Supporting Information

Design of Biodegradable Polyurethanes and the Post-modification with Long Alkyl Chains via Inhibiting Biofilm Formation and Killing Drug-Resistant Bacteria for the Treatment of Wound Bacterial Infection

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Experimental Procedures

Materials: Unless otherwise noted, all chemicals and reagents were obtained commercially and used without further purification. Other organic solvents were used without any further purification. 3-dimethylamino-1,2-propanediol, 1,3-bis-[1-(2-hydroxyethyl)-4-piperidinyl]propane and 2-hydroxyethyl disulfide were purchased from bidepharm, Shanghai, China. DMEM, 0.25 % trypsin EDTA, fetal bovine serum (FBS), and penicillin/streptomycin (P/S) were purchased from Gibco (Grand Island, NY, U.S.A.). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and sodium dodecyl sulfate (SDS) were purchased from Solarbio (Beijing, China). Mueller-Hinton (MH) medium, Luria-Bertani (LB) medium, LB agar, Nutrient Broth (NB) medium were purchased from Beijing Aobox Bio-Technology Co., Ltd., *Staphylococcus aureus* (*S. aureus*) (ATCC6538), *Escherichia coli (E. coli)* (ATCC8099) and MRSA was provided by the Chinese PLA Center for Disease Control and Prevention, Beijing, China. All biological experimentations were tested three times independently.

Characterizations: ^{1}H NMR spectra were measured by a 300 or 400 MHz NMR spectrometer (Bruker). The Size and zeta potential of nanoparticles were measured by a Malvern Zetasizer (Nano ZS, U.K.). The morphology and size of nanoparticles were obtained by transmission electron microscope (TEM) carried out with HT7700. Fluorescence spectra were detected by Steady and transient state fluorescence spectrometer (Edinburgh FLS980). The molecular weight of the polymer was characterized by gel permeation chromatography (GPC) (Waters, USA). The MTT assay conducting and the optical density (OD) reading was recorded using Molecular Device microplate reader (Spetra Max i3X). Fluorescence (FL) spectroscopy was recorded on Lengguang (F97Pro) fluorescence spectrophotometer.

Synthesis of PTA-1~3: 3-dimethylamino-1,2-propanediol (357.5 mg, 3 mmol), 2-hydroxyethyl disulfide (462.7 mg, 3 mmol) and L-lysine diisocyanate (1.5 g, 6.6 mmol) were suspended in 5 mL anhydrous DMF. After magnetic stirring for 12 h, $mPEG_{5000}$-OH (6 g, 1.2 mmol) was added to the reaction mixture and the temperature was raised to 50 ℃. After magnetic stirring for another 6 h, 5 mL mixture was added into 15 mL of deionized water under sonication, followed by dialysis in a dialysis bag (MWCO: 8000 Da). After 72 h, the solution was freeze-dried under reduced pressure to obtained PTA-1.

By changing the weight of 3-dimethylamino-1,2-propanediol to 476.6 mg (4

mmol) and 2-hydroxyethyl disulfide to 308.5 mg (2 mmol), and keeping the other conditions unchanged, PTA-2 was synthesized. By changing the weight of 3-dimethylamino-1,2-propanediol to 572.0 mg (4.8 mmol) and 2-hydroxyethyl disulfide to 185.1 mg (1.2 mmol), and keeping the other conditions unchanged, PTA-3 was synthesized.

Synthesis of PTA-4~6: 1,3-bis-[1-(2-hydroxyethyl)-4-piperidinyl]propane (447.7 mg, 1.5 mmol), 2-hydroxyethyl disulfide (231.4 mg, 1.5 mmol) and L-lysine diisocyanate (746.6 mg, 3.3 mmol) were suspended in 8 mL anhydrous DMF. After magnetic stirring for 16 h, $mPEG_{5000}$-OH (3 g, 0.6 mmol) was added to the reaction mixture and the temperature was raised to 50 ℃. After magnetic stirring for another 6 h, 5 mL mixture was added into 15 mL of deionized water under sonication, followed by dialysis in a dialysis bag (MWCO: 8000 Da). After 72 h, the solution was freeze-dried under reduced pressure to obtained PTA-4.

By changing the weight of 1,3-bis-[1-(2-hydroxyethyl)-4-piperidinyl]propane to 596.9 mg (2 mmol) and 2-hydroxyethyl disulfide to 154.2 mg (1 mmol), and keeping the other conditions unchanged, PTA-5 was synthesized. By changing the weight of 1,3-bis-[1-(2-hydroxyethyl)-4-piperidinyl]propane to 716.2 mg (2.4 mmol) and 2-hydroxyethyl disulfide to 92.5 mg (0.6 mmol), and keeping the other conditions unchanged, PTA-6 was synthesized.

