An Effective Approach to Obtain Functional Poly-β-Peptides for Combating Drug-Resistant Bacterial Infections

Sheng Chen,[¶,1] Zhengjie Luo,[¶,1] Min Zhou,[*,1,2] Ximian Xiao,[2] Zihao Cong,[2] Jiayang Xie,[2,3] Yueming Wu,[2] Haodong Zhang,[2] Xuebin Zhao,[2] Gonghua Song,[4] and Runhui Liu[*,1,2,3,5]

[1]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, P. R. China

[2]Shanghai Frontiers Science Center of Optogenetic Techniques for Cell Metabolism, Frontiers Science Center for Materiobiology and Dynamic Chemistry, Key Laboratory for Ultrafine Materials of Ministry of Education, Engineering Research Center for Biomedical Materials of Ministry of Education, Key Laboratory of Specially Functional Polymeric Materials and Related Technology (Ministry of Education), School of Materials Science and Engineering, East China University of Science and Technology, Shanghai 200237, P. R. China

[3]Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Sciences, Suzhou 215163, P. R. China

[4]Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, P. R. China

[5]Shanghai Shyndec Pharmaceutical Co., Ltd., 378 Jian-Lu Road, Shanghai 201203, P. R. China

Correspondence should be addressed to R.L. (rliu@ecust.edu.cn) and M.Z. (minzhou@ecust.edu.cn).

¶ S.C. and Z.L. have equally contributed to the manuscript.

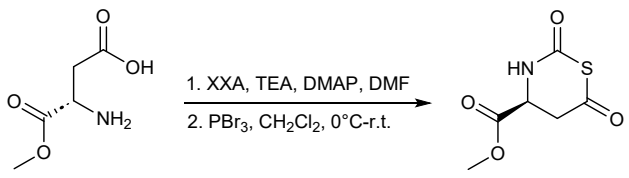
Materials and methods

Materials

Phosphate buffered saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Thermo Fisher Scientific. Solvents used in β-*N*-thiocarboxyanhydride (β-NTA) purification were freshly distilled dichloromethane and/or anhydrous magnesium sulfate ($MgSO_4$)-treated ethyl acetate (EtOAc) and *n*-hexane. Methyl thiazolyl tetrazolium (MTT) was purchased from Shanghai Macklin Biochemical Co., Ltd. (4-tert-butylphenyl)methanamine (*t*BuBnNH$_2$) was purchased from Bidepharm. Potassium trifluoroacetate was purchased from Sigma-Aldrich. *N*,*N*-dimethylformamide (DMF) was purchased from J&K Scientific. Propylamine was purchased from Energy Chemical Co. Melittin was purchased from TargetMol (TargetMol, USA). All other reagents and solvents were purchased from Shanghai Adamas-beta® and without further purification unless otherwise specified.

The synthesized intermediates were purified using a SepaBean machine equipped with Sepaflash columns produced by Santai Technologies Inc. in China. Nuclear magnetic resonance (NMR) spectra were collected on a Bruker spectrometer at 400 MHz or 600 MHz. Gel permeation chromatography (GPC) characterization was performed on a Waters GPC instrument equipped with an isocratic HPLC pump (Waters 1515), a Brookhaven BI-MwA multi-angle light scattering detector and a refractive index detector (Waters 2414) using DMF (supplemented with 0.01 M LiBr) as the mobile phase at a flow rate of 1 mL/min at 50 °C. The Tosoh TSKgel Alpha-2500 column (particle size 7 μm), the Tosoh TSKgel Alpha-3000 column (particle size 7 μm) and the Tosoh TSKgel Alpha-4000 column (particle size 10 μm) connected in series were used for the separation of polymers. GPC characterization was performed on a Waters GPC instrument equipped with an isocratic HPLC pump (Waters 1515), a Brookhaven BI-MwA multi-angle light scattering detector and a refractive index detector (Waters 2414) using hexafluoroisopropanol (HFIP, supplemented with 0.02 M potassium trifluoroacetate) as the mobile phase at a flow rate of 0.3 mL/min. The Tosoh TSKgel Alpha-3000 column (particle size 7 μm) was used for the separation of polymers. These two instruments were chosen according to the solubility of the polymer in DMF and HFIP. The number-average molecular weight (M_n), degree of polymerization (DP) and dispersity index ($Đ$) were calculated from a calibration curve using polymethylmethacrylate (PMMA) as standards. The Fourier transform infrared (FTIR) spectra of poly-β-peptides were collected in ATR mode on Thermo Electron Nicolet is50 FTIR spectrometer. Time-lapse fluorescence images were taken on a Leica confocal laser scanning microscope (CLSM) (LEICA TCS SP8). Zeta potential of bacteria was characterized using a Zetasizer Nano-ZS instrument. The optical density values and fluorescence values were recorded on a SpectraMax® M2 plate reader. The morphology of bacteria was performed on a field emission scanning electron microscope (FESEM, FEI, Nova NanoSEM 450).

Synthesis of β^3-Methyl-L-carboxyhomoglycine *N*-thiocarboxyanhydride (Me-β^3-LCHG NTA)

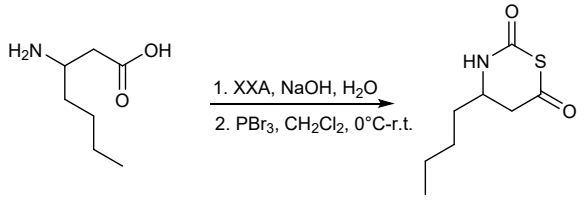


Scheme S1. Synthesis of Me-β^3-LCHG NTA

1-methyl L-aspartate (2.94 g, 20.0 mmol), S-ethoxythiocarbonyl mercapto-acetic acid (XAA,

