Supporting information

OBOC screening high activity and low-toxic polymyxin analogs against MCR-1 resistance strain

Wenhong Zheng †[a,c], Xin Wang †[ac] , Hao Lian †[cd], Pengfei Zou †[bc], Tongyi Sun *[a], Hao Wang*[ac], Li-Li Li*[ab]

[a] *School of Bioscience and Technology Shandong Second Medical University Weifang, Shandong 261053, China*

[b] *School of Material Science and Engineering, Beijing Institute of Technology, Beijing 100081, China.*

[c] *CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology (NCNST), No. 11 Beiyitiao, Zhongguancun, Beijing 100190, China*

[d] *College of Chemical Engineering, Beijing University of Chemical Technology, Beijing 100029, China*

Experimental section

Materials

All chemicals were purchased from commercially available sources. DMF, TIS, Ethanol absolute and DCM were provided by Modern Oriental Technology Development Co., Ltd. 4-Methylmorpholine and Fmoc-Homo-Phe-OH were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. DBU was purchased from Shanghai Aiko Biotechnology Co., Ltd. Dde-Dab(Fmoc)-OH was purchased from Beijing Zhonghao Xinsheng Technology Co., Ltd.Cyanogen bromide was purchased from Beijing InnoChem Science & Technology Co., Ltd. Vitamin C, phenol, tris(2-carboxyethyl)phosphine hydrochloride, Fmoc-phenylglycine-OH, iso-nonanoic acid, 6-methylheptanoic acid, glycerol, and triethylamine were purchased from Shanghai Macklin Biochemical Co., Ltd. TFA, anhydrous methanol, and HBTU were purchased from Sigma (USA). Fmoc-Dab(Boc)-OH, Fmoc-Met-OH, Fmoc-Thr(Tbu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-D-Phe-OH, and Fmoc-Leu-OH were purchased from GL Biochem (Shanghai) Ltd. Ethanedithiol, acetonitrile, and glacial acetic acid were purchased from Beijing Innochem Technology Co., Ltd. Fmoc-6-aminohexanoic acid-OH was purchased from Shanghai Tengzhun Biotechnology Co., Ltd. Polymyxin B sulfate was purchased from Beijing Solarbio Technology Co., Ltd. PMBN was purchased from Tianjin Baibei Biotechnology Co., Ltd. TSB and TSA were purchased from Beijing Wokai Biotechnology Co., Ltd. PBS (pH=7.4) was purchased from Beijing Solarbio Technology Co.,Ltd. The experimental strain *E. coli* ATCC 25922 was obtained from the frozen stock collection of the National Center for Nanoscience and Technology, Chinese Academy of Sciences.

Cell culture

EDTA anticoagulant tube was purchased from Beijing Yihong Guangjie Biotechnology Co., Ltd. DMEM high glucose medium was purchased from Beijing Solarbio Technology Co., Ltd. Trypsin cell digestion solution was purchased from Biyuntian Biotechnology Co., Ltd. Cell culture plates were purchased from Costar. Cell culture dishes were purchased from Corning. Decanoic acid was purchased from Shanghai Dibo Biotechnology Co., Ltd.

Synthesis of Peptides

All peptides were synthesized by solid-phase synthesis method (SPPS) based on standard Fmoc-chemistry.[1] DMF: DBU = 98:2 (v: v) was used as deprotectant, and 5 g of piperazine was added. The ninhydrin test (ninhydrin : phenol : ascorbic acid, 1:1:1, v/v) confirmed complete deprotection. The next amino acid was coupled with the coupling agent DMF:NMM = 19:1 (v: v), and the same process was repeated until the next Chloroacetic acid was coupled with palmitic acid.Next, add 2/3 of the synthetic tube methanol, shake on the shaker for 1 minute, and perform suction filtration three times to remove the methanol. The final product was obtained by lysis of a mixture of TFA (95%, v/v), triisopropylsilane (2.5%, v/v), and water (2.5%, v/v) for 2.5 h in an ice bath. All the deprotectioned side-chains were removed by the mixture of 95% TFA (v/v), 2.5% H2O (v/v), 2.5% EDT (v/v), and 2.5% TIPS (v/v) for 2.5 h in ice bath. The cyclization solution prepared by mixing resin with 5 ml DMSO, 200 μl aqueous solution of 29 mg tris(2-carboxyethyl)-phosphine hydrochloride, and 50 μL triethylamine was placed in a reaction vessel and stirred on a magnetic stirrer at 500 rpm/min for 1 hour to allow the N-terminal chloroacetyl group of the peptide and the thiol group of the cysteine side chain to undergo cyclization. Then, 30 μL of trifluoroacetic acid was added to complete the quenching reaction. The final product was obtained by lysing a mixture of 9.1% acetonitrile (v/v), 72.7% acetic acid (v/v), and 18.2% water (v/v), with 30 mg of HBr added per 1 mL, followed by static lysis at 4°C overnight. The resulting solid was washed three times with ice ether and air dried to obtain the final product. The relative molecular mass and purity were determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF, Autoflex-Max) and Liquid Chromatography-Mass Spectrometry (Bio-LCMS8050, LCMS-8050), respectively.[2]