General procedure for alkylation of PQA-1~15 using different types of alkyl halide: PTA-1~3 (500 mg) and alkyl halide were suspended in 5 mL anhydrous DMF, protected by nitrogen gas. After magnetic stirring for 24 h at 60 ℃, 5 mL mixture was added into 15 mL of deionized water under sonication, followed by dialysis in a dialysis bag (MWCO: 8000 Da). After 72 h, the solution was freeze-dried under reduced pressure to obtained PQA. The corresponding relationship between PQA and PTA-1~3, as well as the types and amounts of alkyl halide, can be found in Table S1.

Preparation of PQA-1~15 and PTA-4~6 aqueous solutions: PQA or PTA (20 mg) was dissolved in DMF (1 mL) to prepare an organic solution by bath sonication which was further filtrated through a 0.22 μm filter. Then, slowly trickle the organic solution (almost dropwise) into a stirred deionized water. After adding and stirring for five minutes, the above mixture was dialyzed in a dialysis bag (MWCO: 14000 Da) for 24 h. Then PQA or PTA aqueous solutions were obtained.

General procedure for DTT experiments: To simulate degradation in bacterial microenvironment, DL-dithiothreitol (30.8 mg) and PTA-1~6 (200 mg) were dissolved

in DMF (20 mL). After magnetic stirring for 24 h at 37 ℃, the solution was characterized by GPC.

Bacterial cultivation: *S. aureus* and *E. coli* were cultured in NB medium in a shaking incubator (200 rpm) at 37°C and harvested at the logarithmic growth phase by centrifugation at 4500 rpm for 5 min. After washing with PBS for three times, the bacteria were resuspended in PBS for further use. The concentration of bacteria was monitored by measuring the optical density at 600 nm (OD600) using a Bio-Rad microplate reader.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assay: Bacteria in logarithmic phase of growth were diluted in MH medium to 1×10^6 colony-forming units (CFU) per milliliter as the working suspension. The PQA-1~15 and PTA-4~6 aqueous solutions were diluted to the desired concentration (6.25-1000 μg/mL) in MH medium by two-fold serial dilution in a 96-well plate. Then, the equal volumes of bacterial suspension (100 μL) were added into each well, which was incubated at 37 °C. After 18 h, the OD value was recorded at 600 nm on a Bio-Rad microplate reader. MH medium without bacteria was considered as the blank and that with bacteria suspension was used as the positive control. The percentage of bacterial cells survival was calculated according to the equation:

$$\%\ cell\ grouth = \frac{OD^{sample} - OD^{blank}}{OD^{positive\ control} - OD^{blank}} \times 100$$

After MIC assay, 5 μL of bacterial suspension with no visible growth was spread onto LB agar plates, which was incubated at 37 °C. After 18 h, the final value was determined by visually observing the viability of the bacteria.

Cytotoxicity assay: Cytotoxicity of PQA-12 and PTA-6 against Vero cells was valued using live/dead staining assay and methyl thiazolyl tetrazolium (MTT) assay. About 2×10^4 Vero cells (Vero cells were provided by the Chinese PLA Center for Disease Control and Prevention) were seeded into each well of 96well plate in DMEM medium with 10% fetal bovine serum, 1 % penicillin/streptomycin, and cultured for 24h in a humidified 5% CO_2 in air incubator. Then, 100 μL of polymer solution in DMEM was diluted to the desired concentration, and replaced the original medium in 96-well plate. The plate was incubated for 24h in a humidified 5% CO_2 in air incubator.

For MTT assay, 10 μL MTT solution in PBS (5 mg/mL) was added to each well of 96-well plate and the plate was incubated for 4 h in a humidified 5% CO_2 in air incubator. After aspirating original solution, 150 μL of DMSO was added into each well of each well of 96well plate. The plate was shaken for 30 min in order to completely dissolve the purple solid.

Cells in Tween 20 solution (20 μL Tween 20 per 100 μL DMEM) was used as negative control. Cells in DMEM were used as the positive control. The OD values was detected at a wavelength of 490 nm on a Molecular Device microplate reader. The percentage of cell viability was calculated by the equation:

$$\%\ cell\ viability = \frac{OD^{sample} - OD^{negative\ control}}{OD^{positive\ control} - OD^{negative\ control}} \times 100$$

Study on bacterial killing kinetics: Bacteria in logarithmic phase of growth were washed three times with PBS, and diluted to 1×10^6 CFU/mL as the working suspension. The PQA-12 or PTA-6 aqueous solution was diluted to the desired concentration in 96-well plate in PBS. Then, the equal volumes of bacterial suspension (50 μL) were added into the 96-well plate, which was incubated at 37 °C. The bacterial suspension was sampled at different time intervals and diluted for plating on NB agar plates. After incubation for 18 h at 37 °C, viability calculation was applied.