3.6 g, 20.0 mmol), triethylamine (TEA) (10.1 g, 100.0 mmol) and 4-dimethylaminopyridine (DMAP) (1.8 g, 2.0 mmol) were added to a reaction flask containing 100 mL anhydrous *N*,*N*-dimethylformamide (DMF). The reaction mixture was stirred vigorously at room temperature under a nitrogen environment for 48 h. The reaction mixture was concentrated and dissolved in 100 mL EtOAc followed by washing with deionized water (3 × 50 mL) and saturated brine (1 × 50 mL) successively. The obtained organic layer was then dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give a crude intermediate that was purified through a silica gel column chromatography to give the pure intermediat. The intermediate was then dissolved in anhydrous dichloromethane (200 mL) and treated with PBr_3 (4.7 g, 17.3 mmol) at 0°C under nitrogen. The reaction mixture was allowed to stir at r.t. for 6 h, and then was washed sequentially with ice water (3 × 100 mL) and saturated brine (1 × 100 mL). The obtained organic phase was dried over $MgSO_4$, filtered and concentrated to afford the crude product that was purified by recrystallization from the mixture of EtOAc and *n*-hexane (1:1 EtOAc: *n*-hexane) to give the pure white fluffy solid (2.31 g, 61.2% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$, Figure S1): δ 6.78 (s, 1H), 4.45–4.41 (m, 1H), 3.88 (s, 3H), 3.24 (dd, J = 16.4, 3.2 Hz, 1H), 2.96 (dd, J = 16.4, 10.5 Hz, 1H). EI-MS (Figure S2): m/z calculated for $C_6H_7NO_4S[M]^+$: 189.0; Found 189.0. The characterization of Me-β^3-LCHG NTA was consistent with our previously reported result[1].

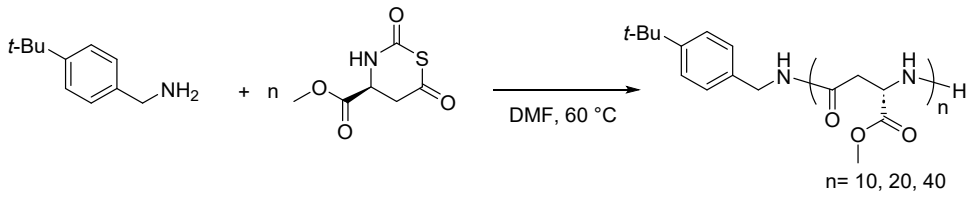
Synthesis of β^3-D/L-homonorleucine *N*-thiocarboxyanhydride (β^3-HNL NTA)



Scheme S2. Synthesis of β^3-HNL NTA

To a solution of NaOH (1.0 g, 25.0 mmol) in deionized (DI) water (20 mL) was added 3-aminoheptanoic acid (1.45 g, 10.0 mmol) and XAA (1.8 g, 10.0 mmol). The reaction was stirred vigorously at room temperature for two days followed by acidification with 2 N HCl to pH = 3 and extraction with ethyl acetate (3 × 40 mL). The combined organic phase was washed with saturated brine (1 × 100 mL), dried over anhydrous $MgSO_4$ and concentrated to give the intermediate. The intermediate was dissolved in anhydrous dichloromethane (50 mL) and treated with PBr_3 (2.7 g, 10.0 mmol) at 0 °C under nitrogen. The reaction mixture was allowed to stir at room temperature for 6 h and then washed sequentially with DI water (1 × 100 mL) and saturated brine (1 × 100 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated to afford the crude product. After purification with recrystallization from the mixture of EtOAc and *n*-hexane (1:3 EtOAc: *n*-hexane), a white fluffy solid was obtained (0.96 g, 54.3% yield over two steps). ^{1}H NMR (400 MHz, $CDCl_3$, Figure S3): δ 6.45 (brs, 1H), 3.79–3.60 (m, 1H), 2.88 (dd, J = 16.1, 2.9 Hz, 1H), 2.65 (dd, J = 16.1, 10.0 Hz, 1H), 1.80–1.59 (m, 2H), 1.46–1.31 (m, 4H), 0.94 (t, J = 7.0 Hz, 3H). EI-MS (Figure S4): m/z calculated for $C_8H_{13}NO_2S$ $[M]^+$: 187.1; Found 187.1. The characterization of β^3-HNL NTA was consistent with our previously reported result[2].

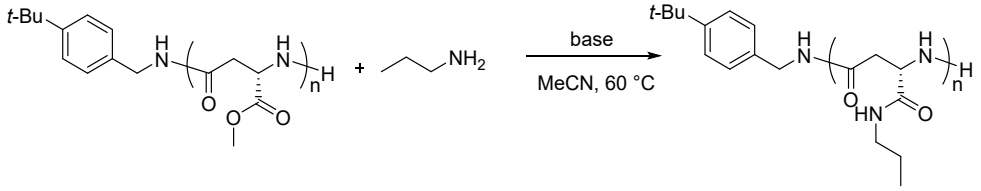
Synthesis of poly(Me-β^3-LCHG)$_n$



Scheme S3. Synthesis of poly(Me-β^3-LCHG)$_n$

For poly-β-peptides with different chain lengths, the synthesis method was similar. The chain lengths of poly-β-peptides were controlled via the ratio of β-NTA to initiator. Take the synthesis of poly(Me-β^3-LCHG)$_{20}$ for example. At ambient condition, Me-β^3-LCHG NTA (47.3 mg, 0.25 mmol) was weighed out and dissolved in DMF (0.5 M), in a reaction vial equipped with a magnetic stir bar. Then a solution of (4-tert-butylphenyl)methanamine (*t*BuBnNH$_2$) in DMF (0.5 M, 25 μL) was added to the reaction, and the reaction mixture was stirred at 60 °C overnight. The resulting poly-β-peptide was precipitated out by pouring the solution into cold methyl tertiary butyl ether (MTBE) (45 mL). The precipitate was collected after centrifugation and dried under air flow. The collected solid was re-dissolved in THF (0.5 mL) and then was precipitated out again by adding MTBE (45 mL) to the solution. This dissolution-precipitation process (0.5 mL THF, 45 mL MTBE) was repeated thrice to obtain the polymer (29.6mg, 86.2% yield). DMF-soluble poly-β-peptide was analyzed by GPC at 2 mg/mL in DMF.