Minimal inhibitory concentration (MIC)

The MIC of each peptide against each strain was determined using clinical laboratory standards, and the culture was shaken (180-200 rpm) until the logarithmic growth phase was reached, then collected by centrifugation and washed three times with 1× PBS. The concentration of suspended bacteria was determined by reading the optical density at 600 nm (OD_{600}). The bacterial suspension was diluted to ensure that the bacterial concentration in the 96-well plate was approximately 7.5×10^5 CFU/ mL ,and 100 μL of this suspension was added to a 96-well tissue culture plate containing different concentrations of drugs (100 μL).[3] In the 96-well tissue culture plate,50 μL of bacterial suspension and 100 μL of liquid tryptic soy broth (TSB) medium were added to each well in the experimental groups, and then 50 μL of polypeptide solutions with different concentrations were added correspondingly. The bacteria were cultured at 37℃ for 18-24 h and their growth was observed by reading at OD_{600}.[4] The MIC was determined as the treatment concentration at which no microbial growth was observed. Each test was carried out in 3 replicates.

Minimum Bactericidal Concentration (MBC)

The experimental method for MBC is based on the results of MIC. Each test was carried out in 3 replicates. After diluting different groups of bacterial suspension cultured for 24 h above, 50 μL was coated on an TSA AGAR plate and cultured in a 37 ℃ incubator for 24 h to calculate colony formation units.[5]

Measurement of Hemolysis

Fresh whole blood of mice eyeballs was taken, and 5 mL was mixed with 120 mL of PBS and centrifuged at 8000 rpm for 5 min to remove the supernatant. The collected erythrocytes were further washed twice to three times with PBS until the supernatant was clarified. Mix 500 μL of peptide solution and 500 μL of red blood cell suspension each in a 2 mL sterile centrifuge tube. Mix 500μL PBS with 500 μL of red blood cell suspension to serve as the negative control group. Mix 500μL H_2O with 500 μL of red blood cell suspension to serve as the positive control group. After pipetting evenly, incubate at 37°C for 2 h, the supernatant was collected by centrifugation at 4000 rpm for 2 min. Finally, the absorption of the supernatant at 540 nm was measured by an enzyme marker and the hemolysis rate was calculated as follows: [6]

$$Hemolysis\% = \frac{(OD_t - OD_n)}{(OD_p - OD_n)} \times 100\%$$

Cytotoxicity Assay

Cytotoxicity evaluation was performed using CCK-8. HepG2 and HEK 293 T cells were incubated in DMEM medium with 5% CO_2 at 37 °C for 24h. Then, after digesting with cell trypsin, dilute it with DMEM to a concentration of 7×10^4 cells per milliliter. HUVECs and HEK 293 T cells (7×10^3 cell $well^{-1}$, 100 μL) were seeded in a 96-well cell culture plate and incubated in DMEM medium with 5% CO_2 at 37 °C for 24 h. Then, the peptide drug solution was removed, and 100 μL of the CCK-8/DMEM solution (with a volume ratio of 10/90) was added. Then, the plate was incubated at 37 °C for 2 hours. Subsequently, at OD_{450} was measured using Multifunctional microplate detector. The relative cell survival rate was calculated using the following equation:

$$Relative\ cell\ survival\ rate\ \% = \frac{(A_{drug} - A_{medium})}{(A_{cintrol} - A_{medium})} \times 100\%$$