Cytoplasmic membrane permeabilization assay: Bacteria in logarithmic phase of growth were washed three times with HEPES buffer (5 mM HEPES, 20 mM glucose), and diluted in HEPES buffer to 3×10^8 CFU/mL. The propidium iodide (PI) at a final concentration of 10 μM was added to the bacterial suspension, and the suspension was incubated for 15 min. Then, 1.0 mL the suspension was added to a cuvette and detected the fluorescence intensity by FL spectroscopy (excitation $\lambda = 535$ nm; emission $\lambda = 617$ nm), and maintained nearly constant. 0.02 mL polymer aqueous solution was added to achieve desired concentration. *S. aureus* and *E. coli*, fluorescence signals were immediately recorded continuously for further 15 min.

Bacterial Morphology Assays: Bacteria in logarithmic phase of growth were washed with PBS for three times, and diluted to 3×10^8 CFU/mL. The PQA-12 or PTA-6 aqueous solution was added to achieve desired concentration, followed by incubation at 37 °C for 60 min. After centrifugation, the bacteria were washed with PBS for three times. Subsequently, 2.5% glutaraldehyde was added to a centrifuge tube, thoroughly shaken, and stored in a refrigerator at 4 ℃ for 12 hours to fix the bacterial form. After fixation, centrifuge and discard the supernatant, add 0.5 mL of sterile water, shake and mix evenly. Take 10 μL of the treated bacterial solution and drop it onto the copper mesh. Dry the copper mesh in a 30 ℃ oven, and observe the bacterial morphology of the bacterial sample using transmission electron microscopy (TEM).

Inhibition of *S. aureus* biofilm formation: *S. aureus* in logarithmic phase of growth were diluted in MH medium containing 1% glucose to 3×10^6 CFU/mL as the working suspension. The PQA-12 and PTA-6 aqueous solutions were diluted to the desired concentration in MH medium containing 1% glucose by two-fold serial dilution in a 96-well plate. Then, equal volumes of bacterial suspension (50 μL) were added into each well, followed by well-shaking. After incubating for 24 h at 37 °C, the original medium was gently aspirated and 100 μL MTT solution in PBS (0.25 mg/mL) was added into each well, which was incubated for 4 h at 37 °C. After aspirating MTT solution, 150 μL of DMSO was added into each well. After shaking for 30 min, the OD

value was recorded at 570 nm on Molecular Device microplate reader. MH mediums with and without bacteria suspension were considered as the blank and the positive control, respectively. The percentage of bacterial cells survival was calculated according to the equation, which was used for evaluating the ability of the sample in inhibiting *S. aureus* biofilm formation:

$$\%\ cell\ grouth = \frac{OD^{sample} - OD^{blank}}{OD^{positive\ control} - OD^{blank}} \times 100$$

Expressions of genes of the outer membrane in *E. coli*: Place PQA-12 (50 μg/mL) and PTA-6 (25 μg/mL) respectively treated *E. coli* was washed three times with PBS, and centrifuged to collect precipitation. Purified RNA and reverse transcription followed the protocol.The gene expressions were determined by the standard procedures as reported.[1]

Evaluation of antimicrobial resistance: The MIC at each passage was measured following the MIC assay procedure. The bacteria was cultured in NB medium containing drug with the previous passage 1/2 MIC concentration at 37 °C for overnight. The each 5 passage MIC was recorded and development of drug resistance was valued by the ratio of each passage MIC to the first passage.

In vivo anti-infective studies: Male Sprague-Dawley rats (8 weeks old) were used in the MRSA infected full-thickness wound model. The rats were randomly divided into three groups (n = 3 per group): PBS, vancomycin, PQA-12 and PTA-6. After anesthesia and removing the dorsal hair, two 6-mm-diameter full-thickness wounds were generated on either side of the back using a biopsy punch. The wounds were infected by a 10 μL MRSA suspension (1×10^7 CFU/mL) per wound and covered with transparent dressing for 4 h. After removing the transparent dressing, treatment was applied by using 15 μL PBS, vancomycin (4 μg/mL, 4×MIC), PQA-12 (1.2 mg/mL, 4×MIC) and PTA-6 (0.8 mg/mL, 4×MIC), respectively. After being treated for 24 h, the rats were sacrificed and the wounds were harvested using a scalpel blade. After homogenized, samples were plated on agar plates to determine CFU. On day 11 post infection, the wound and surrounding skin were harvested and fixed with 4% paraformaldehyde overnight. Then, the wound and skin were embedded in paraffin and sectioned was stained with hematoxylin & eosin.

