, E_2, E_3, E_4, E_7, E_1-Ile, E_1-Nva, E_1-Val, E_2-Ile, E_2-Nva, E_2-Val and E_8-Ile were isolated and characterized from polymyxin E, whereas the stereochemistry of the fatty acyl chain of E_8-Ile was unidentified. In the case of polymyxin B, a total of 39 possible components were speculated in the fermentation products using liquid chromatography coupled to mass spectrometry, with only six of them having defined chemical structures. As for polymyxin E, a total of 36 possible components were speculated, with only eleven of them having determined chemical structures (Table 1).[15-17]

Since the available polymyxins B and E are both complex mixtures, there has been concern about whether these individual constituents have identical pharmacological effects. The percentages of these major and minor components of polymyxins B and E can vary between different fermentation products. Different components and contents may not exhibit an

equivalent pharmacological activity and toxic propensity.[18-20] Tam et al were the first to purify and investigate the *in vitro* potency of five individual polymyxin B components, including polymyxins B_1, B_2, B_3, B_4, and B_1-Ile.[21] Kassamali et al obtained and evaluated the *in vitro* potencies of four polymyxin B components individually and in combination, including B_1, B_2, B_3 and B_1-Ile.[22] Roberts et al purified and investigated the *in vitro* and *in vivo* antimicrobial activities and renal toxicities of four major components of polymyxins B and E, including B_1, B_2, E_1, and E_2.[23] Polymyxins B_1 and E_1 showed a significantly higher (>3-fold) *in vitro* cytotoxicity to HK-2 cells than polymyxins B_2 and E_2, respectively. Sakura et al estimated the antimicrobial activities of the synthetic polymyxins B_1, B_2, B_3, B_4, B_5, B_6 and B_1-Ile.[24] Kline et al synthesized and evaluated the *in vitro* antimicrobial activities of polymyxins E_1, E_7, and E_1-Ile.[25] However, too little is understood about the pharmacological differences between the individual constituents, especially their toxicity differences. Therefore, we have synthesized and evaluated the antimicrobial activities and toxicities of the components of polymyxins B and E, including B_1, B_2, B_3, B_4, B_5, B_1-Ile, E_1, E_2, E_3, E_4, E_7, E_1-Ile, E_1-Nva, E_1-Val, E_2-Ile, E_2-Nva and E_2-Val.

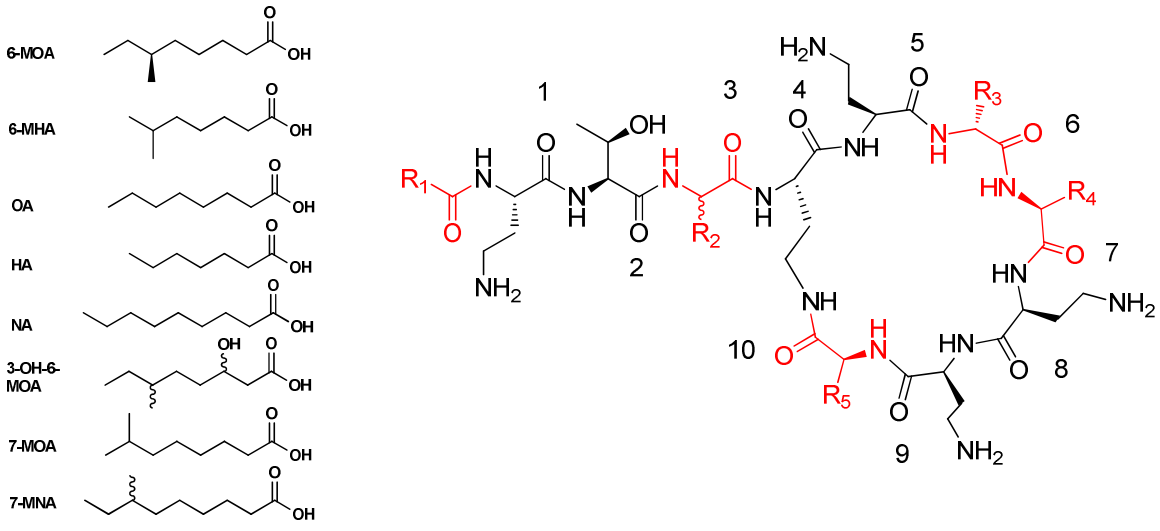
In addition to polymyxins B and E, we have also included the components of the polymyxins A, D, M, S, and T in our studies. Jones et al reported the chemical basis for the classification of polymyxins A, B, C, D, and E,[26] while Brownlee et al reported the chemotherapy and pharmacology of polymyxins A, B, C, D, and E.[27-28] Polymyxin B and polymyxin E were introduced into clinical medicine due to their lower renal toxicity compared with polymyxin A and polymyxin D.[27-28] The amino acid configurations in polymyxins C and F were undetermined.[13-14] The structure of the hydroxy fatty acyl chain of polymyxin K was also unidentified.[13-14] Moreover, Niu et al concluded that polymyxins P_1 and P_2 from *Paenibacillus polymyxa* M-1 contained the same fatty acyl chain, which is consistent with the data reported by

Kimura et al.[29-30] Polymyxins A and M were initially considered identical, but ultimately proven to be different.[31-32] Shoji et al reported the differences in the structure and the pharmacology between polymyxins S_1 and T_1 (containing T_2).[13]

Therefore, polymyxins A, D, M, S, and T have known structures, but almost all of the antimicrobial activities or toxicities were tested using unspecified mixtures. Thus, we have also synthesized and studied the antimicrobial activities and toxicities of polymyxin A, D, M, S, and T individual components, including A_1, A_2, D_1, D_2, M_1, M_2, S_1, T_1, and T_2 as well as a new derivative S_2.

All of the synthesized individual components were initially evaluated for their *in vitro* antibacterial activity and renal toxicity. A subset of the different lipopeptide components was then examined to determine the acute toxicity and nephrotoxicity in ICR mice. In addition, we have summarized the SAR and structure-toxicity relationship (STR) of polymyxins based on natural individual components.

Table 1. Chemical Structures of the Individual Components of Polymyxins



polymyxins	fatty acid (R_1)	position 3 amino acid (R_2)	position 6 amino acid (R_3)	position 7 amino acid (R_4)	position 10 amino acid (R_5)
B_1[a]	6-MOA	L-Dab	D-Phe	L-Leu	L-Thr
B_2[a]	6-MHA	L-Dab	D-Phe	L-Leu	L-Thr
B_3[a]	OA	L-Dab	D-Phe	L-Leu	L-Thr
B_4[a]	HA	L-Dab	D-Phe	L-Leu	L-Thr
B_5[a]	NA	L-Dab	D-Phe	L-Leu	L-Thr
B_6[b]	3-OH-6-MOA	L-Dab	D-Phe	L-Leu	L-Thr
B_1-Ile[a]	6-MOA	L-Dab	D-Phe	L-Ile	L-Thr
E_1[a]	6-MOA	L-Dab	D-Leu	L-Leu	L-Thr
E_2[a]	6-MHA	L-Dab	D-Leu	L-Leu	L-Thr
E_3[a]	OA	L-Dab	D-Leu	L-Leu	L-Thr
E_4[a]	HA	L-Dab	D-Leu	L-Leu	L-Thr
E_7[a]	7-MOA	L-Dab	D-Leu	L-Leu	L-Thr
E_1-Ile[a]	6-MOA	L-Dab	D-Leu	L-Ile	L-Thr
E_1-Nva[a]	6-MOA	L-Dab	D-Leu	L-Nva	L-Thr
E_1-Val[a]	6-MOA	L-Dab	D-Leu	L-Val	L-Thr
E_2-Ile[a]	6-MHA	L-Dab	D-Leu	L-Ile	L-Thr
E_2-Nva[a]	6-MHA	L-Dab	D-Leu	L-Nva	L-Thr
E_2-Val[a]	6-MHA	L-Dab	D-Leu	L-Val	L-Thr
E_8-Ile[b]	7-MNA	L-Dab	D-Leu	L-Ile	L-Thr
A_1[a]	6-MOA	D-Dab	D-Leu	L-Thr	L-Thr
A_2[a]	6-MHA	D-Dab	D-Leu	L-Thr	L-Thr
M_1[a]	6-MOA	L-Dab	D-Leu	L-Thr	L-Thr
M_2[a]	6-MHA	L-Dab	D-Leu	L-Thr	L-Thr

D_1[a]	6-MOA	D-Ser	D-Leu	L-Thr	L-Thr
D_2[a]	6-MHA	D-Ser	D-Leu	L-Thr	L-Thr
S_1[a]	6-MOA	D-Ser	D-Phe	L-Thr	L-Thr
S_2[a,c]	6-MHA	D-Ser	D-Phe	L-Thr	L-Thr
T_1[a]	6-MOA	L-Dab	D-Phe	L-Leu	L-Leu
T_2[a]	6-MHA	L-Dab	D-Phe	L-Leu	L-Leu

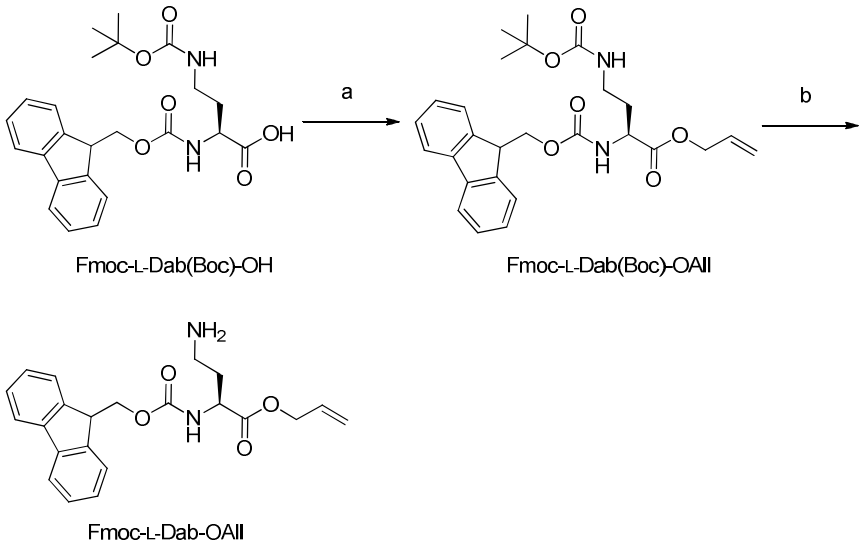
[a] The synthesized compound. [b] Chemical structure of an undetermined compound. [c] New compound. Dab, 2,4-diaminobutyric acid; L-Val, L-valine; 6-MOA, (*S*)-6-methyloctanoic acid; 6-MHA, 6-methylheptanoic acid; OA, octanoic acid; HA, heptanoic acid; NA, nonanoic acid; 3-OH-6-MOA, 3-hydroxy-6-methyloctanoic acid; 7-MOA, 7-methyloctanoic acid; 7-MNA, 7-methylnonanoic acid.

RESULTS AND DISCUSSION

Chemistry. We used the recently reported method employing on-resin cyclization to synthesize the target compounds (Scheme 4).[33] The first step was the O-allyl ester protection of the C-terminal amino acid Fmoc-L-Dab(Boc)-OH (Scheme 1). The (*S*)-6-methyloctanoic acid was synthesized according to the literature (Scheme 2).[34] The 7-methyloctanoic acid was synthesized from 7-methyl-7-octenoic acid (Scheme 3). The side-chain amine of Fmoc-L-Dab-OAll was anchored on the 2-chlorotrityl chloride resin (2CTC resin) in the presence of DIPEA in DCM. The linear peptide was synthesized via standard solid-phase peptide synthesis using a consecutive Fmoc group deprotection strategy followed by an amino acid coupling reaction. In addition, a Dde group was employed as the protecting group for the side-chain amino of Dab4 due to its stability in the Fmoc strategy. After capping the peptide with a fatty acid and removing the Dde group, Fmoc-L-Thr(tBu)-OH or Fmoc-L-Leu-OH was coupled to the side-chain amino of

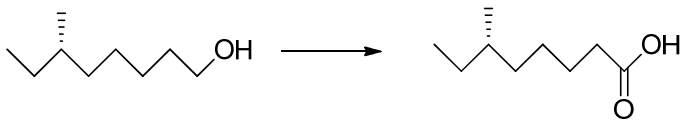
Dab4. Subsequently, on-resin cyclization was conducted following the deprotection of the allyl group on Dab9 and Fmoc group on Thr10, followed by cyclization using PyAOP, HOAt and NMM in DMF at room temperature. Finally, the cyclic peptide was cleaved from the resin using a solution of TFA:TIS:H_2O (95:2.5:2.5). The crude peptide was purified by using semi-preparative HPLC to afford the polymyxin TFA salt, and the total yield was approximately 30% based on the initial resin with a purity >95%. Polymyxin S_2 is a new compound not reported in the literature.

Scheme 1. Synthetic Route of Fmoc-L-Dab-OAll



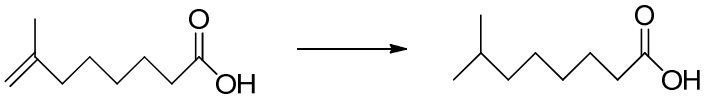
Reagents and conditions: (a) $NaHCO_3$, H_2O, allyl bromide, Aliquat 336, DCM; (b) CF_3COOH, DCM.