Construction of the Peptide Library

Tenta Gel MB-NH_2 resin was employed for peptide synthesis and library construction. The peptide sequence was modified by substituting 9, 8, 8, and 2 different amino acids at the 3rd, 6th, 7th, and 10th positions, respectively. The resin was swollen, and after coupling the amino acid Met as a linker, amino acids were sequentially coupled at the designated positions. During coupling at the screening sites, the resin was divided into separate synthesis tubes based on the number of substituted amino acids, and different amino acids were coupled in each tube. After the final amino acid was coupled and deprotected, chloroacetic acid was introduced to react with the free amino group at the N-terminus, achieving chloroacetylation and facilitating the subsequent cyclization reaction with the thiol group. The resin was washed with methanol, cleavage solution, and nitrogen gas to shrink, cleaved, and the peptides were

then dried. Cyclization was carried out for 1 hour by adding the cyclization solution. This process exposed the thiol group of cysteine in the peptide chain upon removal of side-chain protecting groups, enabling cyclization on the resin. Finally, 30 μL of trifluoroacetic acid was added to quench the reaction. The OBOC (One-Bead-One-Compound) peptide library of polymyxin B analogs was thus successfully constructed, with each resin bead carrying a distinct peptide. Individual peptides were subsequently isolated by separating the resin beads, providing the basis for downstream screening.

Energy minimization

When performing energy minimization experiments in ChemDraw, first draw the structure of the molecule. After drawing, convert the structure into a three-dimensional model through 3D View. Then, import the three-dimensional structure into Chem3D software and perform energy minimization in Chem3D. Through Energy Minimization, select the force field MM2 and set the optimization accuracy. [7, 8]Start energy minimization until the lowest energy state is reached. Save the optimized structure in pdb format and observe it in pymol.[9]

Supplementary figures

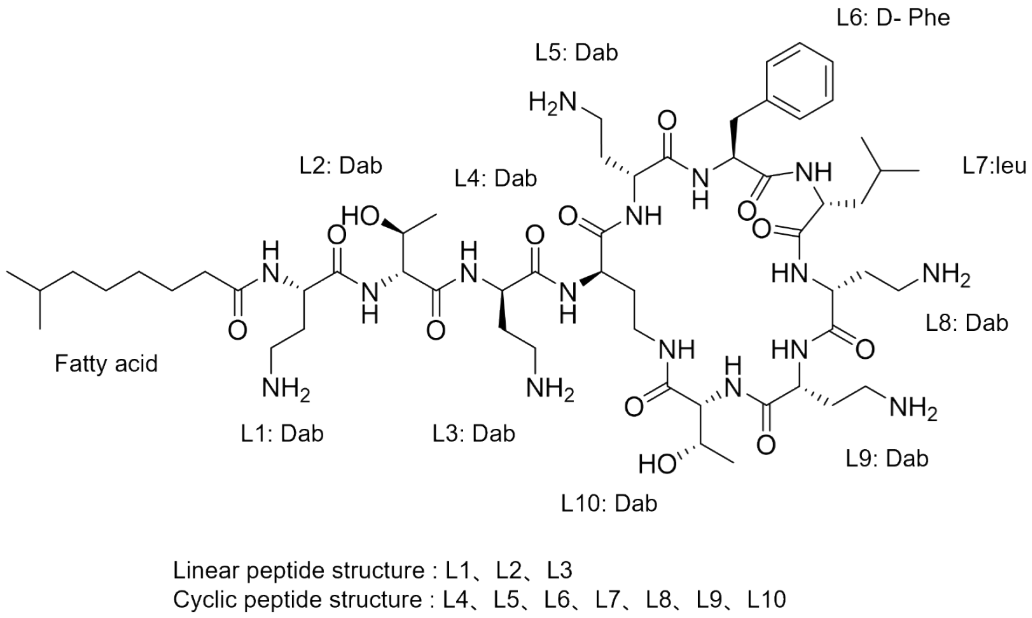

Fig. S1. The structural formula of polymyxin B (PMB).