Supplemental Figures and Tables

Table S1 Synthesis of PQA-1~15

Sample	Precursor	Alkyl halide	Volume (μL)
PQA-1	PTA-1	1-bromobutane	140
PQA-2		1-bromohexane	180
PQA-3		1-bromooctane	230
PQA-4		1-bromodecane	275
PQA-5		1-bromododecane	320
PQA-6	PTA-2	1-bromobutane	280
PQA-7		1-bromohexane	360
PQA-8		1-bromooctane	445
PQA-9		1-bromodecane	530
PQA-10		1-bromododecane	620
PQA-11	PTA-3	1-bromobutane	215
PQA-12		1-bromohexane	280
PQA-13		1-bromooctane	345
PQA-14		1-bromodecane	410
PQA-15		1-bromododecane	475

Table S2. The Zeta potential of PQA-1~15 and PTA-4~6 in aqueous solutions.

Sample	Zeta (mv)	Sample	Zeta (mv)
PQA-1	+48.3	PQA-10	+24.8
PQA-2	+41.8	PQA-11	+26.4
PQA-3	+38.6	PQA-12	+5.53
PQA-4	+39.3	PQA-13	+10.7
PQA-5	+44.2	PQA-14	+19.9
PQA-6	+33.5	PQA-15	+19.6
PQA-7	+8.90	PTA-4	+25.9
PQA-8	+19.5	PTA-5	+22.5
PQA-9	+21.3	PTA-6	+16.5

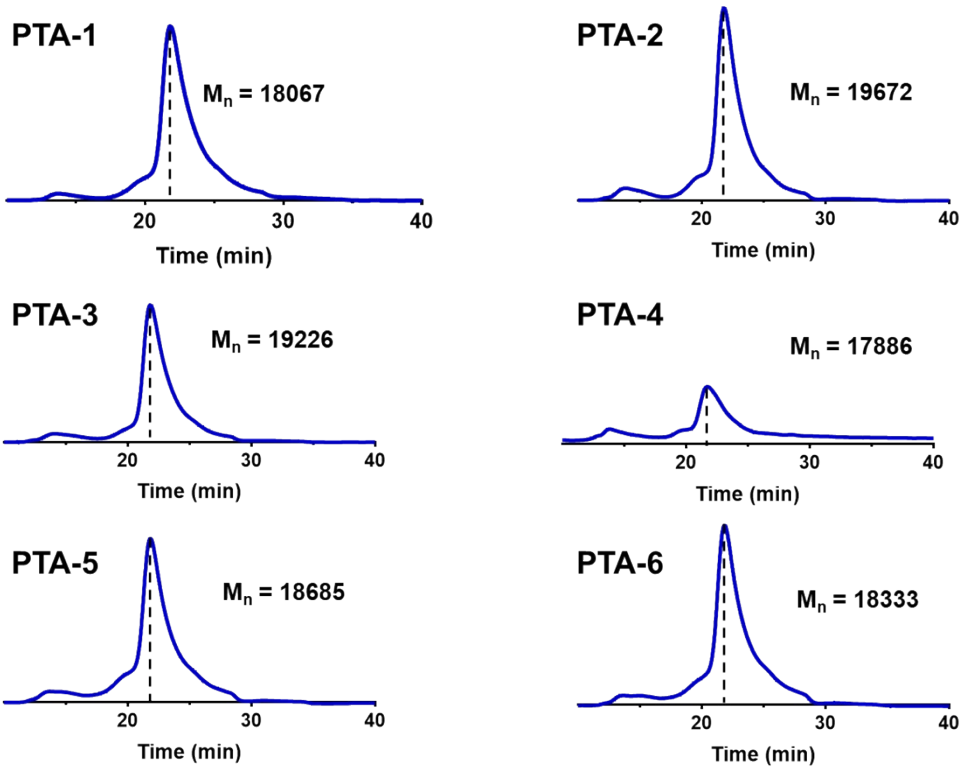

Figure S1 The GPC of PTA-1~6

Table S3 The GPC results of PTA-1~6

Sample	Mn	Mw	MP	Mz	Mz+1	Mz/Mw
PTA-1	18067	31413	28194	179173	727518	5.703768
PTA-2	19672	38969	28274	266699	767613	6.843842
PTA-3	19226	40422	28088	282756	758778	6.995138
PTA-4	17886	35332	27861	226982	714167	6.424240
PTA-5	18685	42233	28062	279223	746967	6.611446
PTA-6	18333	39990	27999	260834	731938	6.522560