Scheme 2. Synthetic Route of (*S*)-6-methyloctanoic acid



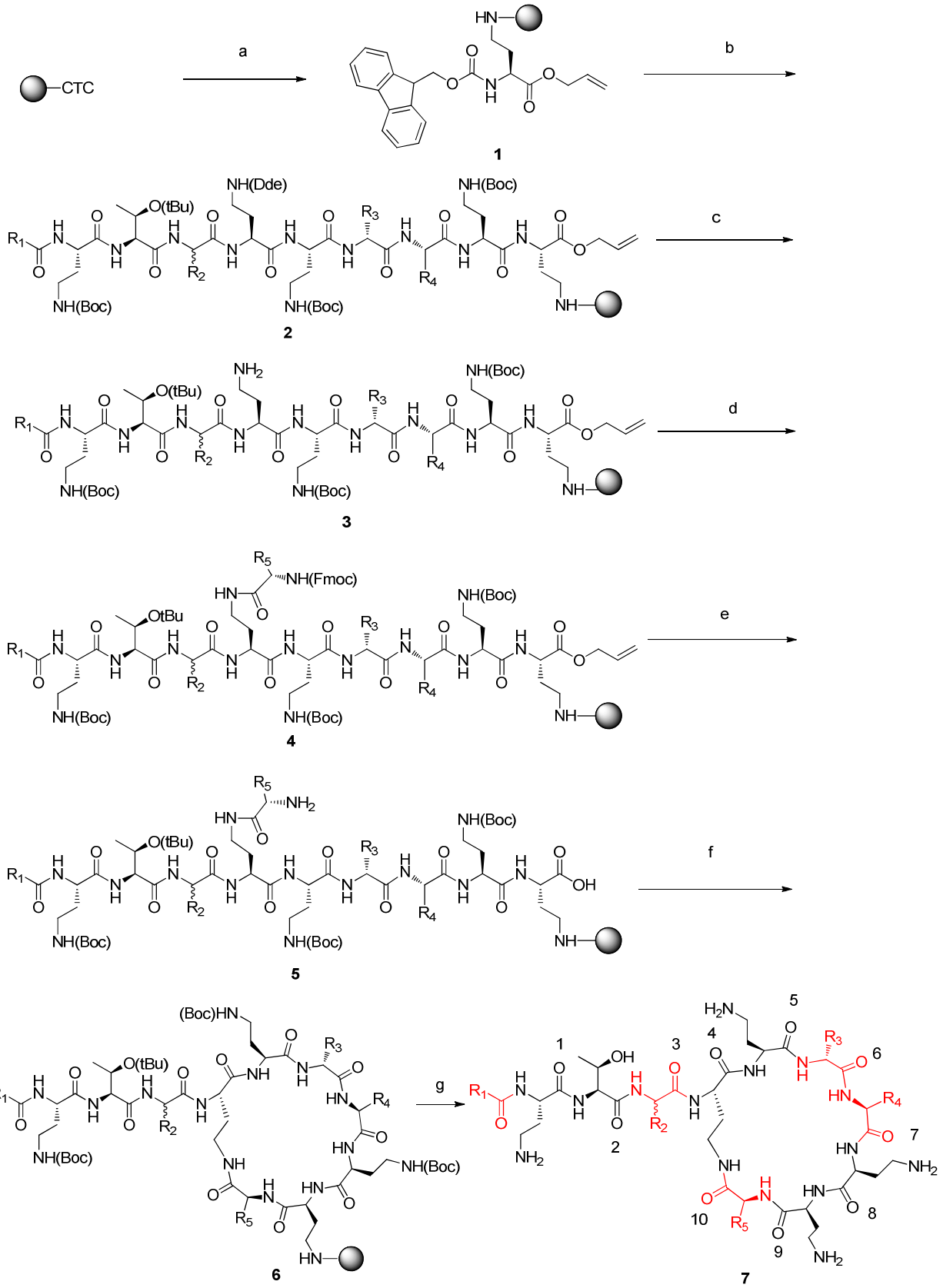
Reagents and conditions: TEMPO, NaClO, $NaClO_2$, CH_3CN, H_2O.

Scheme 3. Synthetic Route of 7-methyloctanoic acid



Reagents and conditions: H_2, Pd/C, MeOH.

Scheme 4. Synthetic Route of the Individual Components of Polymyxins


CTC
a
b
1
NH(Dde)
O(tBu)
NH(Boc)
c
2
NH2
d
3
OtBu
NH(Fmoc)
e
4
f
5
OH
(Boc)HN
g
6
7

Polymyxin B: R_2 = L-Dab, R_3 = D-Phe, R_5 = L-Thr. Polymyxin E: R_2 = L-Dab, R_3 = D-Leu, R_5 = L-Thr.
Polymyxin T: R_2 = L-Dab, R_3 = D-Phe, R_5 = L-Leu.
B_1 , E_1 and T_1: R_1 = 6-MOA, R_4 = L-Leu; B_2 , E_2 and T_2: R_1 = 6-MHA, R_4 = L-Leu;
B_3 and E_3: R_1 = OA, R_4 = L-Leu; B_4 and E_4: R_1 = HA, R_4 = L-Leu;
B_5: R_1 = NA, R_4 = L-Leu; E_7: R_1 = 7-MOA, R_4 = L-Leu;
B_1-Ile and E_1-Ile: R_1 = 6-MOA, R_4 = L-Ile; E_2-Ile: R_1 = 6-MHA, R_4 = L-Ile;
E_1-Nva: R_1 = 6-MOA, R_4 = L-Nva; E_2-Nva: R_1 = 6-MHA,R_4 = L-Nva;
E_1-Val: R_1 = 6-MOA, R_4 = L-Val; E_2-Val: R_1 = 6-MHA, R_4 = L-Val;
Polymyxin A, D and M: R_3 = D-Leu, R_4 = L-Thr, R_5 = L-Thr. Polymyxin S: R_3 = D-Phe, R_4 = L-Thr, R_5 = L-Thr.
A_1: R_1 = 6-MOA, R_2 = D-Dab; A_2: R_1 = 6-MHA, R_2 = D-Dab;
D_1 and S_1: R_1 = 6-MOA, R_2 = D-Ser; D_2 and S_2: R_1 = 6-MHA, R_2 = D-Ser;
M_1: R_1 = 6-MOA, R_2 = L-Dab; M_2: R_1 = 6-MHA, R_2 = L-Dab;
fatty acid (R_1), position 3 amino acid (R_2), position 6 amino acid (R_3), position 7 amino acid (R_4), position 10 amino acid (R_5)

Reagents and conditions: (a) Fmoc-L-Dab-OAll, DIPEA, DCM, 2 h, MeOH; (b) (i) 20% piperidine in DMF, 5 min and 10 min; (ii) appropriate amino acid, HCTU, DIPEA, DMF, 1 h; (iii) corresponding fatty acid, HCTU, DIPEA, DMF, 1 h; (c) 3% hydrazine in DMF, 2×30 min; (d) Fmoc-L-Thr(tBu)-OH or Fmoc-L-Leu-OH, HCTU, DIPEA, DMF, 1 h; (e) (i) $PhSiH_3$, $Pd(PPh_3)_4$, 50% DCM/50% DMF, 2 h; (ii) 20% piperidine in DMF, 5 min and 10 min; (f) PyAOP, HOAt, NMM, DMF, 3 h; (g) TFA:TIS:H_2O (95:2.5:2.5), 2 h; followed by reverse phase HPLC purification.

The *in vitro* antimicrobial activity. The *in vitro* antimicrobial activities of individual polymyxin components were assessed against a panel of the key Gram-negative pathogens (Table 2). Drug-susceptible and drug-resistant ATCC reference strains and clinical isolates were used, including *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*. Overall, almost all of the individual components showed effective activity against the tested Gram-negative pathogens. Additionally, all of the polymyxin B individual components had comparable MICs (0.25-2 μg/mL) against the tested Gram-negative bacteria compared with clinically used polymyxin B (0.5-2 μg/mL), except B_5 (2-4 μg/mL). Meanwhile, all of the polymyxin E individual components had comparable MICs (0.25-2 μg/mL) with that of clinically used polymyxin E (0.5-1 μg/mL). These results suggested that the fatty acyl chain length of the natural components appeared to be C7-C9, as

component B_5 with a longer fatty acyl chain displayed a noticeably reduced antimicrobial activity (approximately 2-fold). Additionally, whether position 6 was occupied by D-Phe or D-Leu, or Leu-7 was substituted with other hydrophobic amino acids such as L-Ile7, L-Nva7 and L-Val7, did not affect the antimicrobial potency. Polymyxins M_1 (≤0.03-1 μg/mL) and M_2 (0.25-0.5 μg/mL) showed noticeably enhanced antimicrobial activities (approximately 2-fold) against *E. coli*, *K. pneumoniae*, and *P. aeruginosa* compared with polymyxins E_1 (0.5-2 μg/mL) and E_2 (0.5-2 μg/mL). Meanwhile, polymyxins A_1 (0.5-1 μg/mL) and A_2 (0.25-1 μg/mL) showed similar antimicrobial activities in comparison with M_1 (≤0.03-1 μg/mL) and M_2 (0.25-1 μg/mL). These results suggested that the substitution of L-Leu7 with a more polar amino acid such as L-Thr could noticeably increase the activity, and the configuration of Dab3 would not affect the antimicrobial activity. Polymyxins D_1 (0.12-1 μg/mL), D_2 (0.12-0.5 μg/mL), S_1 (0.25-1 μg/mL), and S_2 (0.25-0.5 μg/mL) with four positive charges showed similar antimicrobial potencies against *E. coli* and *K. pneumoniae* but noticeably reduced potencies (2-4 μg/mL) against *P. aeruginosa* compared with polymyxins A and M. Additionally, polymyxins D_1 (0.06 μg/mL), D_2 (0.12 μg/mL), S_1 (0.12 μg/mL), and S_2 (0.25 μg/mL) showed noticeably enhanced antimicrobial activity (≥2-fold) against *A. baumannii* compared with polymyxins A and M. These results showed that the substitution of the positive charge of L-Dab3 or D-Dab3 with a polar amino acid such as D-Ser3 could noticeably enhance the antimicrobial activity against *A. baumannii* but noticeably decrease the antimicrobial activity against *P. aeruginosa*. Meanwhile, charges of five Dab residues seemed to be more important for the antimicrobial potency against *P. aeruginosa*. Polymyxins T_1 (2-8 μg/mL) and T_2 (4-8 μg/mL), with an L-Leu10 residue, showed noticeably decreased antimicrobial potencies (approximately 4-fold) compared with polymyxins B_1 (0.25-2 μg/mL) and B_2 (0.5-2 μg/mL). The results indicated that the substitution of L-Thr10 with a more

hydrophobic amino acid such as L-Leu could noticeably reduce the antimicrobial activity against all of the tested Gram-negative bacteria.

The *in vitro* nephrotoxicity. We assessed the renal cell cytotoxicity (CC_{50}) of the synthesized individual components against immortalized Vero kidney cells of the African green monkey (Table 2). The renal toxicities of these different types of individual components were obviously different. Polymyxin E (154±13 μg/mL) showed noticeably reduced renal cytotoxicity ($P<0.01$) compared with polymyxin B (103±13 μg/mL). Polymyxins B_2 (159±14 μg/mL) and B_4 (160±14 μg/mL) displayed noticeably reduced toxicities (approximately 2-fold) compared with polymyxins B_1 (71±6 μg/mL) and B_3 (86±8 μg/mL). However, polymyxin B_5 (33±3 μg/mL) showed significantly increased renal cytotoxicity (>4-fold) compared with polymyxin B_2. Polymyxins E_2 (244±28 μg/mL) and E_4 (216±16 μg/mL) also displayed noticeably reduced toxicities (>2-fold) to Vero cells compared with polymyxins E_1 (95±8 μg/mL) and E_7 (87±7 μg/mL). Polymyxins E_2-Ile, E_2-Nva, and E_2-Val showed comparable toxicities with polymyxin E_2. These results indicated that the polymyxin fatty acyl chain length of C7 showed lower renal cytotoxicity than a chain length of C8 or C9. Additionally, Leu-7 was substituted with other hydrophobic amino acids such as L-Ile7, L-Nva7 and L-Val7 did not remarkably affect the renal cytotoxicity. Polymyxins M_2 (276±17 μg/mL) and A_2 (288±23 μg/mL) showed similar renal cytotoxicities compared with polymyxin E_2. These results suggested that the substitution of L-Leu7 with a more polar amino acid such as L-Thr and the configuration of Dab3 would not affect the renal cytotoxicity. Polymyxins D_2 (318±26 μg/mL) and S_2 (>500 μg/mL) with four positive charges showed decreased renal cytotoxicities compared with polymyxins M_2 and A_2. These results showed that the substitution of the positive charges of L-Dab3 or D-Dab3 with a polar amino acid such as D-Ser3 could decrease the renal cytotoxicity. However, polymyxins T_1 (17±2

μg/mL) and T_2 (30±3 μg/mL) showed significantly increased toxicities (>4-fold) compared with polymyxins B_1 (71±6 μg/mL) and B_2 (159±14 μg/mL), respectively. These results indicated that the substitution of L-Thr10 with a more hydrophobic amino acid such as L-Leu10 could significantly increase the renal cytotoxicity.