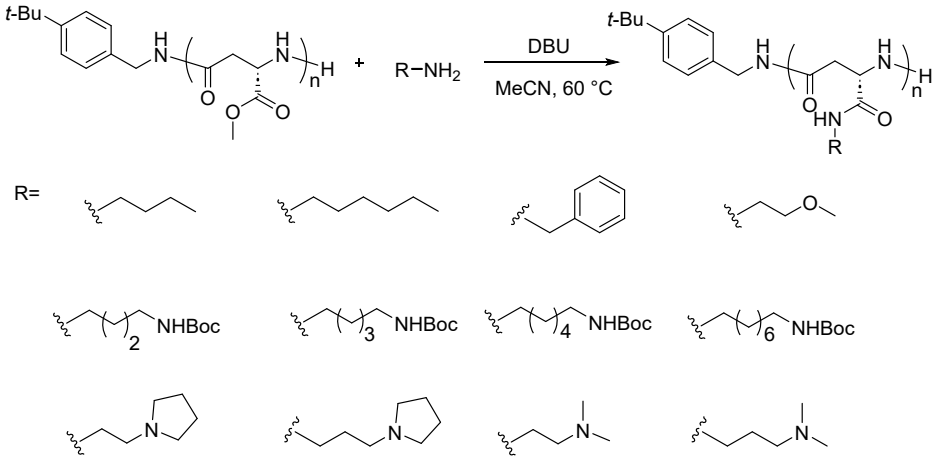
Reaction condition screening of poly-β-peptide synthesis



Scheme S4. Reaction condition screening of poly-β-peptide synthesis

Poly(Me-β^3-LCHG)$_{20}$ (10 mg) was dissolved in acetonitrile (MeCN) (0.5mL), to which propylamine (3 equivalents to methyl ester of poly-β-peptide) was added and stirred, followed by the addition of base (1.2 equivalents to methyl ester), and the reaction was carried out for six hours at 60 °C. Different bases were selected for the screening conditions, including triethylamine (TEA), *N*,*N*-Diisopropylethylamine (DIEA), pyridine, 4-dimethylaminopyridine (DMAP), imidazole, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,5,7-Triazabicylo[4.4.0]dec-5-ene (TBD). At the end of the reaction, all the reaction solution was poured into cold MTBE (45 mL) and the solid was precipitated by centrifugation. The collected solid was dissolved in MeCN (0.5 mL) and precipitated out again by adding MTBE (45 mL). The process was repeated thrice. The preferred reaction conditions were selected by analyzing the GPC trace and calculating the conversion rates of post-modification. The purity of polymer synthesized from the optimal condition was characterized by FTIR and ^{1}H NMR.

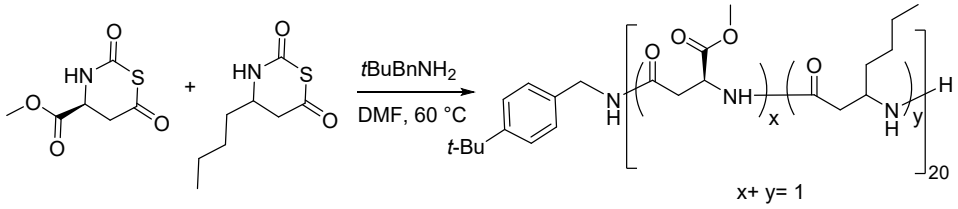
Synthesis of poly-β-peptides with variable side chain structures



Scheme S5. Synthesis of poly-β-peptides with variable side chain structures

For different chain length polymers reacting with different primary amines the conditions were similar. Take Poly(Me-β^3-LCHG) with 20-mer chain length for example. Poly(Me-β^3-LCHG)$_{20}$ (10 mg) was dissolved in MeCN (0.5 mL), to which R-NH_2 (3 equivalents to methyl ester) was added and stirred, followed by the addition of DBU (1.2 equivalents to methyl ester), and the reaction was carried out for six hours at 60 °C. The resulting polymer was precipitated with MTBE (45mL) and the collected solid was dissolved in MeCN (0.5 mL) and precipitated out again by adding MTBE (45 mL). The process was repeated thrice to obtain the poly-β-peptide (75.2–81.5% yield). Poly-β-peptide was analyzed by GPC at 2 mg/mL in DMF or HFIP according to the solubility of polymers.

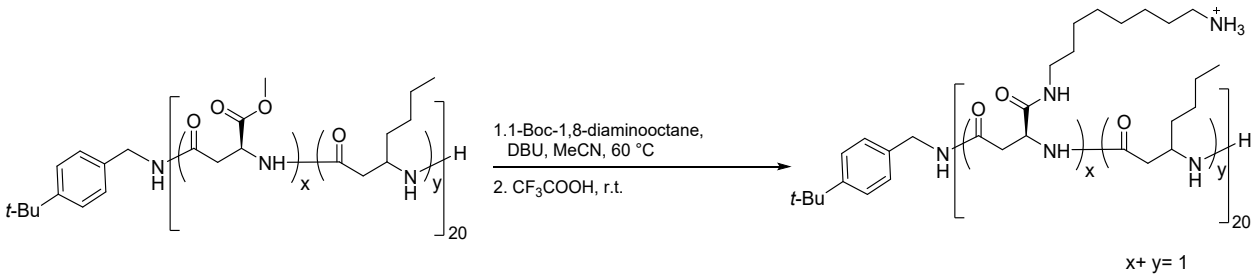
Synthesis of poly(Me-β^3-LCHG)$_x$-*r*-poly(β^3-HNL)$_y$



Scheme S6. Synthesis of poly(Me-β^3-LCHG)$_x$-*r*-poly(β^3-HNL)$_y$

The synthesis of poly(Me-β^3-LCHG)$_x$-*r*-poly(β^3-HNL)$_y$ was followed by the synthesis of poly(Me-β^3-LCHG)$_{20}$, using Me-β^3-LCHG NTA and β^3-HNL NTA with varying ratio as the polymerisation monomer. At ambient condition, a solution of (4-tert-butylphenyl)methanamine (*t*BuBnNH_2) in DMF (0.5 M, 25 μL) was added to the mixture of Me-β^3-LCHG NTA and β^3-HNL NTA in DMF (0.5 M, 500 μL), and the reaction mixture was stirred at 60 °C overnight. The resulting poly-β-peptide was precipitated out by pouring the solution into cold MTBE (45 mL). The precipitate was collected after centrifugation and dried under air flow. The collected solid was re-dissolved in THF (0.5 mL) and then was precipitated out again by adding MTBE (45 mL) to the solution. This dissolution-precipitation process (0.5 mL THF, 45 mL MTBE) was repeated thrice to obtain the polymer (84.3–86.2% yield). Polymers were analyzed by GPC at 2 mg/mL in HFIP.