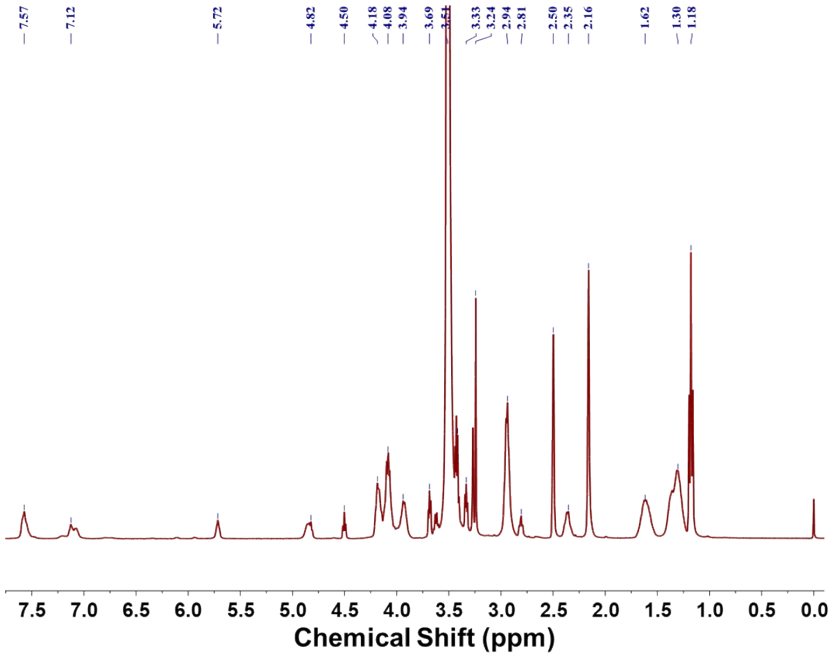

Figure S2 ^{1}H NMR spectrum of PTA-1 in dimethyl sulfoxide-d6

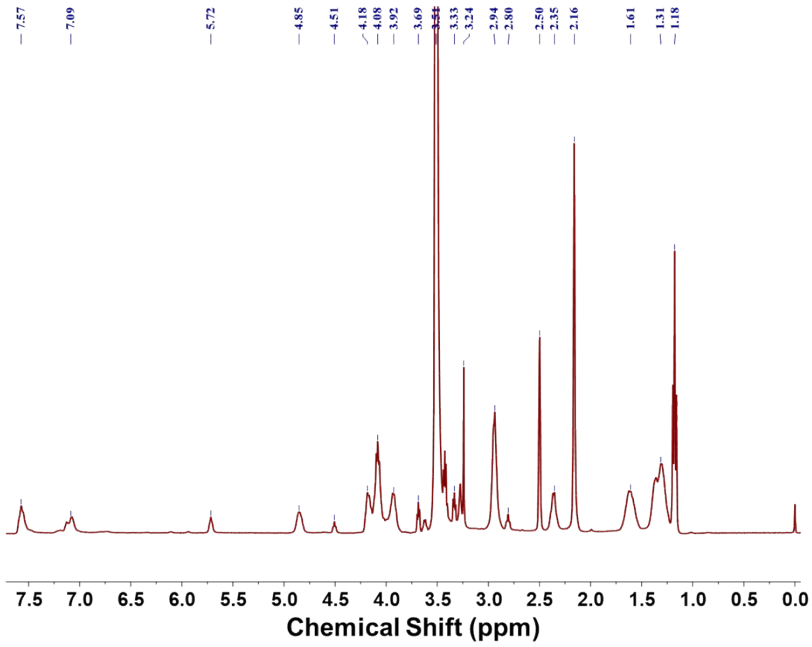

Figure S3 ^{1}H NMR spectrum of PTA-2 in dimethyl sulfoxide-d6

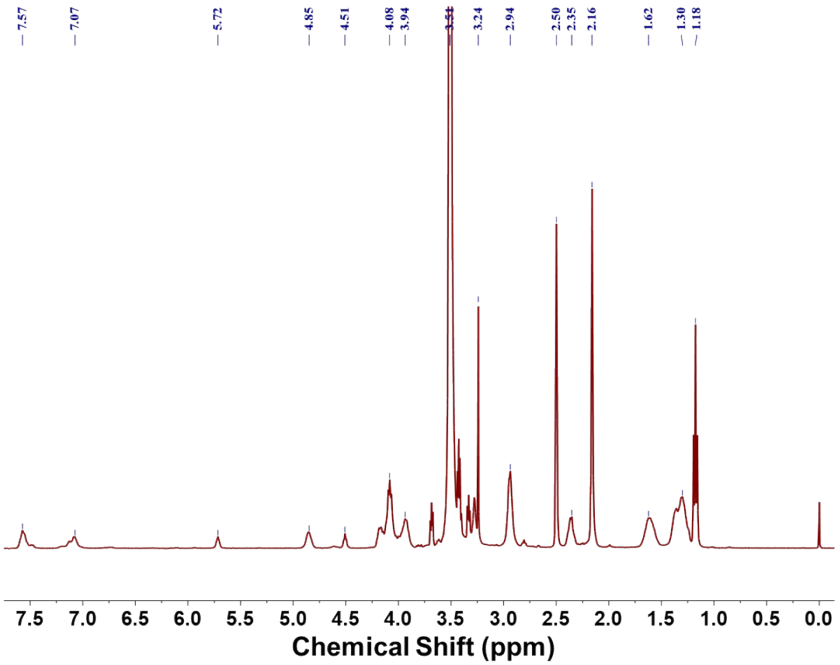