Table 2. Minimum Inhibitory Concentrations (MICs) and Cytotoxicity (CC_{50}) of the Individual Components of Polymyxins

compound	MICs (μg/mL)						CC_{50}[b] (μg/mL)		
	E. coli		*K. pneumoniae*		*P. aeruginosa*	*A. baumannii*	Vero cells	P value, *vs*	
	ATCC 25922	14-11[a] ESBLs	ATCC 700603 ESBLs	ATCC BAA-2146 NDM-1	ATCC 27853	ATCC 19606		E	B
B	1	0.5	1	1	2	0.5	103±13	-	-
E	0.5	0.5	1	0.5	1	1	154±13	-	-
B_1	1	0.5	2	0.25	2	0.25	71±6	<0.01	<0.01
B_2	0.5	0.5	1	1	2	0.5	159±14	0.653	<0.01
B_3	0.5	1	1	1	1	0.5	86±8	<0.01	0.147
B_4	0.5	0.5	1	0.5	1	0.5	160±14	0.571	<0.01
B_5	2	2	2	4	4	2	33±3	<0.01	<0.01
B_1-Ile	1	2	2	1	2	1	75±6	<0.01	<0.05
E_1	1	0.5	1	1	2	0.5	95±8	<0.01	0.454
E_2	0.5	0.5	1	1	2	0.5	244±28	<0.01	<0.01
E_3	0.5	0.5	1	0.5	1	0.5	150±18	0.793	<0.05
E_4	1	0.5	0.5	0.5	1	2	216±16	<0.01	<0.01

E_7	1	1	1	1	2	1	87±7	<0.01	0.086
E_1-Ile	1	0.5	1	1	2	1	176±15	0.115	<0.01
E_1-Nva	1	1	2	1	1	1	109±9	<0.01	0.434
E_1-Val	0.5	0.5	1	0.5	2	0.5	131±9	0.060	<0.05
E_2-Ile	1	0.5	0.5	1	2	1	199±13	<0.05	<0.01
E_2-Nva	0.5	0.25	0.5	0.5	1	0.5	168±12	0.225	<0.01
E_2-Val	0.5	0.25	0.5	0.5	2	1	225±17	<0.01	<0.01
A_1	0.5	1	1	1	1	1	145±12	0.427	<0.01
A_2	0.5	0.25	1	0.5	1	0.5	288±23	<0.01	<0.01
M_1	0.25	≤0.03	0.25	0.5	1	0.5	96±9	<0.01	0.444
M_2	0.5	0.25	0.5	0.5	0.5	1	276±18	<0.01	<0.01
D_1	0.12	0.25	1	0.5	2	0.06	189±11	<0.05	<0.01
D_2	0.25	0.12	0.5	0.5	4	0.12	318±26	<0.01	<0.01
S_1	0.25	0.5	1	0.5	4	0.12	160±23	0.686	<0.05
S_2	0.25	0.25	0.5	0.5	2	0.25	>500	<0.01	<0.01
T_1	4	4	8	4	4	2	17±2	<0.01	<0.01
T_2	8	4	4	4	4	4	30±3	<0.01	<0.01

[a] Clinical isolate. [b] 50% cytotoxicity concentration. ESBLs, extended-spectrum β-lactamases; NDM-1, New Delhi Metallo-beta-lactamase-1. The MICs were the representative results of at least two independent experiments, in the case of different results for the two experiments, a third experiment was conducted, and the confirmed results were given. The CC_{50} values were indicated as mean ± SD values and calculated from three independent experiments, each performed in triplicate. Statistical analysis was performed with the Student's t-test using SPSS 16.0.

Acute toxicity. All animal experiments were performed in full compliance with the protocols approved by the Animal Ethics Committee of the Institute of Medicinal Biotechnology (Beijing, China). The acute toxicity using ICR mice was evaluated after the intravenous injection of varying doses of components into four to five groups (weight, 18 to 22 g; n=10 mice per group; 5 male, 5 female) for one compound. The LD_{50} values (Table 3) were calculated according to the Bliss test protocol. One-way analysis of variance (ANOVA) was used for comparisons of LD_{50} values. Near-lethal doses caused immediate signs of the pallor of the ears, convulsions, muscular incoordination, and respiratory distress. Death from respiratory failure occurred within 5 min with lethal doses. The different types of these lipopeptide components with similar or improved antibacterial activity were examined to determine their acute toxicities. In summary, all of the tested individual components displayed lower acute toxicity than polymyxin B mixtures and polymyxin E mixtures. Polymyxins B_2 (LD_{50}, 12.6 mg/kg), E_2 (LD_{50}, 11.3 mg/kg), E_2-Ile (LD_{50}, 12.9 mg/kg), E_2-Nva (LD_{50}, 12.4 mg/kg), and E_2-Val (LD_{50}, 13.2 mg/kg) displayed significantly reduced acute toxicities ($P<0.01$) compared with polymyxins B (LD_{50}, 6.9 mg/kg) and E (LD_{50}, 8.5 mg/kg), respectively. These results indicated that the individual synthetic components were less toxic than the natural mixtures used clinically. In addition, the differences in the acute toxicities between mixtures and individual components were probably associated with impurities. Additionally, whether position 6 was occupied by D-Phe or D-Leu, or Leu-7 was substituted with other hydrophobic amino acids such as L-Ile7, L-Nva7 and L-Val7, did not affect the acute toxicity. Polymyxins M_1 (LD_{50}, 11.4 mg/kg) and M_2 (LD_{50}, 13.0 mg/kg) showed the similar acute toxicities compared with polymyxin E_2. Polymyxin A_2 (LD_{50}, 12.6 mg/kg) showed a similar acute toxicity compared with polymyxin M_2. These results suggest that a fatty acyl chain length of C7-C8 of the natural components, the configuration of Dab3, and the substitution

of L-Leu7 with a more polar amino acid such as L-Thr would not affect the acute toxicity. Additionally, polymyxins D_2 (LD_{50}, 20.5 mg/kg) and S_2 (LD_{50}, 17.0 mg/kg) with four positive charges showed significantly reduced acute toxicities ($P<0.01$) compared with polymyxins A_2 and M_2. These results suggest that the substitution of the positive charges of Dab3 or D-Dab3 with polar amino acid such as D-Ser3 could significantly reduce the acute toxicity.

Table 3. Acute Toxicity (LD_{50}) of the Individual Components of Polymyxins

compound	Acute toxicity			
	LD_{50} value (mg/kg)	95% Confidence interval (mg/kg)	P value, *vs*	
			E	B
B	6.9	6.5−7.3	-	-
E	8.5	8.0−9.0	-	-
B_2	12.6	11.9−13.5	<0.01	<0.01
E_2	11.3	10.8−11.8	<0.01	<0.01
E_2-Ile	12.9	11.9−13.9	<0.01	<0.01
E_2-Nva	12.4	11.7−13.1	<0.01	<0.01
E_2-Val	13.2	12.5−14.0	<0.01	<0.01
A_2	12.6	11.9−13.5	<0.01	<0.01
M_1	11.4	10.8−12.1	<0.01	<0.01
M_2	13.0	12.0−14.0	<0.01	<0.01
D_2	20.5	18.8−22.4	<0.01	<0.01
S_2	17.0	15.8−18.2	<0.01	<0.01

The LD_{50} values were calculated according to the Bliss test protocol. One-way analysis of variance (ANOVA) was used for comparisons of LD_{50} values.

The *in vivo* nephrotoxicity. The *in vivo* renal toxicities of the different types of these major lipopeptide components with similar or improved antibacterial activity and cytotoxicity were examined in a mouse nephrotoxicity model reported by Yousef et al.[35-36] The ICR mice (weight, 18 to 22 g; n=8 mice per group; 4 male, 4 female) were intravenously administered the component at a dose of 2.5 mg/kg twice daily for 7 days (an accumulated dose of 35 mg/kg), followed by histological examination (Table 4, Figure 1). In preliminary studies, histological examination of the kidneys from the mice injected with polymyxins A_2 (SQS +1), B_2 (SQS +1), and D_2 (SQS +1) showed mild damage compared with those injected with polymyxin E and the saline controls. Meanwhile, polymyxins E_2 (SQS 0→+1), M_2 (SQS 0→+1), and S_2 (SQS 0→+1) showed no significant change or mild damage compared with the saline control.

Table 4. Histological Examination Results of the Individual Components of Polymyxins

compound	n	SQS kidney histology score[a]
Polymyxin E	8	0→+1
saline	8	0
B_2	8	+1
E_2	8	0→+1
A_2	8	+1
M_2	8	0→+1
D_2	8	+1
S_2	8	0→+1

[a] SQS 0=no significant change; SQS +1=mild damage; SQS +2=mild to moderate damage.

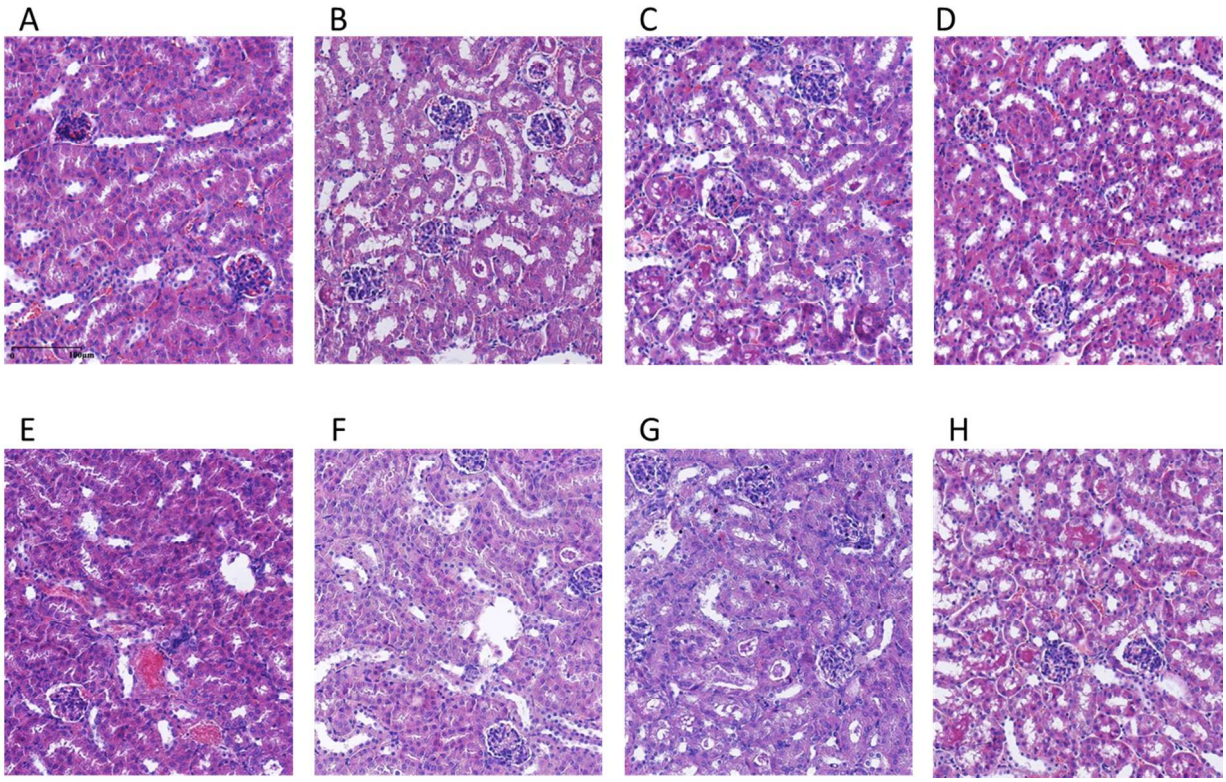


Figure 1. Representative histological images of: (A) mouse kidney from the saline control group showing no histological damage; (B), (C), (D), (E), (F), and (G) mice kidneys after exposure to polymyxins A_2, B_2, D_2, E_2, M_2, and S_2 with grade 1 lesions (SQS=+1), showing tubular dilatation, prominent nuclei, and a few pale tubular casts; (H) mouse kidney after exposure to the polymyxin E control group with grade 1 lesions (SQS=+1). Original magnification: x200 for all panels.

CONCLUSIONS

In this paper, a systematic study on the synthetic preparation and biological activity of different components of polymyxins was reported for the first time. Twenty-six reported natural individual components, including B_1, B_2, B_3, B_4, B_5, B_1-Ile, E_1, E_2, E_3, E_4, E_7, E_1-Ile, E_1-Nva, E_1-Val, E_2-Ile, E_2-Nva, E_2-Val, A_1, A_2, D_1, D_2, M_1, M_2, S_1, T_1, T_2, and a new derivative S_2 were

synthesized, and the differences in efficacy and toxicity between the individual components were investigated.