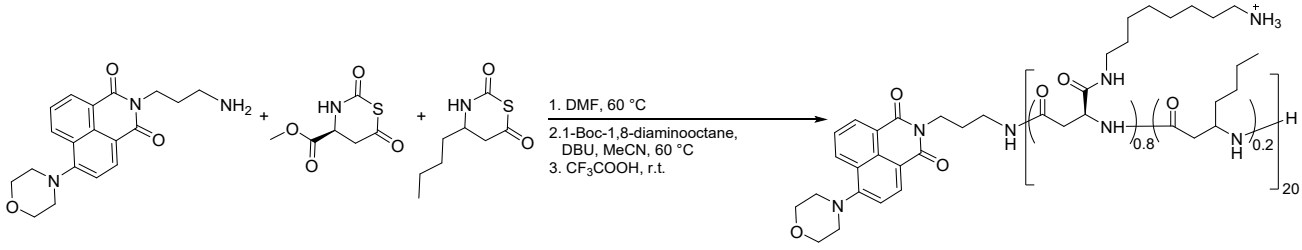
Synthesis of poly(AOc-β^3-LAHG)$_x$-*r*-poly(β^3-HNL)$_y$ (AOc$_x$HNL$_y$)



Scheme S7. Synthesis of AOc$_x$HNL$_y$

Synthesis of poly(AOc-β^3-LAHG)$_x$-*r*-poly(β^3-HNL)$_y$ was followed by the abovementioned process, using poly(Me-β^3-LCHG)$_x$-*r*-poly(β^3-HNL)$_y$ (10 mg) and 1-Boc-1,8-diaminooctane as reactants. The polymer obtained was dissolved in trifluoroacetic acid (TFA) and shaked for 2 hours to remove the *N*-Boc protection of the side chain amino group. After removing the solvent under the airflow, the mixture was dissolved in methanol (0.5 mL), subsequently adding cold MTBE (45 mL) to the mixture to precipitate powder. After centrifuging, the supernatant was removed and then the obtained polymer was dried under airflow. The dissolution-precipitation cycle was repeat twice again to provide purified polymer. Polymer was dissolved in Milli-Q water and subjected to lyophilization to give powder in the form of TFA salt (79.6–82.3% yield). Poly(AOc-β^3-LAHG)$_x$-*r*-poly(β^3-HNL)$_y$ were characterized by ^{1}H NMR.

Synthesis of dye-labeled poly(AOc-β^3-LAHG)$_{0.8}$-*r*-poly(β^3-HNL)$_{0.2}$ (dye-AOc$_{0.8}$HNL$_{0.2}$)



Scheme S8. Synthesis of dye-AOc$_{0.8}$HNL$_{0.2}$

The synthesis of 2-(3-aminopropyl)-6-morpholino-1H-benzo[de]isoquinoline-1,3(2H)-dione (dye-NH$_2$) was reported in our previous study[2]. The synthesis of dye-labeled poly(Me-β^3-LCHG)$_{0.8}$-*r*-poly(β^3-HNL)$_{0.2}$ was followed by the process of synthesis of poly(Me-β^3-LCHG)$_x$-*r*-poly(β^3-HNL)$_y$. At an ambient condition, a solution of dye-NH$_2$ in DMF (0.5 M, 25 μL) was added to the mixture of Me-β^3-LCHG NTA and β^3-HNL NTA in DMF (0.5 M, 500 μL) , and the reaction mixture was stirred at 60 °C overnight.

The synthesis of dye-AOc$_{0.8}$HNL$_{0.2}$ was followed by the process of synthesis of poly(AOc-β^3-LAHG)$_x$-*r*-poly(β^3-HNL)$_y$ to obtain the dye-polymer (43.7 mg, 74% yield). Dye-AOc$_{0.8}$HNL$_{0.2}$ was characterized by GPC at 2 mg/mL in HFIP (Figure S5).

Antibacterial activity assay

The bacterial strains evaluated in this study comprised *Staphylococcus aureus* (*S. aureus* 2902), *Staphylococcus epidermidis* (*S. epidermidis* 0692), *Enterococcus faecium* (*E. faecium* 1205),

vancomycin-resistant *Enterococcus* (VRE 01, VRE 02), *Pseudomonas aeruginosa* (*P. aeruginosa* 2512), and *Escherichia coli* (*E. coli* ATCC25922, *E. coli* 2904, *E. coli* 1602).

The bacterial strains were cultured in Luria-Bertani (LB) medium at 37 °C with shaking at 200 rpm for 10 hours. Subsequently, the bacterial cells were washed with PBS and diluted to a concentration of 2×10^5 CFU/mL in Mueller-Hinton (MH) medium. In a 96-well plate, the antimicrobial compounds were serially diluted two-fold and then mixed with an equal volume of bacterial suspension. The plate was incubated at 37 °C for 9 hours. MH medium with bacteria only served as the positive control, while MH medium without bacteria served as the blank control. The optical density (OD) at 600 nm was recorded using a SpectraMax® M2 plate reader. The bacterial viability in each well was calculated by the equation: $(\%\ bacterial\ viability = \frac{A_{600}^{polymer} - A_{600}^{blank}}{A_{600}^{positive} - A_{600}^{blank}} \times 100)$. The minimum inhibitory concentration (MIC) was defined as the lowest concentration that completely inhibited the growth of bacteria. The test was performed in duplicate.

To evaluate the impact of lipopolysaccharide (LPS) on the antibacterial activity of the tested compounds, the compounds were serially diluted two-fold in MH medium supplemented with varying concentrations of LPS. All other procedures were conducted according to the MIC assay protocol.

To evaluate the impact of lipids on the antibacterial activity of the tested compounds, the compounds were serially diluted two-fold in MH medium supplemented with varying concentrations of phosphatidyl ethanolamine (PE), phosphatidyl glycerol (PG), and cardiolipin (CL). All other procedures were conducted according to the MIC assay protocol.