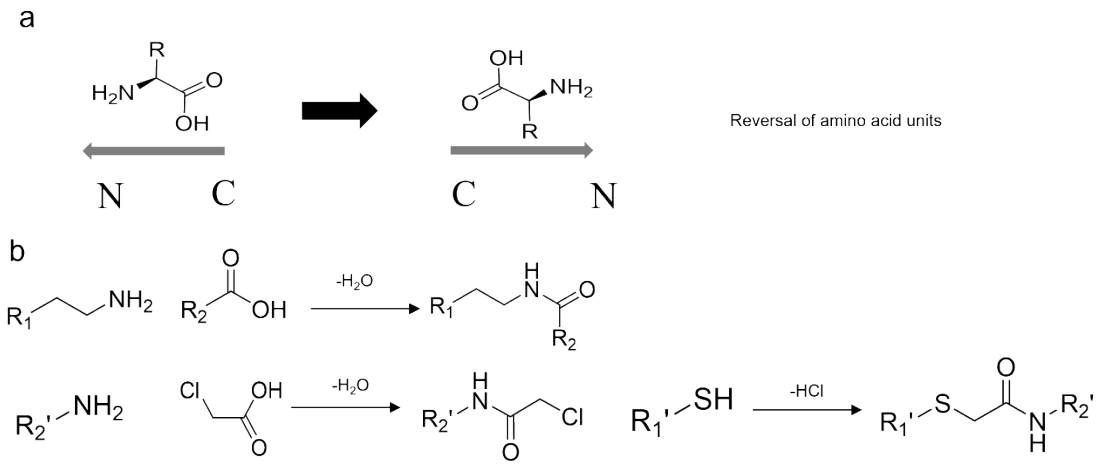

Fig. S2. The change of amino acid unit and the cyclization pattern. a. From L1 to L10, N-terminal and C-terminal reversal of the amino acid unit. b. The cyclization method changes from the original amide reaction to a thiol substitution reaction.

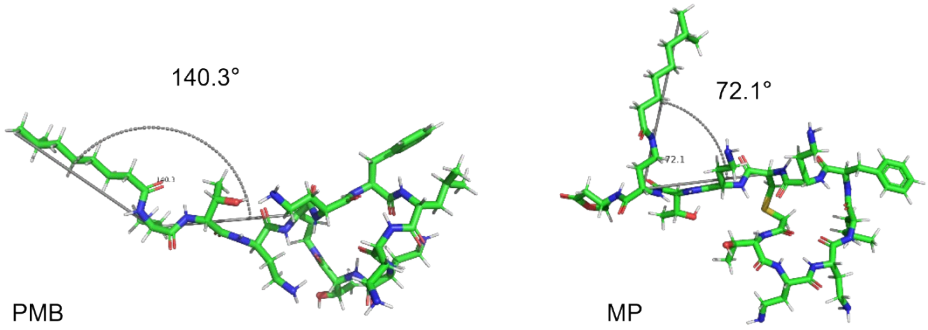

Fig. S3. Detect the angle by minimizing energy. the angle between PMB and the mother peptide (MP), the fatty acid chain and the peptide ring.

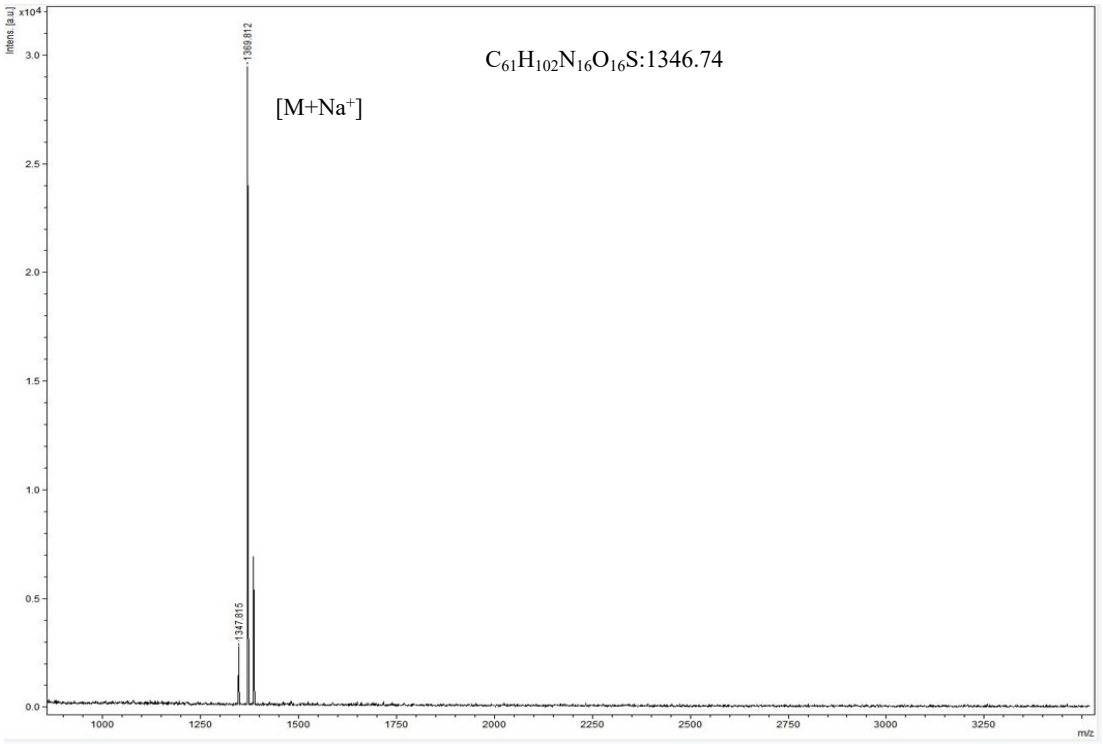

Fig. S4. Molecular weight of mother peptide.