The antimicrobial activity was tested against ATCC reference strains and clinical isolates. Almost all of the synthesized individual components of polymyxins showed very strong antimicrobial activity against *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*, except for polymyxins B_5 (NA), T_1 (L-Leu10), and T_2 (L-Leu10). Additionally, polymyxins A_1, A_2, M_1 and M_2 with L-Thr7 showed much stronger activity against *E. coli*, *K. pneumoniae*, and *P. aeruginosa* than the controls polymyxins B and E. Polymyxins D_1, D_2, S_1, and S_2 with D-Ser3 showed noticeably enhanced antimicrobial activity against *E. coli*, *K. pneumoniae*, and *A. baumannii*, but noticeably reduced potency against *P. aeruginosa* compared with the controls. The increased hydrophobicity at the fatty acyl or position 7, position 10 amino acid residues could decrease the *in vitro* activity. The renal cytotoxicities of E_2, E_4, E_2-Val, A_2, M_2, D_2, and S_2 were significantly lower ($P<0.01$) than those of polymyxins B and E. In contrast, the cytotoxicities of B_5, T_1, and T_2 were significantly higher ($P<0.01$) than those of the controls. The increased hydrophobicity at the fatty acyl or position 10 amino acid residue could increase the renal cytotoxicity. The different types of these major lipopeptide components (B_2, E_2, A_2, M_2, D_2, and S_2) and other different types of these components (E_2-Ile, E_2-Nva, E_2-Val, and M_1) with equal or better antibacterial activity were examined to determine their acute toxicities in ICR mice. The acute toxicities of all the tested compounds were significantly lower ($P<0.01$) than those of the controls polymyxins B (6.9 mg/kg) and E (8.5 mg/kg). The different types of these major lipopeptide components (B_2, E_2, A_2, M_2, D_2, and S_2) with equal or better antibacterial activity and lower cytotoxicity were examined to determine the nephrotoxicity in ICR mice. The *in vivo* renal toxicities of polymyxins E_2, M_2, and S_2 in ICR mice showed no significant change

or mild damage compared with the saline control. Polymyxin individual components with reduced renal cytotoxicities showed reduced acute toxicities compared with polymyxins B and E, especially polymyxins D_2 and S_2. However, differences in their *in vivo* renal toxicity were observed, and further studies are urgently needed to investigate this phenomenon.

Overall trends of SAR and STR can be summarized according to the *in vitro* activity, renal cytotoxicity and acute toxicity. A fatty acyl chain length of C9 (B_5) with high hydrophobicity noticeably reduced the antimicrobial activity compared with fatty acyl chain lengths of C7-C8 (B_1, B_2, B_3, B_4), and the fatty acyl chain lengths of C8-C9 (B_1, B_3, B_5) obviously increased the renal cytotoxicity compared with a chain length of C7 (B_2, B_4). Additionally, whether position 6 was occupied by D-Phe (B) or D-Leu (E), or Leu-7 was substituted with other hydrophobic amino acids such as L-Ile7, L-Nva7 and L-Val7, did not substantially affect the antimicrobial potency and acute toxicity. However, the substitution of L-Leu7 (E) with a more polar amino acid L-Thr7 (M) noticeably increased the activity but did not affect the renal cytotoxicity and acute toxicity. The configuration of Dab3 (A, M) did not affect the antimicrobial potency, renal cytotoxicity and acute toxicity. Substitution of the positive charges of Dab3 or D-Dab3 with a polar amino acid D-Ser3 (D, S) enhanced the antimicrobial activity against *E. coli*, *K. pneumoniae*, and *A. baumannii* but noticeably decreased the antimicrobial activity against *P. aeruginosa*. Additionally, the individual components with four positive charges (D, S) decreased the renal cytotoxicity and acute toxicity. However, the five positive charges of Dab residues were very important for the potency against *P. aeruginosa*. The substitution of L-Thr10 with a more hydrophobic amino acid L-Leu10 (T) significantly reduced the antimicrobial activity and increased renal cytotoxicity.

The results of this study showed that there were obvious differences in biological activity between the individual components, especially differences in toxicity. More importantly, this

research found that the new compound S_2 with four positive charges displayed a much more potent activity against *E. coli*, *K. pneumoniae*, and *A. baumannii* as well as a noticeably reduced toxicity compared with polymyxin B or E. This discovery that components with lower positive charges could simultaneously increase the efficacy and decrease the toxicity is very exciting. In addition, polymyxins A_2, M_2, and D_2 also showed stronger activities and lower renal cytotoxicities than the control. These results indicate that S_2 could be a new drug candidate with similar or better antimicrobial potency and lower toxicity than the current clinical polymyxins B and E. The initial SAR and STR studies indicate that further structural modifications could concentrate on polymyxin S with four positive charges rather than the previous lead compound polymyxin B with five positive charges.

EXPERIMENTAL SECTION

Synthetic materials and methods

Materials. The protected amino acids and HCTU were purchased from GL Biochem (Shanghai, China). PyAOP, HOAt, and $Pd(PPh_3)_4$ were purchased from Meryer (Shanghai, China). Acetonitrile (HPLC grade), TFA (HPLC grade), allyl bromide, Aliquat 336, TEMPO, (*S*)-(+)-6-methyl-1-octanol, 7-methyl-7-octenoic acid, NMM, TIS, $PhSiH_3$, and DIPEA were purchased from J&K Scientific (Beijing, China). Piperidine, diethyl ether anhydrous, hydrazine hydrate solution (40%), $NaHCO_3$, AcOEt, PE, MeOH, and DCM were purchased from Beijing Chemical Works (Beijing, China). DMF was purchased from Beijing Bomaijie (Beijing, China). 2CTC resin (loading=0.53 mmol/g) was purchased from Tianjin Nankaihecheng (Tianjin, China).

Fmoc-L-Dab-OAll. Fmoc-L-Dab(Boc)-OH (38.7 g, 87.8 mmol) was added to water (180 mL) containing $NaHCO_3$ (7.4 g, 87.8 mmol). Aliquat 336 (10 g, 23 mmol) and allyl bromide (38 mL, 439 mmol) were dissolved in DCM (180 mL). The mixture was then added slowly into the Fmoc-L-Dab(Boc)-OH aqueous solution. The reaction was stirred overnight at room temperature. Subsequently, 180 mL water was added, and the aqueous phase was extracted by DCM (3×100 mL). After drying over anhydrous Na_2SO_4 and concentration under reduced pressure, Fmoc-L-Dab(Boc)-OAll was obtained without purification. Then, the obtained residue was dissolved in 30% TFA/DCM (200 mL) and stirred under room temperature for 2 h. Finally, the organic solvent was removed, and the Fmoc-L-Dab-OAll was purified by flash chromatography (DCM:MeOH=20:1) with an 89.8% yield (30 g) as a white powder. ^{1}H NMR (400 MHz, DMSO) δ (ppm): 8.12 (s, 2H), 7.98 (d, *J* = 7.9 Hz, 1H), 7.90 (d, *J* = 7.5 Hz, 2H), 7.72 (dd, *J* = 7.3, 2.5 Hz, 2H), 7.43 (t, *J* = 7.4 Hz, 2H), 7.38 – 7.30 (m, 2H), 5.90 (ddt, *J* = 17.2, 10.5, 5.3 Hz, 1H), 5.32 (dd, *J* = 17.3, 1.6 Hz, 1H), 5.21 (dd, *J* = 10.5, 1.4 Hz, 1H), 4.61 (d, *J* = 5.0 Hz, 2H), 4.39 – 4.28 (m, 2H), 4.28 – 4.18 (m, 2H), 2.92 – 2.78 (m, 2H), 2.17 – 1.85 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ (ppm): 171.11, 156.08, 143.74, 143.70, 140.73, 132.24, 127.67, 127.09, 125.20, 125.18, 120.15, 117.79, 65.75, 65.06, 51.55, 46.59, 35.97, 28.46. LC-MS (ESI): $[M+H]^+$ 381, calcd 381.

(*S*)-6-Methyloctanoic Acid. Sodium hypochlorite (8 wt % in water, 15 mL) and a solution of $NaClO_2$ (5.2 g, 57.6 mmol) in water (15 mL) were added to a mixture of (*S*)-(+)-6-methyl-1-octanol (4.15 g, 28.8 mmol), TEMPO (0.45 g, 2.88 mmol), aqueous NaH_2PO_4 (0.67 M, 150 mL), and acetonitrile (150 mL). The mixture was stirred for 12 h at room temperature, and a saturated aqueous solution of $Na_2S_2O_3$ (60 mL) was added. The resulting mixture was extracted with AcOEt (2×100 mL), and the combined extracts were washed with 1 N HCl (50 mL), dried over

anhydrous Na_2SO_4, filtered, and concentrated under reduced pressure. The obtained residue was purified by flash chromatography (AcOEt:PE=1:10) with a 92% yield (4.2 g) as a colorless oil. $[\alpha]_D^{20}$ +8.4° (c=1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 11.64 (br s, 1H), 2.35 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.55 (m, 2H), 1.39 – 1.25 (m, 5H), 1.20 – 1.05 (m, 2H), 0.87 – 0.84 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 180.71, 36.20, 34.25, 34.22, 29.46, 26.60, 25.03, 19.15, 11.39. LC-MS (ESI): $[M-H]^-$ 157, calcd 157.

7-Methyloctanoic Acid. 7-Methyl-7-octenoic acid (0.81 g, 5.2 mmol) was dissolved in MeOH (20 mL), and palladium on carbon (10%, 200 mg) was added to the vessel. The hydrogenation vessel was subjected to H_2 (50 psi) at room temperature for 2 h. The resulting mixture was filtered and concentrated under reduced pressure. The obtained residue was dried under vacuum at room temperature for 1 h to give the compound (0.81 g, 98% yield) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 11.50 (s, 1H), 2.39 (t, *J* = 7.5 Hz, 2H), 1.73 – 1.63 (m, 2H), 1.56 (dp, *J* = 13.2, 6.7 Hz, 1H), 1.41 – 1.29 (m, 4H), 1.27 – 1.16 (m, 2H), 0.90 (d, *J* = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ (ppm): 180.57, 38.76, 34.16, 29.32, 27.93, 27.01, 24.72, 22.61. LC-MS (ESI): $[M-H]^-$ 157, calcd 157.

Synthesis of polymyxins A_1 to T_2. Step 1: 0.5 mmol 2CTC resin (0.95 g, 0.53 mmol/g) was swelled in 50% DCM/50% DMF for 5 min before use. Then, Fmoc-L-Dab-OAll (2 mmol, 4 eq.), DIPEA(4 mmol, 8 eq.) and DCM (8 mL) were added to react with the resin for 2 h. The resin was capped with 0.5 mL MeOH to quench the remaining 2-chlorotrityl chloride, and compound **1** was obtained.

Step 2: Linear peptide chains were assembled by standard solid-phase synthesis using a CS136XT CSBio Peptide Synthesizer (CSBio, America). The Fmoc group was removed by

treatment with 20% piperidine in DMF twice (5 min, 10 min), followed by washing with DMF and DCM. Generally, the subsequent coupling was carried out using a solution of 4 eq. of the appropriate amino acid or corresponding fatty acid, 4 eq. of HCTU, and 8 eq. of DIPEA in DMF at room temperature after Fmoc removal. Each coupling step required 1 h, and the resin was washed by DMF and DCM. The peptide was capped with the corresponding fatty acid to achieve compound **2**.

Step 3: The Dde group was removed by treatment with 3% hydrazine hydrate in DMF for 30 min two times, and compound **3** was obtained.

Step 4: Fmoc-L-Thr(tBu)-OH or Fmoc-L-Leu-OH was coupled to the side-chain amino of Dab4 to achieve compound **4**. The subsequent coupling was carried out using a solution of 4 eq. of the appropriate amino acid, 4 eq. of HCTU, and 8 eq. of DIPEA in DMF at room temperature for 1 h after the removal of the Dde group.

Step 5: The Fmoc group was removed by 20% piperidine in DMF twice (5 min, 10 min), and the allyl group was removed by treatment with $PhSiH_3$ (7 eq.) and $Pd(PPh_3)_4$ (0.2 eq.) in 50% DCM/50% DMF for 2 h. After deprotection, the resin was washed with 0.5% sodium diethyldithiocarbamate in DMF and DMF several times, and compound **5** was obtained.

Step 6: The final cyclization step was carried out with 4 eq. of PyAOP, 4 eq. of HOAt and 8 eq. of NMM for 3 h, and compound **6** was obtained.

Step 7: The cleavage reagent TFA:TIS:H_2O (95:2.5:2.5) was added into the dry resin prewashed with DMF and DCM, and the cleavage was carried out for 2 h. The crude peptides were obtained by precipitation with cold diethyl ether and then purification by using semi-

preparative HPLC to afford the polymyxin TFA salt **7**. Total yields were approximately 30% based on the initial 2CTC resin.

Purification and Analyses. Analytical HPLC was performed on an Agilent Technologies 1200 Series Instrument. The peptides were analyzed using an Eclipse XDB-C18 (4.6×150 mm, 5 μm) under a 214 nm UV detector. The gradient mode was 10-70% solvent B over 25 min, 70-10% solvent B over 0.5 min, and 10% solvent B over 4.5 min at a flow rate of 0.8 mL/min (solvent A was 0.08% TFA/water and solvent B was 0.08% TFA/acetonitrile). The semi-preparative HPLC was performed on a Shimadzu Prominence HPLC LC-20AT system. The peptides were purified by using a Shim-pack PREP-ODS (20×250 mm ID, 10 μm) under a 214 nm UV detector. The gradient was 10-70% solvent B over 30 min at a flow rate of 6.0 mL/min (solvent A was 0.08% TFA/water and solvent B was 0.08% TFA/acetonitrile). The synthesized peptide was purified by using semi-preparative HPLC to afford the polymyxin TFA salt with a purity >95%. LC-MS analysis was carried out with a Shimadzu LC-MS 2020 system using an electrospray ionization source (ESI) and a single-quadrupole mass analyzer. A Shim–pack VP-ODS (2.0×150 mm, 5 μm) was used with a gradient of 10-90% solvent B over 5 min at a flow rate of 0.5 mL/min (solvent A was 0.1% formic acid in water and solvent B was 0.1% formic acid in acetonitrile). Mass spectra were acquired in the negative or positive ion mode with a scan range of m/z 100-800. The optical rotations of individual components were measured by a Rudolph Research Analytical Autopol IV. High resolution mass spectra (HRMS) were taken on a LTQ Orbitrap XL instrument (Thermo Scientific, USA). All purified peptides were analyzed using a Bruker 500 MHz spectrometer. Small molecule intermediates were analyzed with a Bruker 400 MHz spectrometer. (Bruker Bioscience, USA).