Hemolysis assay

Fresh murine blood was washed three times with Tris-buffered saline (TBS) to prepare a 5% (v/v) working suspension of murine red blood cells (RBCs). The test compounds were serially diluted two-fold in a 96-well plate and then mixed with an equal volume of RBC suspension. Following incubation at 37 °C for 1 hour, the plate was centrifuged at 3700 rpm for 5 minutes. Subsequently, 80 µL of supernatant from each well was carefully transferred to a new 96-well plate. Wells containing 0.1% TX-100 and RBCs served as the positive control, whereas wells with RBC suspension alone served as the blank control. The OD at 405 nm was recorded using a SpectraMax® M2 plate reader, and the percentage of hemolysis was calculated by the equation: $(\%\ hemolysis = \frac{A_{405}^{polymer} - A_{405}^{blank}}{A_{405}^{positive} - A_{405}^{blank}} \times 100)$. The HC_{50} value was defined as the lowest concentration causing 50% RBC lysis. The test was performed in triplicate.

Cytotoxicity assay

African Chlorocebus sabaeus kidney fibroblast cells (COS-7) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator containing 5% CO_2. Upon reaching approximately 90% confluence, cells were detached using trypsin, collected by centrifugation, and diluted to a concentration of 1×10^5 cells/mL in fresh DMEM. 100 µL of cell suspension was added to each well of a 96-well plate and incubated for 24 hours. After removing the spent medium, the test compounds were serially diluted two-fold in

DMEM and transferred to the plate pre-cultured with cells, followed by a further 24-hour incubation. Subsequently, 10 μL of methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution was added to each well to achieve a final concentration of 0.5 mg/mL, and the plate was incubated in the dark for 4 hours. After removing the supernatant, 150 μL of DMSO was added to dissolve the formazan crystals. Wells containing cells and DMEM served as the positive controls, whereas wells containing only DMEM were used as the blank control. The OD at 570 nm was recorded using a SpectraMax® M2 plate reader, and the cell viability was calculated by the equation: $(\%\ cell\ viability = \frac{A_{570}^{polymer} - A_{570}^{blank}}{A_{570}^{positive} - A_{570}^{blank}} \times 100)$. The IC_{50} value was defined as the lowest concentration causing 50% cell death. The test was performed in triplicate.

Time-lapse fluorescence confocal imaging

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 2×10^7 CFU/mL in PBS to serve as the working suspension. 10 μL of the bacterial suspension was pipetted into a confocal dish and incubated for 5 minutes to facilitate bacterial adhesion. Subsequently, 10 μL of PBS containing dye-$AOc_{0.8}HNL_{0.2}$ (8 × MIC, green fluorescence) and propidium iodide (PI, 20 μg/mL, red fluorescence) was added into the bacterial droplets. Green fluorescence (excitation λ = 488 nm, emission λ = 525 nm) and red fluorescence (excitation λ = 561 nm, emission λ = 617 nm) were recorded to monitor the bacterial interactions with fluorescently labeled polymers.

Zeta potential assay

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 1×10^7 CFU/mL in PBS to serve as the working suspension. The bacterial working suspension was subsequently treated with varying concentrations of $AOc_{0.8}HNL_{0.2}$. The treated suspensions were incubated for 30 minutes. Then the zeta potential of bacteria was recorded using a Zetasizer Nano-ZS instrument. The test was performed in triplicate.

Outer membrane permeabilization assay

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 1×10^8 CFU/mL in 5 mM HEPES buffer supplemented with 20 mM glucose (pH = 7.4), serving as the working suspension. A solution of N-phenyl-1-naphthylamine (NPN) was added to 10 mL of the bacterial suspension to achieve a final concentration of 10 μM. Subsequently, 90 μL of the prepared bacterial suspension were transferred into the wells of a 384-well plate. The fluorescence intensity changes (excitation λ = 350 nm, emission λ = 420 nm) were recorded using a SpectraMax® M2 plate reader. Once the fluorescence intensity had stabilized, $AOc_{0.8}HNL_{0.2}$ was added into the suspension at final concentrations of 2 × MIC and 4 × MIC, and the fluorescence intensity was continuously recorded thereafter. Melittin served as a positive control, while the buffer without added compounds served as a blank control. The test was performed in triplicate.

Cytoplasmic membrane depolarization assay

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 1×10^8 CFU/mL in 5 mM HEPES buffer supplemented with 20 mM glucose (pH = 7.4) and 0.2 mM EDTA, serving as the working suspension. A solution of $DiSC_3(5)$ was added to 10 mL of the bacterial suspension to achieve a final concentration of 4 μM. After incubating at 37 °C for 1 hour in the dark, 74.56 mg of KCl was added to a final concentration of 0.1 M. Subsequently, 90 μL of the prepared bacterial suspension were transferred into the wells of a 384-well plate. The fluorescence intensity changes (excitation λ = 622 nm, emission λ = 670 nm) were recorded using a SpectraMax® M2 plate reader. Once the fluorescence intensity had stabilized, $AOc_{0.8}HNL_{0.2}$ was added into the suspension at final concentrations of 2 × MIC and 4 × MIC, and the fluorescence intensity was continuously recorded thereafter. Melittin served as a positive control, while the buffer without added compounds served as a blank control. The test was performed in triplicate.

Cytoplasmic membrane permeabilization assay

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 1×10^8 CFU/mL in PBS to serve as the working suspension. A solution of SYTOX Green was added to 10 mL of the bacterial suspension to achieve a final concentration of 5 μM. Subsequently, 90 μL of the prepared bacterial suspension were transferred into a 384-well plate. The fluorescence intensity changes (excitation λ = 488 nm, emission λ = 530 nm) were recorded using a SpectraMax® M2 plate reader. Once the fluorescence intensity had stabilized, $AOc_{0.8}HNL_{0.2}$ was added into the suspension at final concentrations of 2 × MIC and 4 × MIC, and the fluorescence intensity was continuously recorded. Melittin served as a positive control, while the buffer without added compounds served as a blank control. The test was performed in triplicate.

SEM characterization

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 2×10^7 CFU/mL in MH medium as the working suspension. 500 μL of the bacterial suspension was subsequently mixed with an equal volume of $AOc_{0.8}HNL_{0.2}$ solution (4 × MIC) and incubated at 37 °C for 2 hours. The untreated bacterial suspension was used as the blank control. $AOc_{0.8}HNL_{0.2}$-treated or untreated bacteria were collected, washed with PBS, and fixed overnight at room temperature in PBS containing 4% glutaraldehyde. Subsequently, the bacteria were washed with PBS and dehydrated with graded ethanol solutions (30%, 50%, 70%, 80%, 90%, 95%, and 100%). The dehydrated bacteria were transferred onto gold-coated sheets for characterization using SEM.