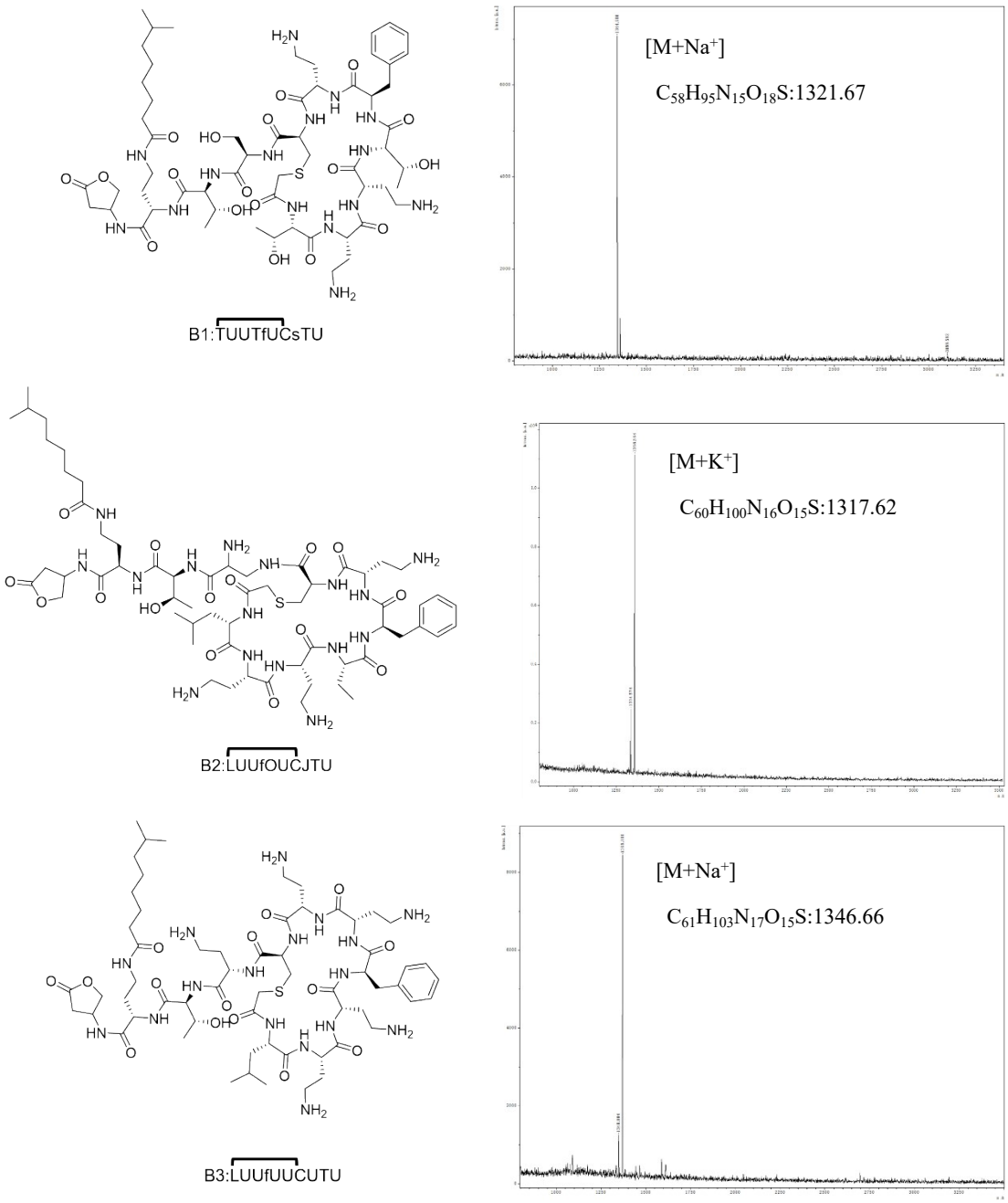

Fig. S5. The structural formulas and corresponding molecular weights of negative candidates B1, B2, and B3.