Minimum Inhibitory Concentrations Determination. Minimum inhibitory concentrations (MICs) of these individual components were determined by the serial two-fold agar dilution method as described by the Clinical and Laboratory Standards Institute. Bacteria used in this study were standard isolates from ATCC or clinical isolates of China. Bacteria were cultured in Mueller-Hinton broth, and the inoculum was about 10^4 colony forming units (cfu)/spot. The final concentrations of these components were ranged from 0.03-128 μg/mL. Culture plates were incubated at 35 °C for 18 h and the MICs were then recorded. The MIC was defined as the lowest concentration of the antimicrobial agent at which visible growth of the bacteria was prevented. The MICs were the representative results of at least two independent experiments, in the case of different results for the two experiments, a third experiment was conducted, and the confirmed results were given.

Cytotoxicity assay. Cytotoxicity to Vero cells was determined using the MTT method. In brief, Vero cells were seeded at $2x10^3$ cells/well into 96-well plates and incubated for 24 h at 37 °C, 5% CO_2. Then, compounds were added into each well. After incubation for 72 h, MTT was added to the medium (final to 50 μL) and incubated for 2 h. After removing the culture medium, 200 μL of dimethyl sulfoxide was added. The plates were read using an enzyme-linked immune-sorbent assay plate reader at 570 nm. The viability of untreated cells was set as 100% and the viability in other groups was calculated by comparing the optical density reading with the control. Cell Cytotoxicity (CC_{50}) was calculated with GraphPad Prism 5. The results were expressed as the mean ± SD from three independent experiments, each performed in triplicate. Polymyxins B and E were used as positive control.

Acute toxicity to mice. The acute toxicity was evaluated by intravenous administration of varying doses of individual polymyxin components as well as polymyxins B and E into ICR

mice. The ICR mice (weight, 18 to 22 g; age=4 weeks; n=10 mice per group; 5 male, 5 female) were purchased from Vital River Laboratories (Beijing, China). Four to five doses (four to five groups) were used for each compound. Survival was monitored daily for 7 days, and the amount of test compounds per kg of mouse body weight that constituted a lethal dose for 50% of the animals (LD_{50}) was calculated. The LD_{50} values were calculated according to the Bliss test protocol. All animal experiments were performed in full compliance with the protocols approved by the Animal Ethics Committee of the Institute of Medicinal Biotechnology (Beijing, China).

Renal toxicity to mice. Solutions of individual polymyxin components and polymyxin E in saline (0.25 mg/mL) were stored at 4 °C before use. The ICR mice (weight, 18 to 22 g; age=4 weeks) were purchased from Vital River Laboratories (Beijing, China). The mice (n=8 mice per group; 4 male, 4 female) were intravenously administered the component at a dose of 2.5 mg/kg twice daily (8 h apart) for 7 days until an accumulated dose of 35 mg was achieved. Two hours after the last dose, the mice were sacrificed. Their kidneys were collected and placed in 10% formalin in a 10 mL plastic tube. The formalin-fixed kidneys were dehydrated and embedded in paraffin wax. The sections (5 mm) were counterstained with hematoxylin and eosin (H&E) and examined using a light microscope. The whole sections were examined by a pathologist (P. A. H.) who was blinded to the treatment groups. The SQS of each group was calculated according the grades and the percentages of the kidney slices affected.[35-36] All animal experiments were performed in full compliance with the protocols approved by the Animal Ethics Committee of the Institute of Medicinal Biotechnology (Beijing, China).

Characterization of synthetic polymyxins A_1 to T_2. Compound A_1. Yield 30%, white solid. Molecular Formula $C_{51}H_{96}O_{14}N_{16}$. HRMS: $[M+H]^+$ 1157.73788, calcd 1157.73647. HPLC: t_R = 10.16 min, purity 97.3%. $[\alpha]_D^{25}$ -30.5° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm):

4.57 – 4.47 (m, 3H), 4.47 – 4.40 (m, 3H), 4.36 (td, *J* = 11.6, 5.2 Hz, 3H), 4.32 – 4.20 (m, 3H), 4.18 (d, *J* = 5.0 Hz, 1H), 3.46 – 3.35 (m, 1H), 3.23 – 2.98 (m, 11H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.32 – 1.95 (m, 11H), 1.91 (dt, *J* = 14.1, 6.5 Hz, 1H), 1.75 – 1.51 (m, 5H), 1.38 – 1.25 (m, 5H), 1.24 – 1.16 (m, 9H), 1.12 (dt, *J* = 14.1, 7.0 Hz, 2H), 0.97 (d, *J* = 5.6 Hz, 3H), 0.92 (d, *J* = 5.5 Hz, 3H), 0.83 (t, *J* = 7.0 Hz, 6H).

Compound A_2. Yield 31%, white solid. Molecular Formula $C_{50}H_{94}O_{14}N_{16}$. HRMS: $[M+H]^+$ 1143.72282, calcd 1143.72082. HPLC: t_R = 9.28 min, purity 95.8%. $[\alpha]_D^{25}$ -33.1° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.55 – 4.31 (m, 9H), 4.30 – 4.22 (m, 3H), 4.16 (d, *J* = 5.0 Hz, 1H), 3.47 – 3.33 (m, 1H), 3.25 – 2.98 (m, 11H), 2.39 – 1.95 (m, 13H), 1.95 – 1.85 (m, 1H), 1.74 – 1.46 (m, 6H), 1.30 (dt, *J* = 15.4, 7.5 Hz, 2H), 1.24 – 1.15 (m, 11H), 0.97 (d, *J* = 5.8 Hz, 3H), 0.92 (d, *J* = 5.7 Hz, 3H), 0.84 (d, *J* = 6.6 Hz, 6H).

Compound B_1. Yield 32%, white solid. Molecular Formula $C_{56}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1203.75736, calcd 1203.75720. HPLC: t_R = 11.56 min, purity 97.7%. $[\alpha]_D^{25}$ -60.0° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.41 (t, *J* = 7.2 Hz, 2H), 7.38 – 7.33 (m, 1H), 7.28 (d, *J* = 7.1 Hz, 2H), 4.58 (t, *J* = 8.3 Hz, 1H), 4.55 – 4.47 (m, 3H), 4.37 (d, *J* = 4.3 Hz, 1H), 4.33 – 4.19 (m, 7H), 3.40 – 3.31 (m, 1H), 3.23 – 3.04 (m, 11H), 2.93 – 2.85 (m, 1H), 2.85 – 2.75 (m, 1H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.29 – 1.86 (m, 12H), 1.66 – 1.55 (m, 2H), 1.50 (ddd, *J* = 13.5, 9.4, 3.9 Hz, 1H), 1.41 (dd, *J* = 18.3, 7.3 Hz, 1H), 1.37 – 1.25 (m, 5H), 1.22 (t, *J* = 6.4 Hz, 6H), 1.12 (td, *J* = 13.1, 7.2 Hz, 2H), 0.84 (t, *J* = 6.9 Hz, 7H), 0.77 (d, *J*= 5.0 Hz, 3H), 0.70 (d, *J* = 5.7 Hz, 3H).

Compound B_2. Yield 32%, white solid. Molecular Formula $C_{55}H_{96}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1189.74287, calcd 1189.74155. HPLC: t_R = 10.87 min, purity 98.3%. $[\alpha]_D^{25}$ -62.6° (c=0.15, 12%

AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.41 (t, *J* = 7.3 Hz, 2H), 7.38 – 7.33 (m, 1H), 7.28 (d, *J* = 7.3 Hz, 2H), 4.60 – 4.46 (m, 4H), 4.39 – 4.34 (m, 1H), 4.33 – 4.15 (m, 7H), 3.42 – 3.31 (m, 1H), 3.24 – 3.00 (m, 11H), 2.95 – 2.87 (m, 1H), 2.87 – 2.77 (m, 1H), 2.34 (t, *J* = 7.2 Hz, 2H), 2.30 – 1.83 (m, 12H), 1.59 (dq, *J* = 13.6, 6.8 Hz, 2H), 1.55 – 1.45 (m, 2H), 1.41 (t, *J* = 12.5 Hz, 1H), 1.31 (dt, *J* = 15.0, 7.6 Hz, 2H), 1.20 (dt, *J* = 15.5, 6.5 Hz, 8H), 0.86 (dd, *J* = 6.6, 1.2 Hz, 6H), 0.77 (s, 4H), 0.70 (s, 3H).

Compound B_3. Yield 33%, white solid. Molecular Formula $C_{55}H_{96}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1189.74238, calcd 1189.74155. HPLC: t_R = 10.98 min, purity 97.6%. $[\alpha]_D^{25}$ -61.5° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.41 (t, *J* = 7.3 Hz, 2H), 7.35 (t, *J* = 7.3 Hz, 1H), 7.28 (d, *J* = 7.1 Hz, 2H), 4.58 (t, *J* = 8.1 Hz, 1H), 4.55 – 4.48 (m, 3H), 4.37 (d, *J* = 4.4 Hz, 1H), 4.33 – 4.19 (m, 7H), 3.41 – 3.32 (m, 1H), 3.21 – 3.04 (m, 11H), 2.94 – 2.86 (m, 1H), 2.85 – 2.76 (m, 1H), 2.33 (t, *J* = 7.4 Hz, 2H), 2.30 – 1.83 (m, 12H), 1.65 – 1.56 (m, 2H), 1.50 (td, *J* = 9.3, 4.4 Hz, 1H), 1.41 (t, *J* = 12.8 Hz, 1H), 1.33 – 1.24 (m, 8H), 1.22 (t, *J* = 6.5 Hz, 6H), 0.87 (t, *J* = 7.0 Hz, 3H), 0.77 (s, 4H), 0.70 (d, *J* = 4.4 Hz, 3H).

Compound B_4. Yield 33%, white solid. Molecular Formula $C_{54}H_{94}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1175.72884, calcd 1175.72590. HPLC: t_R = 10.20 min, purity 95.3%. $[\alpha]_D^{25}$ -65.1° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.41 (t, *J* = 7.2 Hz, 2H), 7.38 – 7.33 (m, 1H), 7.28 (d, *J* = 7.0 Hz, 2H), 4.57 (t, *J* = 8.3 Hz, 1H), 4.55 – 4.49 (m, 3H), 4.37 (d, *J* = 4.3 Hz, 1H), 4.33 – 4.19 (m, 7H), 3.41 – 3.33 (m, 1H), 3.23 – 3.02 (m, 11H), 2.96 – 2.87 (m, 1H), 2.82 (dd, *J* = 18.4, 10.5 Hz, 1H), 2.34 (t, *J* = 7.4 Hz, 2H), 2.30 – 1.84 (m, 12H), 1.66 – 1.55 (m, 2H), 1.54 – 1.45 (m, 1H), 1.40 (t, *J* = 13.0 Hz, 1H), 1.35 – 1.25 (m, 6H), 1.22 (t, *J* = 6.0 Hz, 6H), 0.87 (dd, *J* = 9.2, 4.6 Hz, 3H), 0.77 (s, 4H), 0.70 (s, 3H).

Compound B_5. Yield 32%, white solid. Molecular Formula $C_{56}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1203.75845, calcd 1203.75720. HPLC: t_R = 11.79 min, purity 95.4%. $[\alpha]_D^{25}$ -62.5° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.40 (t, *J* = 7.2 Hz, 2H), 7.35 (t, *J* = 7.3 Hz, 1H), 7.27 (d, *J* = 7.3 Hz, 2H), 4.57 – 4.48 (m, 4H), 4.37 (d, *J* = 4.3 Hz, 1H), 4.33 – 4.22 (m, 5H), 4.20 (dd, *J* = 8.7, 4.3 Hz, 2H), 3.43 – 3.32 (m, 1H), 3.22 – 3.01 (m, 11H), 2.93 (d, *J* = 10.0 Hz, 1H), 2.87 – 2.78 (m, 1H), 2.32 (t, *J* = 7.4 Hz, 2H), 2.29 – 1.84 (m, 12H), 1.65 – 1.55 (m, 2H), 1.53 – 1.44 (m, 1H), 1.37 (dd, *J* = 19.0, 7.4 Hz, 1H), 1.29 (d, *J* = 1.9 Hz, 10H), 1.21 (dd, *J* = 6.0, 4.4 Hz, 6H), 0.86 (t, *J* = 6.8 Hz, 3H), 0.78 – 0.62 (m, 7H).

Compound B_1-Ile. Yield 32%, white solid. Molecular Formula $C_{56}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1203.75939, calcd 1203.75720. HPLC: t_R = 11.29 min, purity 98.2%. $[\alpha]_D^{25}$ -58.6° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.25 (t, *J* = 7.3 Hz, 2H), 7.19 (t, *J* = 7.4 Hz, 1H), 7.13 (d, *J* = 7.1 Hz, 2H), 4.62 – 4.57 (m, 1H), 4.39 – 4.29 (m, 3H), 4.21 (dd, *J* = 9.9, 5.2 Hz, 2H), 4.14 – 4.02 (m, 6H), 3.21 – 3.11 (m, 1H), 3.08 – 2.84 (m, 11H), 2.69 – 2.61 (m, 1H), 2.60 – 2.52 (m, 1H), 2.19 (t, *J* = 7.3 Hz, 2H), 2.17 – 1.89 (m, 8H), 1.87 – 1.62 (m, 5H), 1.51 – 1.38 (m, 2H), 1.22 – 1.10 (m, 5H), 1.10 – 1.02 (m, 7H), 0.98 (dt, *J* = 7.0, 6.6 Hz, 2H), 0.90 – 0.77 (m, 1H), 0.72 – 0.63 (m, 9H), 0.57 (d, *J* = 6.8 Hz, 3H).