Figure S4 ^{1}H NMR spectrum of PTA-3 in dimethyl sulfoxide-d6

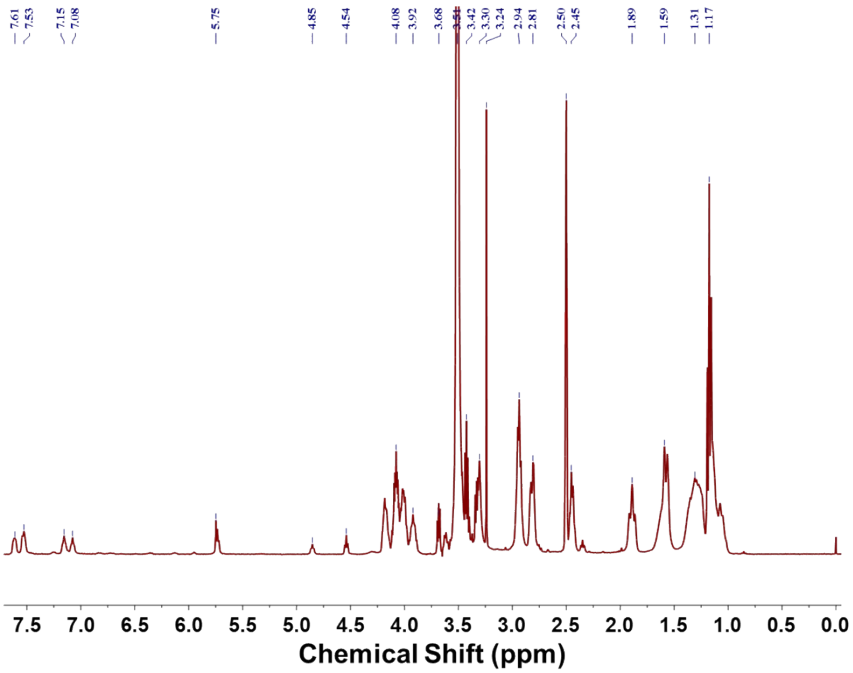

Figure S5 ^{1}H NMR spectrum of PTA-4 in dimethyl sulfoxide-d6

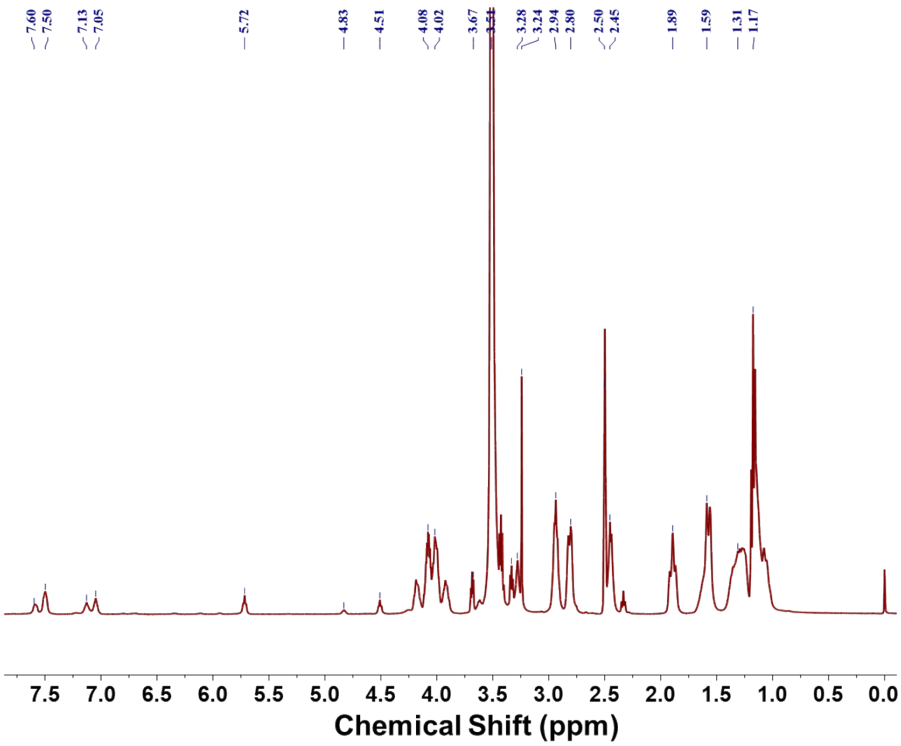