Evaluation of antimicrobial resistance

E. coli ATCC25922 was prepared following the previously described protocol to a concentration of 1×10^5 CFU/mL in MH medium as the working suspension. The working suspension was then treated with either $AOc_{0.8}HNL_{0.2}$ or norfloxacin at a concentration of 0.5×MIC, and the treated mixtures were incubated at 37 °C for 24 hours. Following incubation, the bacteria suspension was diluted 400-fold with fresh MH medium in a new container for another cycle of bacteria-drug incubation. This cycle was repeated every 24 h. The MIC values were examined every 4 days, and the concentration of $AOc_{0.8}HNL_{0.2}$ and the antibiotic were adjusted accordingly to maintain a consistent level of 0.5×MIC. All MIC tests were repeated at least twice at different time.

In vivo wound infection model

All procedures were approved by the Animal Research Bioethics Committee, East China University of Science and Technology (ECUST-2023-012).

The standard strain *E. coli* ATCC25922 and clinically isolated multidrug-resistant strain *E. coli* 2904 were used to evaluated the efficacy of $AOc_{0.8}HNL_{0.2}$ *in vivo*. Female ICR mice (21-23 g) were used for the *E. coli*-induced wound infection model. *E. coli* was cultured following the previously described protocol, washed three times with saline and diluted to 1.0×10^8 CFU/mL as the working suspension. Mice were anesthetized via intraperitoneal injection of sodium pentobarbital at a dose of 75 mg/kg. The dorsal area of the mice was shaved and sterilized using 75% ethanol. Full-thickness wounds with a diameter of 6 mm were created on the dorsal surface of the mice. 10 μL of bacterial working suspension was applied to the wound site, which was subsequently covered with a Tegaderm dressing (3M). 24 hours post-infection, the mice were randomly divided into three groups (6 mice per group). 15 μL of treatment solutions (saline as a negative control, 4 mg/mL $AOc_{0.8}HNL_{0.2}$, and 4 mg/mL imipenem as a positive control) was applied to the infected wound every 4 hours for 3 times. 4 hours after the final dose, the infected wounds were excised and homogenized in 0.1% Triton X-100, then plated onto LB agar plates for subsequent colony counting. Representative wound samples were collected and fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for histological analysis.

In vivo toxicity

Female ICR mice (21−23 g) were used for subcutaneous toxicity evaluation. Mice were anesthetized via intraperitoneal injection of sodium pentobarbital at a dose of 75 mg/kg. The dorsal area of the mice was shaved and sterilized using 75% ethanol. 15 μL of $AOc_{0.8}HNL_{0.2}$ solution (4 mg/mL) was administered via subcutaneous injection beneath the mouse's skin every 4 hours for 3 times. 4 hours after final dose, the skin appearance and the inner surface of the skin were examined. Representative skin samples were collected and fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and stained with H&E for histological analysis.

Statistical Analysis

The significance of multiple comparisons was determined by a one-way ANOVA with Tukey post-test. All results were expressed as mean values ± standard error. A p value > 0.05 was considered not significant (n.s.), and $p < 0.05$ was considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Analysis

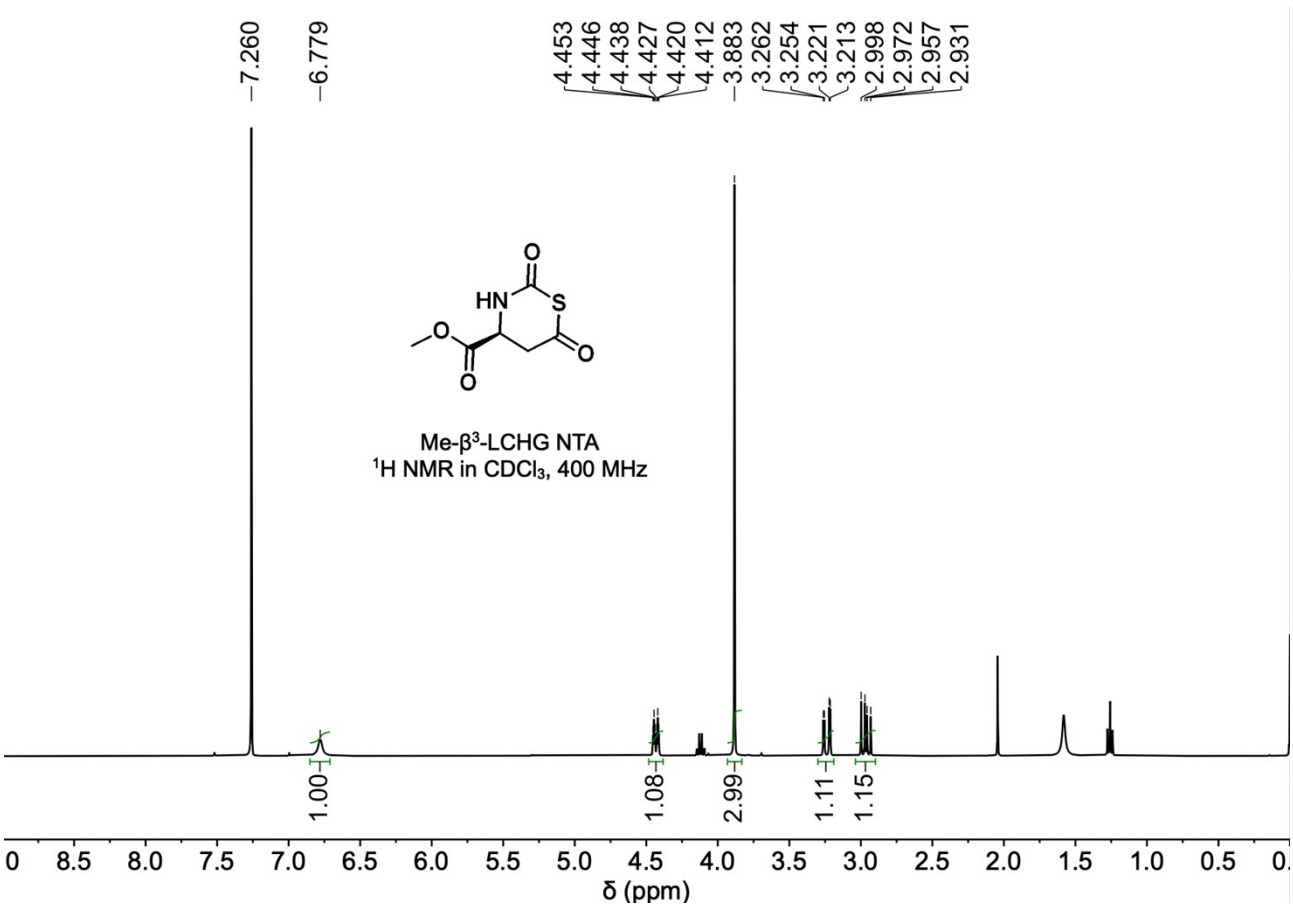


Figure S1. ^{1}H NMR spectrum of Me-β^{3}-LCHG NTA in $CDCl_3$, 400 MHz.