Compound D_1. Yield 29%, white solid. Molecular Formula $C_{50}H_{93}O_{15}N_{15}$. HRMS: $[M+H]^+$ 1144.70561, calcd 1144.70483. HPLC: t_R = 10.55 min, purity 99.1%. $[\alpha]_D^{25}$ -37.7° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.52 (dt, *J* = 9.3, 6.2 Hz, 2H), 4.48 – 4.39 (m, 4H), 4.35 (dd, *J* = 9.0, 5.0 Hz, 3H), 4.32 – 4.18 (m, 3H), 4.16 (d, *J* = 5.1 Hz, 1H), 3.91 (ddd, *J* = 16.0, 11.7, 4.9 Hz, 2H), 3.43 – 3.32 (m, 1H), 3.20 – 2.97 (m, 9H), 2.37 – 1.97 (m, 11H), 1.94 – 1.84 (m, 1H), 1.73 – 1.52 (m, 5H), 1.36 – 1.24 (m, 5H), 1.24 – 1.16 (m, 9H), 1.15 – 1.06 (m, 2H), 0.95 (d, *J* = 5.6 Hz, 3H), 0.91 (d, *J* = 5.5 Hz, 3H), 0.82 (t, *J* = 7.0 Hz, 6H).

Compound D_2. Yield 30%, white solid. Molecular Formula $C_{49}H_{91}O_{15}N_{15}$. HRMS: $[M+H]^+$ 1130.69126, calcd 1130.68918. HPLC: t_R = 9.60 min, purity 95.9%. $[\alpha]_D^{25}$ -36.7° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.57 – 4.51 (m, 2H), 4.49 – 4.40 (m, 4H), 4.37 (dd, *J* = 7.6, 4.9 Hz, 3H), 4.34 – 4.19 (m, 3H), 4.17 (d, *J* = 5.2 Hz, 1H), 3.92 (ddd, *J* = 16.1, 11.7, 4.9 Hz, 2H), 3.45 – 3.34 (m, 1H), 3.22 – 2.98 (m, 9H), 2.34 (t, *J* = 7.4 Hz, 2H), 2.31 – 1.98 (m, 9H), 1.96 – 1.84 (m, 1H), 1.74 – 1.47 (m, 6H), 1.30 (dt, *J* = 15.2, 7.7 Hz, 2H), 1.26 – 1.14 (m, 11H), 0.97 (d, *J* = 5.8 Hz, 3H), 0.92 (d, *J* = 5.7 Hz, 3H), 0.85 (d, *J* = 6.6 Hz, 6H).

Compound E_1. Yield 31%, white solid. Molecular Formula $C_{53}H_{100}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1169.77475, calcd 1169.77285. HPLC: t_R = 11.23 min, purity 95.8%. $[\alpha]_D^{25}$ -46.5° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.58 (dd, *J* = 9.5, 4.8 Hz, 1H), 4.52 (ddd, *J* = 9.4, 5.1, 1.9 Hz, 2H), 4.42 (dd, *J* = 10.8, 4.3 Hz, 1H), 4.37 (d, *J* = 4.4 Hz, 1H), 4.35 – 4.20 (m, 7H), 3.41 – 3.32 (m, 1H), 3.24 – 2.98 (m, 11H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.31 – 1.96 (m, 11H), 1.95 – 1.85 (m, 1H), 1.75 – 1.53 (m, 8H), 1.37 – 1.25 (m, 5H), 1.25 – 1.19 (m, 6H), 1.18 – 1.07 (m, 2H), 0.98 – 0.92 (m, 6H), 0.90 (dd, *J* = 13.7, 6.1 Hz, 6H), 0.84 (t, *J* = 7.0 Hz, 6H).

Compound E_2. Yield 32%, white solid. Molecular Formula $C_{52}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1155.75847, calcd 1155.75720. HPLC: t_R = 10.48 min, purity >99.0%. $[\alpha]_D^{25}$ -50.4° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.58 (dd, *J* = 9.5, 4.8 Hz, 1H), 4.54 – 4.48 (m, 2H), 4.42 (dd, *J* = 10.9, 4.2 Hz, 1H), 4.36 (d, *J* = 4.4 Hz, 1H), 4.34 – 4.20 (m, 7H), 3.41 – 3.32 (m, 1H), 3.24 – 2.99 (m, 11H), 2.33 (t, *J* = 7.3 Hz, 2H), 2.31 – 1.95 (m, 11H), 1.95 – 1.84 (m, 1H), 1.75 – 1.47 (m, 9H), 1.34 – 1.26 (m, 2H), 1.26 – 1.15 (m, 8H), 0.94 (dd, *J* = 6.1, 2.3 Hz, 6H), 0.89 (dd, *J* = 13.4, 6.1 Hz, 6H), 0.85 (d, *J* = 6.6 Hz, 6H).

Compound E_3. Yield 32%, white solid. Molecular Formula $C_{52}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1155.75828, calcd 1155.75720. HPLC: t_R = 10.63 min, purity 95.7%. $[\alpha]_D^{25}$ -51.7° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.58 (dd, *J* = 9.5, 4.8 Hz, 1H), 4.52 (dd, *J* = 9.4, 5.2 Hz, 2H), 4.42 (dd, *J* = 10.7, 4.4 Hz, 1H), 4.37 (d, *J* = 4.4 Hz, 1H), 4.35 – 4.23 (m, 6H), 4.21 (d, *J* = 4.8 Hz, 1H), 3.41 – 3.32 (m, 1H), 3.23 – 2.99 (m, 11H), 2.33 (t, *J* = 7.3 Hz, 2H), 2.31 – 1.95 (m, 11H), 1.89 (td, *J* = 13.8, 8.4 Hz, 1H), 1.75 – 1.53 (m, 8H), 1.33 – 1.25 (m, 8H), 1.22 (d, *J* = 6.4 Hz, 6H), 0.95 (d, *J* = 5.7 Hz, 6H), 0.91 (d, *J* = 6.0 Hz, 3H), 0.87 (dd, *J* = 11.1, 6.7 Hz, 6H).

Compound E_4. Yield 32%, white solid. Molecular Formula $C_{51}H_{96}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1141.74320, calcd 1141.74155. HPLC: t_R = 9.84 min, purity 95.5%. $[\alpha]_D^{25}$ -57.6° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.57 – 4.47 (m, 3H), 4.40 (dd, *J* = 9.2, 6.1 Hz, 1H), 4.37 – 4.32 (m, 2H), 4.31 – 4.20 (m, 5H), 4.18 (d, *J* = 4.7 Hz, 1H), 3.41 – 3.33 (m, 1H), 3.23 – 2.96 (m, 11H), 2.32 (t, *J* = 7.3 Hz, 2H), 2.30 – 1.83 (m, 12H), 1.72 – 1.52 (m, 8H), 1.27 (s, 6H), 1.21 (dd, *J* = 6.2, 1.8 Hz, 6H), 0.93 (d, *J* = 6.3 Hz, 6H), 0.90 (d, *J* = 5.8 Hz, 3H), 0.85 (dd, *J* = 8.6, 6.8 Hz, 6H).

Compound E_7. Yield 30%, white solid. Molecular Formula $C_{53}H_{100}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1169.77479, calcd 1169.77285. HPLC: t_R = 11.33 min, purity 96.1%. $[\alpha]_D^{25}$ -50.8° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.56 (dd, *J* = 9.5, 4.8 Hz, 1H), 4.50 (dd, *J* = 9.4, 5.2 Hz, 2H), 4.40 (dd, *J* = 10.7, 4.5 Hz, 1H), 4.36 (d, *J* = 4.4 Hz, 1H), 4.34 – 4.22 (m, 6H), 4.20 (d, *J* = 4.8 Hz, 1H), 3.40 – 3.31 (m, 1H), 3.22 – 2.97 (m, 11H), 2.32 (t, *J* = 7.3 Hz, 2H), 2.29 – 1.93 (m, 11H), 1.93 – 1.82 (m, 1H), 1.73 – 1.45 (m, 9H), 1.29 (dd, *J* = 9.1, 5.9 Hz, 4H), 1.21 (d, *J* = 6.4 Hz, 6H), 1.15 (dd, *J* = 14.0, 6.8 Hz, 2H), 0.93 (d, *J* = 5.4 Hz, 6H), 0.88 (dd, *J* = 14.8, 6.1 Hz, 6H), 0.84 (d, *J* = 6.6 Hz, 6H).

Compound E_1-Ile. Yield 31%, white solid. Molecular Formula $C_{53}H_{100}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1169.77553, calcd 1169.77285. HPLC: t_R = 11.02 min, purity 96.4%. $[\alpha]_D^{25}$ -43.7° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.56 (dd, *J* = 9.6, 4.7 Hz, 1H), 4.50 (dt, *J* = 9.5, 5.8 Hz, 2H), 4.42 – 4.33 (m, 3H), 4.32 – 4.11 (m, 6H), 3.35 (td, *J* = 14.1, 7.0 Hz, 1H), 3.25 – 2.93 (m, 11H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.30 – 1.96 (m, 12H), 1.95 – 1.83 (m, 1H), 1.70 – 1.53 (m, 5H), 1.43 – 1.26 (m, 6H), 1.22 (d, *J* = 6.2 Hz, 6H), 1.19 – 1.07 (m, 3H), 0.94 (t, *J* = 6.9 Hz, 6H), 0.89 (dd, *J* = 12.5, 6.3 Hz, 6H), 0.84 (t, *J* = 7.0 Hz, 6H).

Compound E_1-Nva. Yield 31%, white solid. Molecular Formula $C_{52}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1155.75873, calcd 1155.75720. HPLC: t_R = 10.74 min, purity 96.3%. $[\alpha]_D^{25}$ -48.3° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.57 (dd, *J* = 9.5, 4.9 Hz, 1H), 4.54 – 4.48 (m, 2H), 4.37 (q, *J* = 5.0 Hz, 2H), 4.31 (dt, *J* = 9.4, 4.7 Hz, 4H), 4.24 (dt, *J* = 11.9, 5.6 Hz, 3H), 3.41 – 3.31 (m, 1H), 3.24 – 2.98 (m, 11H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.31 – 1.95 (m, 11H), 1.94 – 1.82 (m, 2H), 1.76 – 1.53 (m, 6H), 1.42 (td, *J* = 13.5, 8.1 Hz, 1H), 1.37 – 1.25 (m, 6H), 1.25 – 1.18 (m, 6H), 1.18 – 1.07 (m, 2H), 0.97 – 0.87 (m, 9H), 0.84 (t, *J* = 6.9 Hz, 6H).

Compound E_1-Val. Yield 31%, white solid. Molecular Formula $C_{52}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1155.75888, calcd 1155.75720. HPLC: t_R = 10.52 min, purity 95.5%. $[\alpha]_D^{25}$ -52.9° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.56 – 4.48 (m, 3H), 4.37 – 4.22 (m, 8H), 4.18 (d, *J* = 5.1 Hz, 1H), 3.36 (dt, *J* = 14.2, 7.2 Hz, 1H), 3.24 – 2.98 (m, 11H), 2.35 – 2.03 (m, 13H), 1.98 (dt, *J* = 11.9, 7.2 Hz, 1H), 1.87 (td, *J* = 13.9, 6.6 Hz, 1H), 1.69 – 1.51 (m, 5H), 1.35 – 1.24 (m, 5H), 1.21 (dd, *J* = 6.3, 2.9 Hz, 6H), 1.16 – 1.06 (m, 2H), 0.97 – 0.91 (m, 6H), 0.89 (t, *J* = 5.4 Hz, 6H), 0.83 (t, *J* = 7.0 Hz, 6H).

Compound E_2-Ile. Yield 32%, white solid. Molecular Formula $C_{52}H_{98}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1155.75949, calcd 1155.75720. HPLC: t_R = 10.18 min, purity >99.0%. $[\alpha]_D^{25}$ -50.6° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.57 (dd, *J* = 9.8, 4.6 Hz, 1H), 4.50 (dt, *J* = 9.9, 5.1 Hz, 2H), 4.41 – 4.34 (m, 2H), 4.32 (d, *J* = 7.3 Hz, 1H), 4.30 – 4.18 (m, 6H), 3.32 (dt, *J* = 14.0, 7.1 Hz, 1H), 3.21 (dt, *J* = 13.9, 6.9 Hz, 1H), 3.16 – 2.98 (m, 10H), 2.32 (t, *J* = 7.3 Hz, 2H), 2.30 – 1.95 (m, 12H), 1.86 (td, *J* = 14.3, 7.8 Hz, 1H), 1.68 – 1.46 (m, 6H), 1.44 – 1.34 (m, 1H), 1.29 (ddd, *J* = 12.2, 9.8, 7.4 Hz, 2H), 1.25 – 1.13 (m, 9H), 0.95 – 0.85 (m, 12H), 0.84 (t, *J* = 4.6 Hz, 6H).