Figure S6 ^{1}H NMR spectrum of PTA-5 in dimethyl sulfoxide-d6

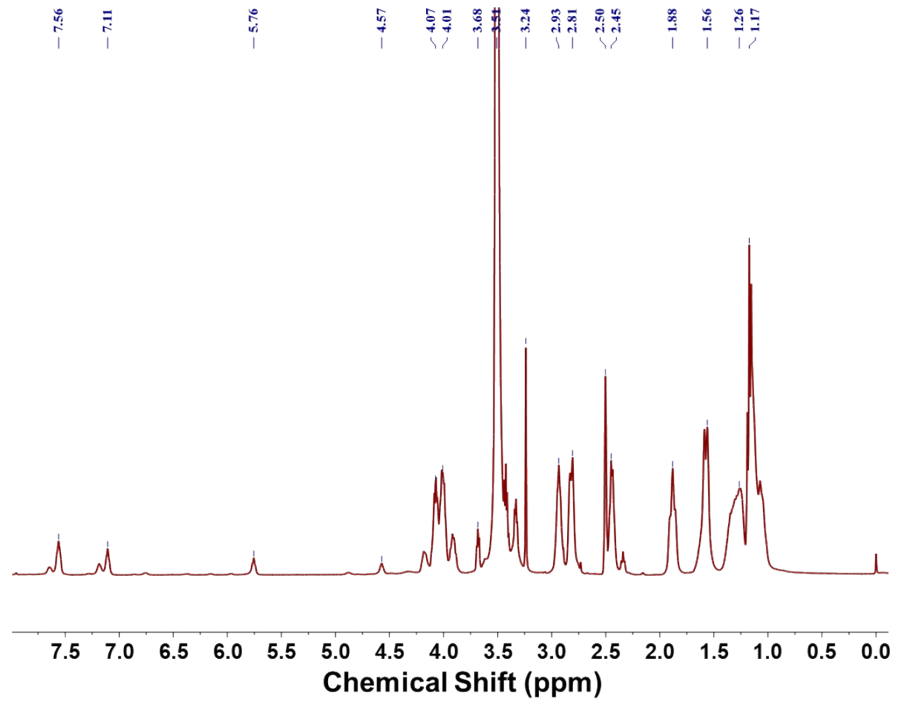

Figure S7 ^{1}H NMR spectrum of PTA-6 in dimethyl sulfoxide-d6

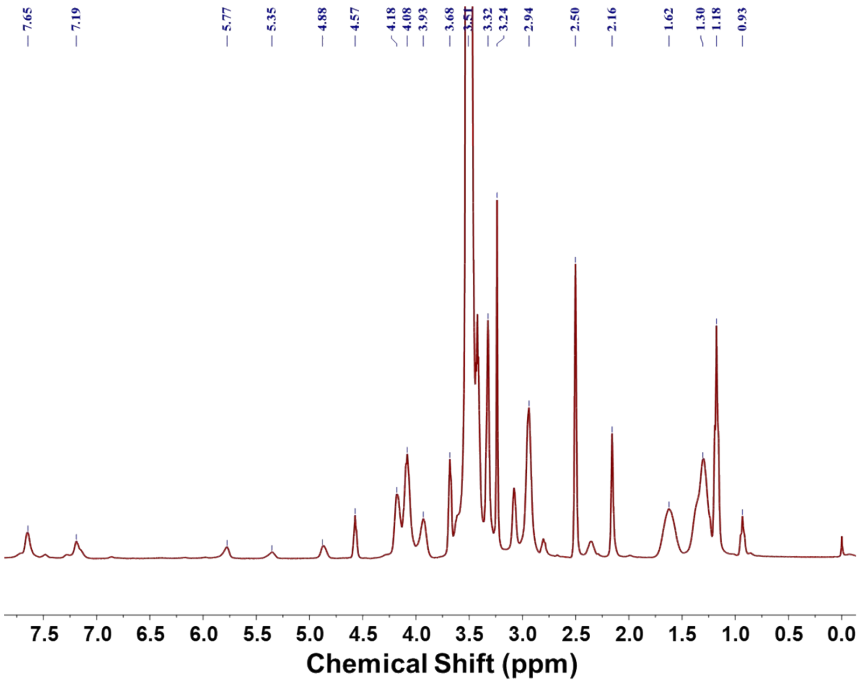

Figure S8 ^{1}H NMR spectrum of PQA-1 in dimethyl sulfoxide-d6