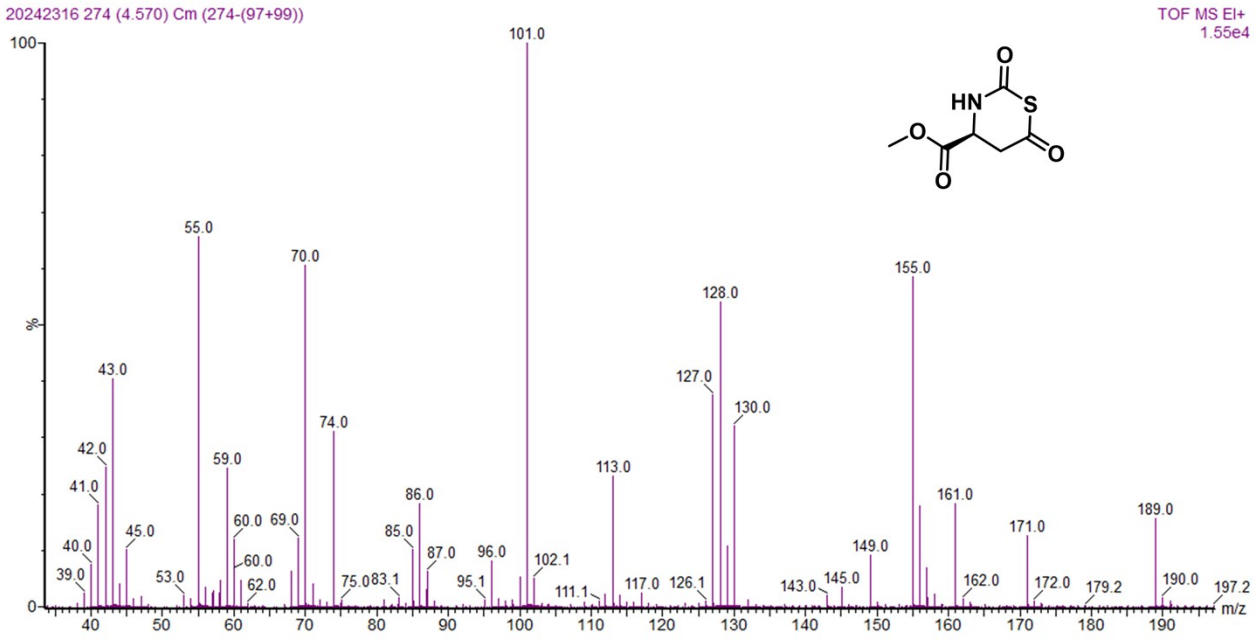


Figure S2. EI-MS spectrum of Me-β^{3}-LCHG NTA.

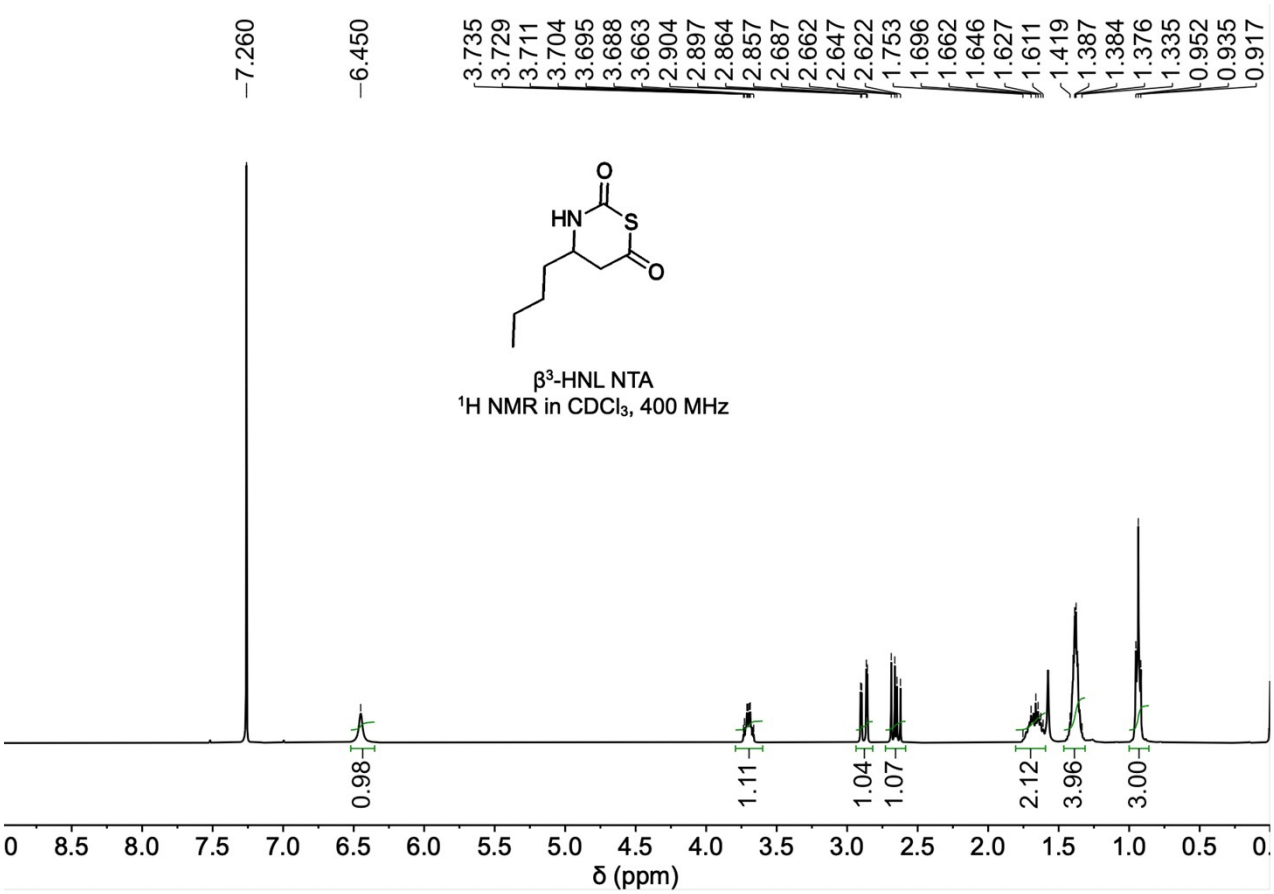


Figure S3. ^{1}H NMR spectrum of β^{3}-HNL NTA in $CDCl_3$, 400 MHz.