Compound E_2-Nva. Yield 32%, white solid. Molecular Formula $C_{51}H_{96}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1141.74289, calcd 1141.74155. HPLC: t_R = 9.94 min, purity >99.0%. $[\alpha]_D^{25}$ -50.0° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.57 (dd, *J* = 9.5, 4.9 Hz, 1H), 4.54 – 4.48 (m, 2H), 4.40 – 4.34 (m, 2H), 4.31 (dd, *J* = 8.5, 5.7 Hz, 4H), 4.25 (dt, *J* = 17.8, 5.6 Hz, 3H), 3.41 – 3.32 (m, 1H), 3.25 – 2.98 (m, 11H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.31 – 1.95 (m, 11H), 1.88 (tt, *J* = 13.6, 9.9 Hz, 2H), 1.76 – 1.47 (m, 7H), 1.47 – 1.36 (m, 1H), 1.31 (dt, *J* = 15.1, 7.6 Hz, 3H), 1.25 – 1.13 (m, 8H), 0.96 – 0.87 (m, 9H), 0.85 (d, *J* = 6.6 Hz, 6H).

Compound E_2-Val. Yield 32%, white solid. Molecular Formula $C_{51}H_{96}O_{13}N_{16}$. HRMS: $[M+H]^+$ 1141.74340, calcd 1141.74155. HPLC: t_R = 9.69 min, purity >99.0%. $[\alpha]_D^{25}$ -52.7° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.56 – 4.47 (m, 3H), 4.34 (dt, *J* = 11.8, 5.4 Hz, 4H), 4.31 – 4.21 (m, 4H), 4.18 (d, *J* = 5.0 Hz, 1H), 3.40 – 3.30 (m, 1H), 3.23 – 2.99 (m, 11H), 2.35 – 2.03 (m, 13H), 2.02 – 1.92 (m, 1H), 1.87 (td, *J* = 14.0, 6.8 Hz, 1H), 1.69 – 1.46 (m, 6H), 1.29 (dt, *J* = 15.3, 7.4 Hz, 2H), 1.24 – 1.13 (m, 8H), 0.94 (d, *J* = 6.3 Hz, 6H), 0.89 (t, *J* = 5.8 Hz, 6H), 0.84 (d, *J* = 6.6 Hz, 6H).

Compound M_1. Yield 31%, white solid. Molecular Formula $C_{51}H_{96}O_{14}N_{16}$. HRMS: $[M+H]^+$ 1157.73823, calcd 1157.73647. HPLC: t_R = 9.96 min, purity >99.0%. $[\alpha]_D^{25}$ -48.6° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.57 (dd, *J* = 9.5, 4.9 Hz, 1H), 4.54 – 4.49 (m, 2H), 4.49 – 4.41 (m, 3H), 4.40 – 4.28 (m, 4H), 4.28 – 4.22 (m, 2H), 4.20 (d, *J* = 5.0 Hz, 1H), 3.43 – 3.33 (m, 1H), 3.23 – 3.00 (m, 11H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.32 – 1.94 (m, 11H), 1.94 – 1.83 (m, 1H), 1.75 – 1.54 (m, 5H), 1.36 – 1.25 (m, 5H), 1.25 – 1.18 (m, 9H), 1.17 – 1.08 (m, 2H), 0.97 (d, *J* = 5.8 Hz, 3H), 0.92 (d, *J* = 5.7 Hz, 3H), 0.84 (t, *J* = 7.0 Hz, 6H).

Compound M_2. Yield 33%, white solid. Molecular Formula $C_{50}H_{94}O_{14}N_{16}$. HRMS: $[M+H]^+$ 1143.72181, calcd 1143.72082. HPLC: t_R = 9.05 min, purity >99.0%. $[\alpha]_D^{25}$ -49.2° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 4.59 – 4.48 (m, 3H), 4.48 – 4.28 (m, 7H), 4.28 – 4.21 (m, 2H), 4.19 (d, *J* = 5.0 Hz, 1H), 3.44 – 3.33 (m, 1H), 3.25 – 2.99 (m, 11H), 2.33 (t, *J* = 7.3 Hz, 2H), 2.31 – 1.93 (m, 11H), 1.88 (td, *J* = 14.1, 8.9 Hz, 1H), 1.75 – 1.47 (m, 6H), 1.35 – 1.26 (m, 2H), 1.26 – 1.14 (m, 11H), 0.97 (d, *J* = 5.6 Hz, 3H), 0.92 (d, *J* = 5.6 Hz, 3H), 0.85 (d, *J* = 6.6 Hz, 6H).

Compound S_1. Yield 29%, white solid. Molecular Formula $C_{53}H_{91}O_{15}N_{15}$. HRMS: $[M+H]^+$ 1178.69151, calcd 1178.68918. HPLC: t_R = 10.89 min, purity >99.0%. $[\alpha]_D^{25}$ -45.2° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.40 (t, *J* = 7.4 Hz, 2H), 7.32 (dd, *J* = 15.5, 7.4 Hz, 3H), 4.73 (t, *J* = 8.2 Hz, 1H), 4.52 (dd, *J* = 9.2, 5.3 Hz, 1H), 4.47 (dt, *J* = 9.6, 5.2 Hz, 2H), 4.41 – 4.32 (m, 3H), 4.31 – 4.19 (m, 5H), 4.17 (d, *J* = 5.1 Hz, 1H), 3.92 (ddd, *J* = 16.1, 11.7, 4.9 Hz, 2H), 3.43 – 3.32 (m, 1H), 3.22 – 3.01 (m, 9H), 2.92 – 2.82 (m, 1H), 2.80 – 2.70 (m, 1H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.32 – 1.84 (m, 10H), 1.66 – 1.54 (m, 2H), 1.39 – 1.26 (m, 5H), 1.22 (dd, *J* = 12.6, 6.3 Hz, 6H), 1.18 – 1.07 (m, 2H), 0.84 (t, *J* = 6.8 Hz, 6H), 0.79 (d, *J* = 5.9 Hz, 3H).

Compound S_2. Yield 31%, white solid. Molecular Formula $C_{52}H_{89}O_{15}N_{15}$. HRMS: $[M+H]^+$ 1164.67546, calcd 1164.67353. HPLC: t_R = 10.00 min, purity >99.0%. $[\alpha]_D^{25}$ -45.7° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.40 (t, *J* = 7.4 Hz, 2H), 7.32 (dd, *J* = 15.6, 7.2 Hz, 3H), 4.75 (t, *J* = 8.2 Hz, 1H), 4.53 (dd, *J* = 9.2, 5.3 Hz, 1H), 4.50 – 4.44 (m, 2H), 4.43 – 4.32 (m, 3H), 4.32 – 4.15 (m, 6H), 3.92 (ddd, *J* = 15.9, 11.6, 4.8 Hz, 2H), 3.43 – 3.31 (m, 1H), 3.22 – 2.98 (m, 9H), 2.91 – 2.81 (m, 1H), 2.80 – 2.68 (m, 1H), 2.34 (t, *J* = 7.3 Hz, 2H), 2.30 – 1.83 (m, 10H), 1.65 – 1.56 (m, 2H), 1.52 (td, *J* = 13.3, 6.6 Hz, 1H), 1.31 (dt, *J* = 15.1, 7.7 Hz, 2H), 1.26 – 1.14 (m, 8H), 0.86 (d, *J* = 6.6 Hz, 6H), 0.81 (d, *J* = 6.2 Hz, 3H).

Compound T_1. Yield 35%, white solid. Molecular Formula $C_{58}H_{102}O_{12}N_{16}$. HRMS: $[M+H]^+$ 1215.79579, calcd 1215.79359. HPLC: t_R = 12.84 min, purity 95.5%. $[\alpha]_D^{25}$ -68.1° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.41 (t, *J* = 7.3 Hz, 2H), 7.38 – 7.33 (m, 1H), 7.28 (d, *J* = 7.1 Hz, 2H), 4.61 – 4.47 (m, 4H), 4.38 (d, *J* = 3.2 Hz, 1H), 4.33 – 4.14 (m, 6H), 3.48 – 3.32 (m, 1H), 3.22 – 3.00 (m, 11H), 3.00 – 2.92 (m, 1H), 2.91 – 2.83 (m, 1H), 2.34 (t, *J* = 7.2 Hz, 2H), 2.30 – 1.86 (m, 12H), 1.72 – 1.52 (m, 5H), 1.51 – 1.42 (m, 1H), 1.35 (dd, *J* = 26.7, 10.6 Hz, 6H), 1.23 (d, *J* = 6.1 Hz, 3H), 1.17 – 1.08 (m, 2H), 0.94 (d, *J* = 6.1 Hz, 3H), 0.89 (d, *J* = 6.1 Hz, 3H), 0.84 (t, *J* = 6.7 Hz, 6H), 0.74 (d, *J* = 4.6 Hz, 3H), 0.66 (s, 4H).

Compound T_2. Yield 36%, white solid. Molecular Formula $C_{57}H_{100}O_{12}N_{16}$. HRMS: $[M+H]^+$ 1201.78017, calcd 1201.77794. HPLC: t_R = 12.25 min, purity 97.0%. $[\alpha]_D^{25}$ -64.2° (c=0.15, 12% AcOH). ^{1}H NMR (500 MHz, D_2O) δ (ppm): 7.39 (t, *J* = 7.3 Hz, 2H), 7.34 (t, *J* = 7.3 Hz, 1H), 7.26 (d, *J* = 7.2 Hz, 2H), 4.53 (ddd, *J* = 15.8, 9.4, 5.8 Hz, 4H), 4.36 (d, *J* = 4.2 Hz, 1H), 4.31 – 4.20 (m, 5H), 4.17 (dd, *J* = 11.5, 3.6 Hz, 1H), 3.44 – 3.34 (m, 1H), 3.20 – 2.92 (m, 12H), 2.91 – 2.82 (m, 1H), 2.32 (t, *J* = 7.3 Hz, 2H), 2.29 – 1.83 (m, 12H), 1.70 – 1.41 (m, 7H), 1.38 – 1.25 (m,

3H), 1.24 – 1.13 (m, 5H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 6.4 Hz, 3H), 0.84 (d, *J* = 6.6 Hz, 6H), 0.72 (d, *J* = 6.1 Hz, 3H), 0.64 (d, *J* = 5.5 Hz, 4H).

ASSOCIATED CONTENT

Supporting Information.

SQS for individual rats and characterization of synthetic individual polymyxin components (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

SAR, structure−activity relationships; STR, structure−toxicity relationships; *E. coli*, *Escherichia coli*; *K. pneumoniae*, *Klebsiella pneumoniae*; *A. baumannii*, *Acinetobacter baumannii*; *P. aeruginosa*, *Pseudomonas aeruginosa*; 2CTC resin, 2-chlorotrityl chloride resin; DIPEA, *N*,*N*-diisopropylethylamine; Fmoc, fluorenylmethyloxycarbonyl; Dde, 1-(4,4-dimethyl-2,6-dioxocylohexylidene)-ethyl; HCTU, 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminiumHexafluorophosphate; PyAOP, (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphoniumhexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; $Pd(PPh_3)_4$, tetrakis(triphenylphosphine)palladium; TFA, trifluoroacetic acid; TEMPO, 2,2,6,6-tetramethylpiperidinooxy; NMM, *N*-methyl morpholine; TIS, triisopropylsilane; $PhSiH_3$, phenylsilane; AcOEt, ethyl acetate; PE, petroleum ether; MeOH, methanol; DCM, dichloromethane; DMF, *N,N*-dimethylformamide; ATCC, American Type Culture Collection; MICs, minimum inhibitory concentrations; LD_{50}, lethal dose for 50% of the animals; SQS, semi-quantitative score; HPLC, high performance liquid chromatography.