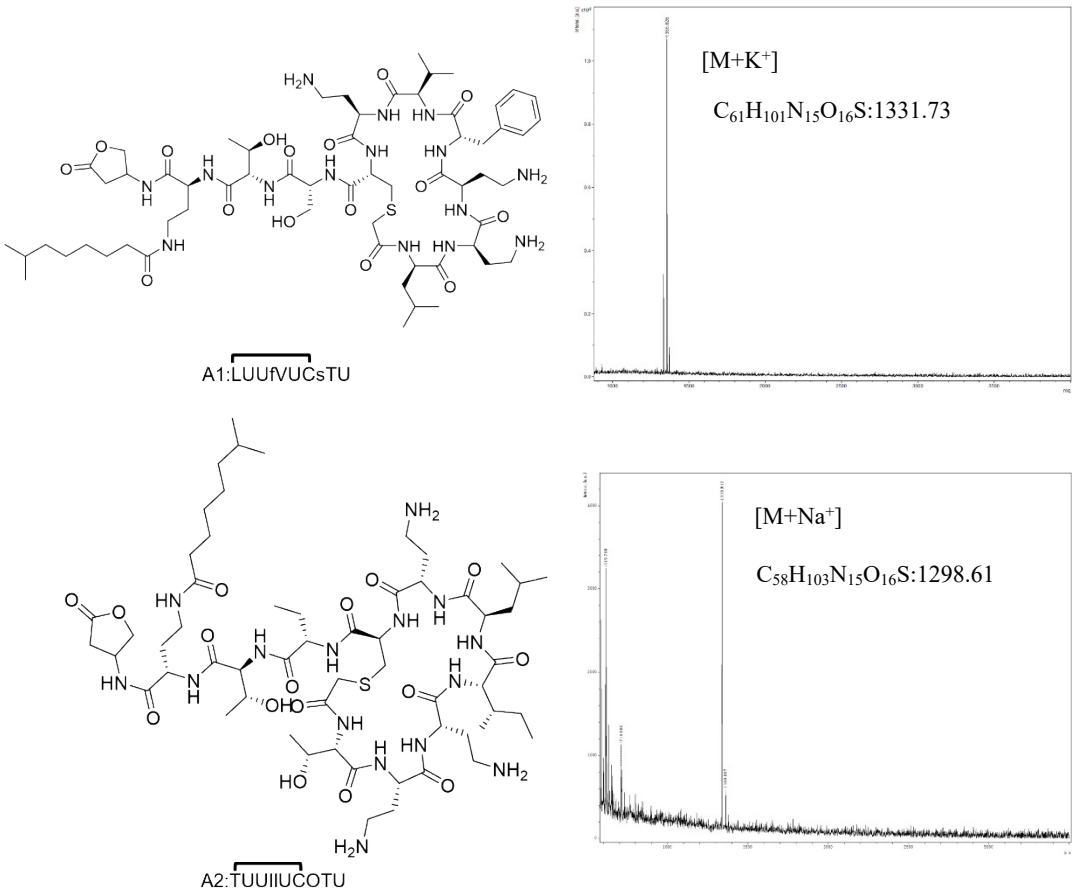

Fig. S6. The structural formulas and corresponding molecular weights of Positive candidates A1, A2.

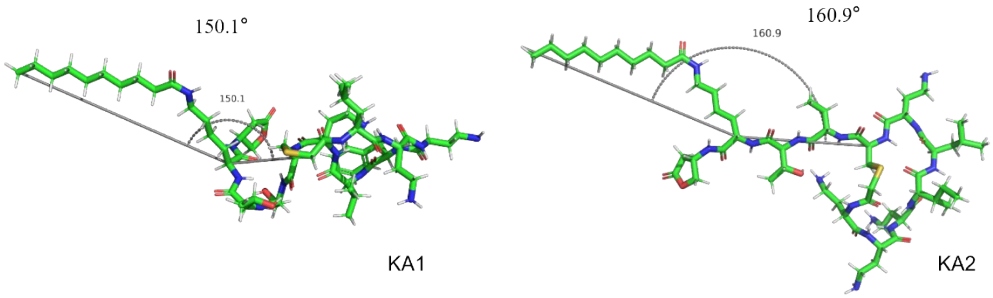

Fig. S7. Detect the angle by minimizing energy. the angle between the fatty chain and the peptide ring in KA1 and KA2.