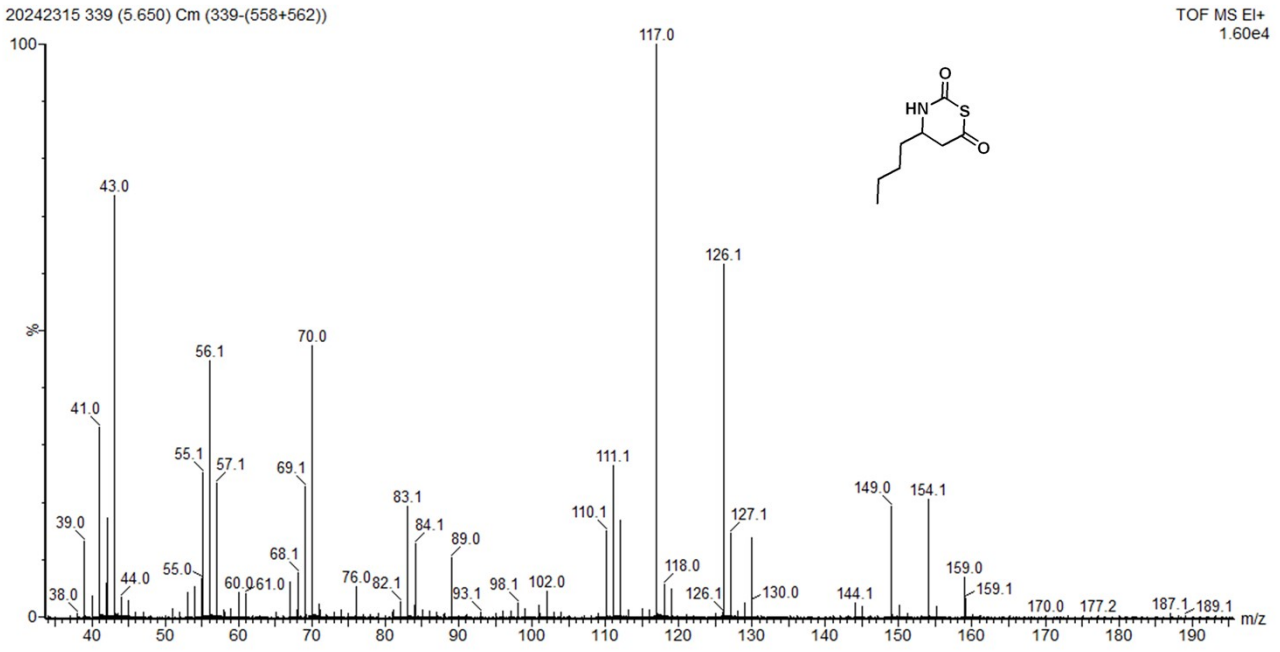


Figure S4. EI-MS spectrum of β^{3}-HNL NTA.

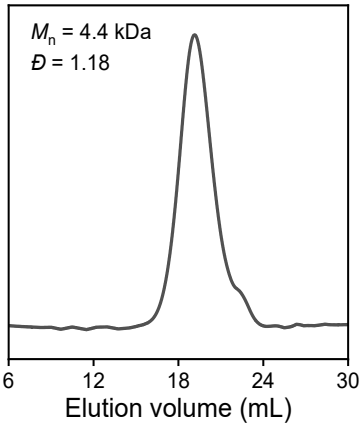


Figure S5. GPC trace of dye-$AOc_{0.8}HNL_{0.2}$.

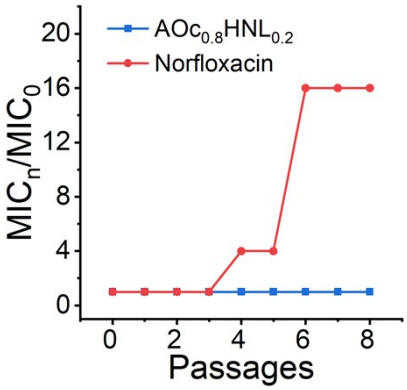


Figure S6. Antibacterial resistance test on $AOc_{0.8}HNL_{0.2}$ and norfloxacin against *E. coli* ATCC25922.

Table S1. Drug susceptibility of clinically isolated multidrug-resistant bacterium *E. coli* 2904. "+" means resistant.

E. coli 2904					
Drug	Susceptibility	Drug	Susceptibility	Drug	Susceptibility
ampicillin	+	cefoxitin	+	levofloxacin	+
ceftriaxone	+	cefazolin	+	amikacin	+
ceftazidime	+	cefuroxime	+	gentamicin	+
cefotaxime	+	ciprofloxacin	+	tobramycin	+

References

1. M. Zhou, J. Zou, L. Liu, X. Xiao, S. Deng, Y. Wu, J. Xie, Z. Cong, Z. Ji and R. Liu, Synthesis of poly-α/β-peptides with tunable sequence via the copolymerization on N-carboxyanhydride and N-thiocarboxyanhydride, *iScience*, 2021, **24**, 103124.
2. M. Zhou, X. Xiao, Z. Cong, Y. Wu, W. Zhang, P. Ma, S. Chen, H. Zhang, D. Zhang, D. Zhang, X. Luan, Y. Mai and R. Liu, Water-Insensitive Synthesis of Poly-β-Peptides with Defined Architecture, *Angew. Chem., Int. Ed.*, 2020, **59**, 7240-7244.