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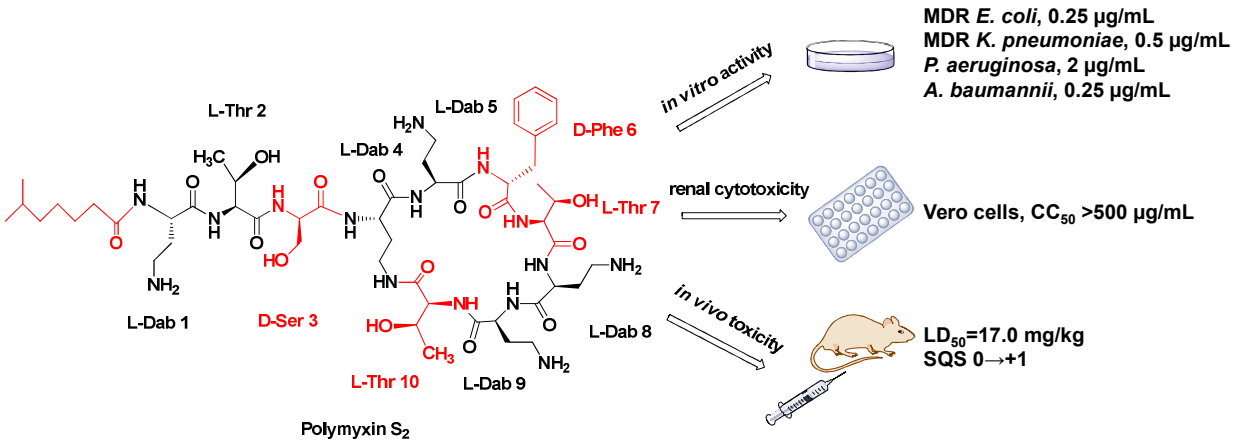
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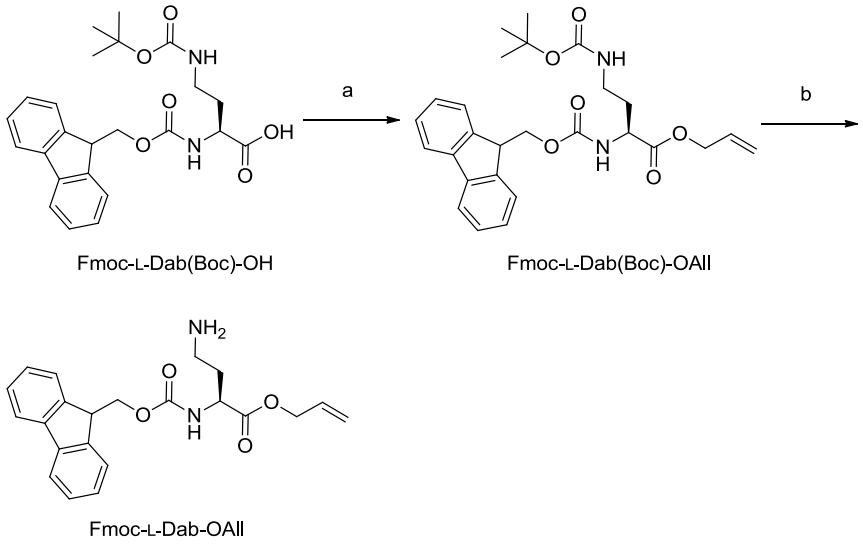
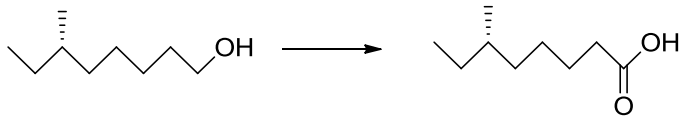
L-Dab 9
Polymyxin S2
in vitro activity
MDR E. coli, 0.25 µg/mL
MDR K. pneumoniae, 0.5 µg/mL
P. aeruginosa, 2 µg/mL
A. baumannii, 0.25 µg/mL
renal cytotoxicity
Vero cells, CC50 >500 µg/mL
in vivo toxicity
LD50=17.0 mg/kg
SQS 0→+1

Scheme 1. Synthetic Route of Fmoc-L-Dab-OAll



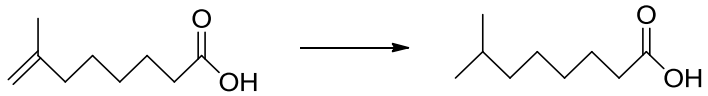
Reagents and conditions: (a) $NaHCO_3$, H_2O, allyl bromide, Aliquat 336, DCM; (b) CF_3COOH, DCM.

Scheme 2. Synthetic Route of (*S*)-6-methyloctanoic acid



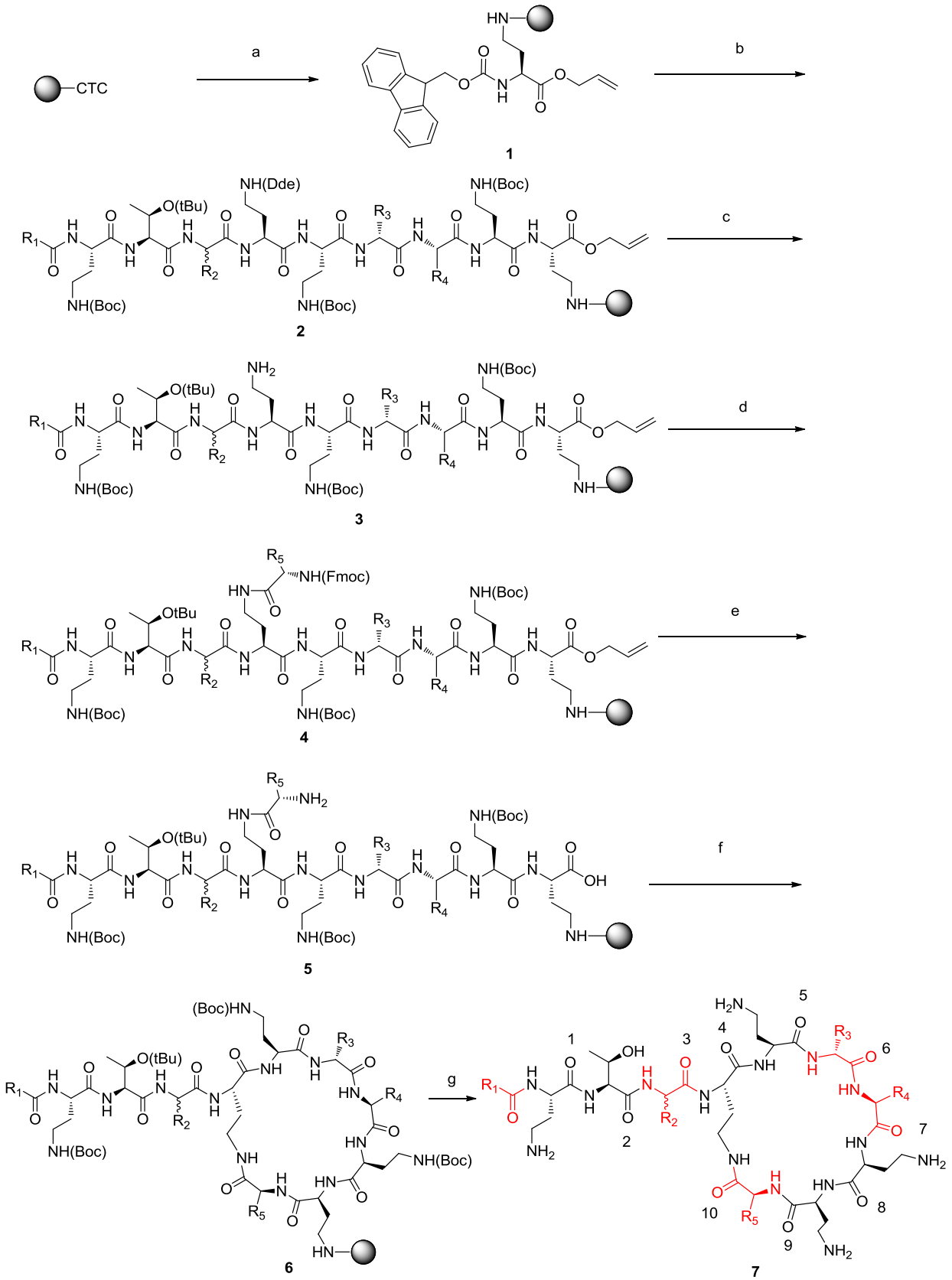
Reagents and conditions: TEMPO, NaClO, $NaClO_2$, CH_3CN, H_2O.

Scheme 3. Synthetic Route of 7-methyloctanoic acid



Reagents and conditions: H_2, Pd/C, MeOH.

Scheme 4. Synthetic Route of the Individual Components of Polymyxins


CTC
a
b
1
NH(Dde)
O(tBu)
NH(Boc)
c
2
NH2
d
3
R5
NH(Fmoc)
OtBu
e
4
f
5
OH
(Boc)HN
g
6
7

Polymyxin B: R_2 = L-Dab, R_3 = D-Phe, R_5 = L-Thr. Polymyxin E: R_2 = L-Dab, R_3 = D-Leu, R_5 = L-Thr.
Polymyxin T: R_2 = L-Dab, R_3 = D-Phe, R_5 = L-Leu.
B_1 , E_1 and T_1: R_1 = 6-MOA, R_4 = L-Leu; B_2 , E_2 and T_2: R_1 = 6-MHA, R_4 = L-Leu;
B_3 and E_3: R_1 = OA, R_4 = L-Leu; B_4 and E_4: R_1 = HA, R_4 = L-Leu;
B_5: R_1 = NA, R_4 = L-Leu; E_7: R_1 = 7-MOA, R_4 = L-Leu;
B_1-Ile and E_1-Ile: R_1 = 6-MOA, R_4 = L-Ile; E_2-Ile: R_1 = 6-MHA, R_4 = L-Ile;
E_1-Nva: R_1 = 6-MOA, R_4 = L-Nva; E_2-Nva: R_1 = 6-MHA,R_4 = L-Nva;
E_1-Val: R_1 = 6-MOA, R_4 = L-Val; E_2-Val: R_1 = 6-MHA, R_4 = L-Val;
Polymyxin A, D and M: R_3 = D-Leu, R_4 = L-Thr, R_5 = L-Thr. Polymyxin S: R_3 = D-Phe, R_4 = L-Thr, R_5 = L-Thr.
A_1: R_1 = 6-MOA, R_2 = D-Dab; A_2: R_1 = 6-MHA, R_2 = D-Dab;
D_1 and S_1: R_1 = 6-MOA, R_2 = D-Ser; D_2 and S_2: R_1 = 6-MHA, R_2 = D-Ser;
M_1: R_1 = 6-MOA, R_2 = L-Dab; M_2: R_1 = 6-MHA, R_2 = L-Dab;
fatty acid (R_1), position 3 amino acid (R_2), position 6 amino acid (R_3), position 7 amino acid (R_4), position 10 amino acid (R_5)

Reagents and conditions: (a) Fmoc-L-Dab-OAll, DIPEA, DCM, 2 h, MeOH; (b) (i) 20% piperidine in DMF, 5 min and 10 min; (ii) appropriate amino acid, HCTU, DIPEA, DMF, 1 h; (iii) corresponding fatty acid, HCTU, DIPEA, DMF, 1 h; (c) 3% hydrazine in DMF, 2×30 min; (d) Fmoc-L-Thr(tBu)-OH or Fmoc-L-Leu-OH, HCTU, DIPEA, DMF, 1 h; (e) (i) $PhSiH_3$, $Pd(PPh_3)_4$, 50% DCM/50% DMF, 2 h; (ii) 20% piperidine in DMF, 5 min and 10 min; (f) PyAOP, HOAt, NMM, DMF, 3 h; (g) TFA:TIS:H_2O (95:2.5:2.5), 2 h; followed by reverse phase HPLC purification.

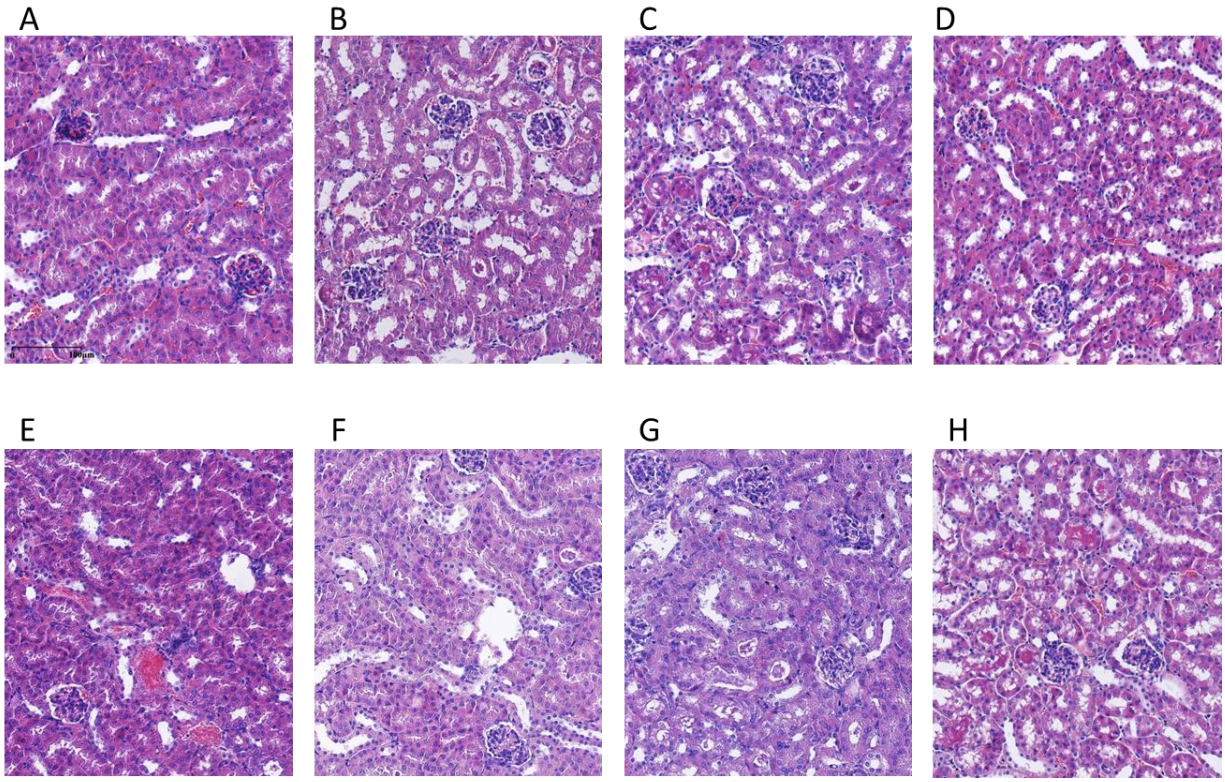


Figure 1. Representative histological images of: (A) mouse kidney from the saline control group showing no histological damage; (B), (C), (D), (E), (F), and (G) mice kidneys after exposure to polymyxins A_2, B_2, D_2, E_2, M_2, and S_2 with grade 1 lesions (SQS=+1), showing tubular dilatation, prominent nuclei, and a few pale tubular casts; (H) mouse kidney after exposure to the polymyxin E control group with grade 1 lesions (SQS=+1). Original magnification: x200 for all panels.