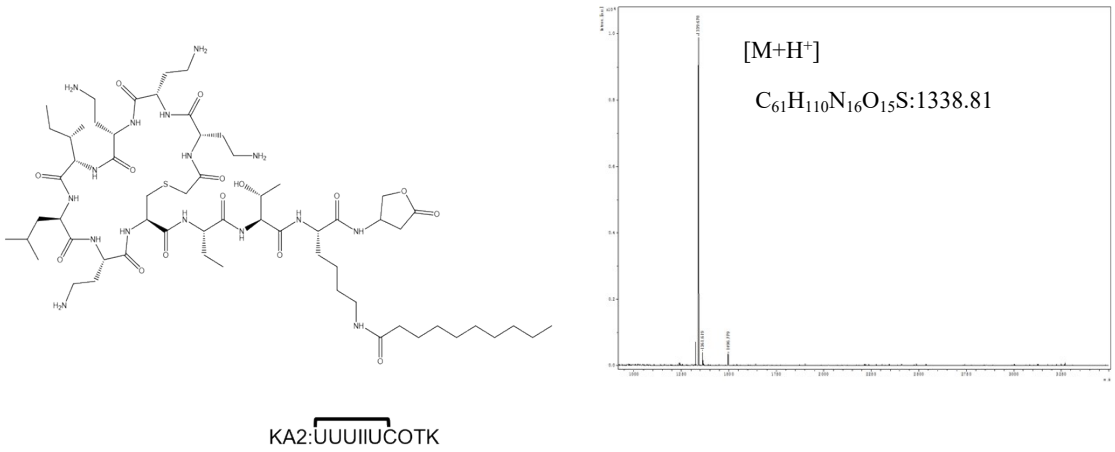

Fig. S8. The structural formulas and corresponding molecular weights of KA1, KA2.

Table S1. MIC of PMB and MP against *E. coli* ATCC 25922 (n=5).

	E. coli ATCC 25922 MIC (μg/mL)
PMB	1
MP	32

Table S2. Subsequent sequence with molecular weight of 1331. (O: Abu, U: Dab, J: Dap, f: D-Phe, s: D-Ser, l: D-Leu)

L3 Hydrophilic	L6, L7 Hydrophilic	L3, L67 Hydrophilic	L10 Hydrophilic
LUUfVUCsTU	LUUfsUCVTU	LUUfUUCJTU	TUUlfUCOTU
LUUfOUCTTU	LUUfTUCOTU	LUUUfUCJTU	TUUlOUCfTU
LUUOfUCTTU	LUUsfUCVTU		TUUIfUCOTU
LUUVfUCsTU	LUUsVUCfTU		TUUIOUCfTU
	LUUTfUCOTU		TUUflUCOTU
	LUUTOUCfTU		TUUflUCOTU
	LUUVsUCfTU		TUUfOUCLTU
	LUUOTUCfTU		TUUfOUCITU
			TUUfVUCVTU
			TUUOlUCfTU
			TUUOIUCfTU
			TUUOfUCLTU
			TUUOfUCITU
			TUUVfUCVTU
			TUUVVUCfTU

Table S2(Continued). Subsequent sequence with molecular weight of 1297. (O: Abu, U: Dab, J: Dap, f: D-Phe, s: D-Ser, l: D-Leu)

L3 Hydrophilic	L67 Hydrophilic	L3、L67 Hydrophilic	L10 Hydrophilic
LUUIVUCsTU	LUUlsUCVTU	LUUUlUCJTU	TUUIlUCOTU
LUUIOUCTTU	LUUlTUCOTU	LUUUIUCJTU	TUUllUCOTU
LUUlVUCsTU	LUUIsUCVTU	LUUUVUCUTU	TUUlIUCOTU
LUUlOUCTTU	LUUITUCOTU	LUUVUUCUTU	TUUlOUCLTU
LUUOlUCTTU	LUUslUCVTU	LUUlUUCJTU	TUUlOUCITU
LUUOIUCTTU	LUUsIUCVTU	LUUIUUCJTU	TUUlVUCVTU
LUUVVUCTTU	LUUsVUCLTU		TUUIIUCOTU
LUUVlUCsTU	LUUsVUCITU		TUUIOUCLTU
LUUVIUCsTU	LUUTlUCOTU		TUUIOUCITU
	LUUTIUCOTU		TUUIVUCVTU
	LUUTOUCITU		TUUOlUCLTU

Table S3. MIC of A group (A1, A2) and B group (B1, B2, B3) for *E. coli* ATCC 25922, *E. coli* MDR ESBL (clinical), *E. coli* 3CZ30E (n=5).

	MIC(μg/mL)		
	E. coli ATCC 25922	*E. coli* MDR ESBL	*E. coli* 3CZ30E
A1	16	16	16
A2	16	16	16
B1	32	32	32
B2	32	32	32
B3	32	32	32

Table S4. Additive summary of structure-activity relationship analysis of amino acids involved in mutations in groups A and B.

group	A	A	B	B	B
classification	A1	A2	B1	B2	B3
vr3_dzp	9.223583295	10.1954916	8.164956538	9.618967	4.466129
vr3_dzi	10.15179879	11.25013563	8.341231123	11.54973	5.893128
vr3_dzare	9.617539484	10.30761747	8.544276261	10.03094	5.14492
vr2_dzp	29.40932306	30.02506791	6.47720853	49.6754	3.855697
vr2_dzi	34.53717874	35.34247512	4.048474155	61.74733	4.453955
vr1_dzp	68.20599068	69.51221929	57.76227669	62.66791	29.60092
vr1_dzi	69.88254824	70.79836334	59.78579391	69.01153	27.71793
vr1_dzare	66.74374676	68.82902511	56.04796676	61.63222	28.77742
ve3_dzare	13.42319496	14.77948997	4.557800839	24.29622	2.709325
spmax_dzse	36.491893	34.93264344	30.85231703	22.68726	19.72921
spmax_dzi	36.18778414	35.96992197	30.32534619	21.8831	22.61635
spmax_dzare	38.52228953	37.85210724	29.49895558	27.20527	21.68587
spmad_dzse	9.46176185	9.321547291	7.202967316	7.31967	4.448007
spdiam_dzse	53.65349937	50.62673871	44.74018515	32.04497	30.41977
spad_dzse	70.17018832	67.77764906	58.79155908	40.31294	39.95198
spad_dzi	70.71105003	69.43495759	61.15647526	41.04959	42.46487
spabs_dzp	106.4003511	110.6062985	93.9670738	76.23177	54.40357
spabs_dzi	81.28374652	80.12370024	57.72861554	63.35678	42.56657
spabs_dzare	70.66178723	68.11413715	57.14046582	44.05633	40.34848
sm1_dzi	3.585021945	4.922385831	16.31031808	5.581095	3.174183
sm1_dzare	12.40164531	13.71627406	13.36532898	22.75535	2.322721
rpcg	13.70040432	13.86033294	4.91810999	25.11903	4.947164
rncg	24.83544758	25.46587088	24.18467804	47.0298	4.272591
logee_dzse	36.83318181	35.81531578	29.13214773	23.63746	20.54212
bertzct	205.7183795	209.8926451	182.7544448	130.0965	103.2177
balabanj	8.159873846	8.252177227	7.10773284	6.059283	3.402123

Table S5. MBCs of the KA group (KA1, KA2) and PMB against *E. coli* [a] ATCC 25922, *E. coli* [b] MDR ESBL, and *E. coli* [c] 3CZ30E, as well as the HC_{10} (10% hemolysis concentration) of the KA group and PMB.

	MBC(μg/mL)			HC_{10}(μg/mL)
	E. coli [a]	*E. coli* [b]	*E. coli* [c]	
PMB	1	1	8	8
KA1	1	1	8	64
KA2	1	1	8	64

References

1 P. Cherkupally, G. A. Acosta, S. Ramesh, B. G. De la Torre, et al., *Amino Acids*, 2014, **46**, 1827-1838.
2 P. Zou, L. Huang, Y. Li, D. Liu, et al., *Adv. Mater.*, 2024, **36**, 2407152.
3 I. Wiegand, K. Hilpert and R. E. W. Hancock, *Nat. Protoc.*, 2008, **3**, 163-175.
4 C. Dai, M. Zhou, W. Jiang, X. Xiao, et al., *J Mater Sci Technol.*, 2020, **59**, 220-226.
5 J. M. Andrews, *J. Antimicrob. Chemother.*, 2001, **48 Suppl 1**, 5-16.
6 A. G. Elliott, J. X. Huang, S. Neve, J. Zuegg, et al., *Nat. Commun.*, 2020, **11**, 3184.
7 A. Pilipović, D. Mitrović, S. Obradović and M. Poša, *Eur. Rev. Med. Pharmacol. Sci.*, 2021, **25**, 7501-7507.
8 M. Zamani, P. Mokarram, M. Jamshidi, M. Siri, et al., *Curr. Comput. Aided Drug Des.*, 2024, **20**, 943-954.
9 P. Zou, L. Huang, Y. Li, D. Liu, et al., *Adv. Mater.*, 2024, **36**, e2407152.