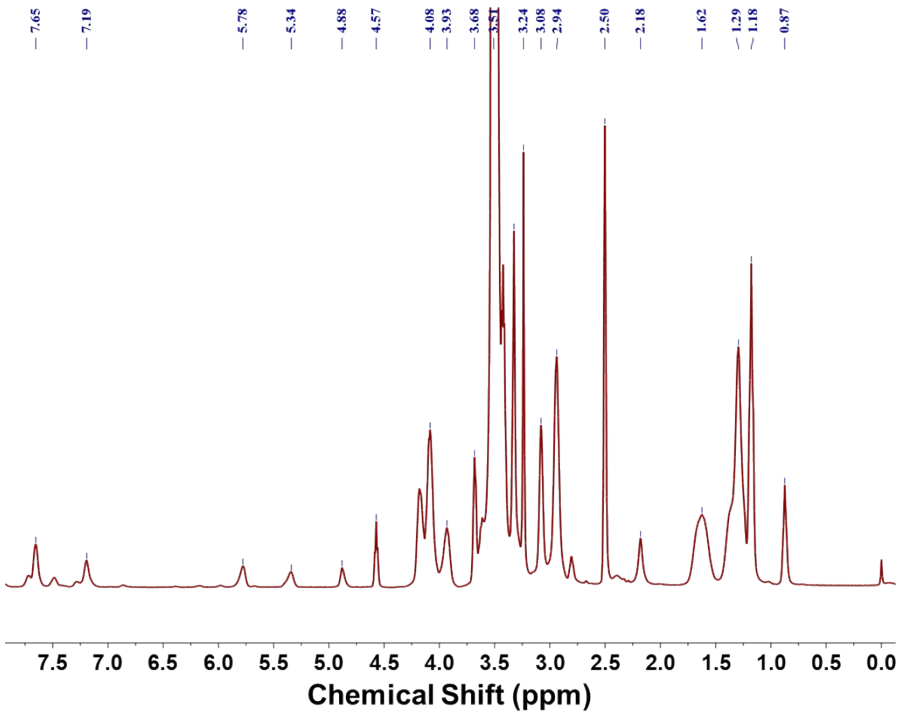

Figure S9 ^{1}H NMR spectrum of PQA-2 in dimethyl sulfoxide-d6

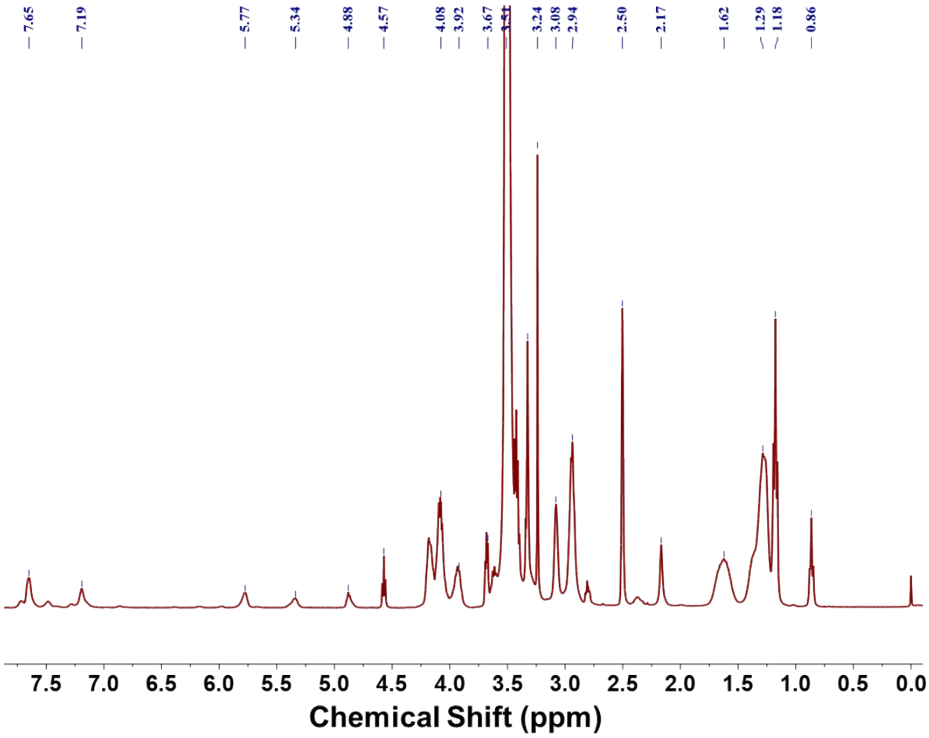

Figure S10 ^{1}H NMR spectrum of PQA-3 in dimethyl sulfoxide-d6

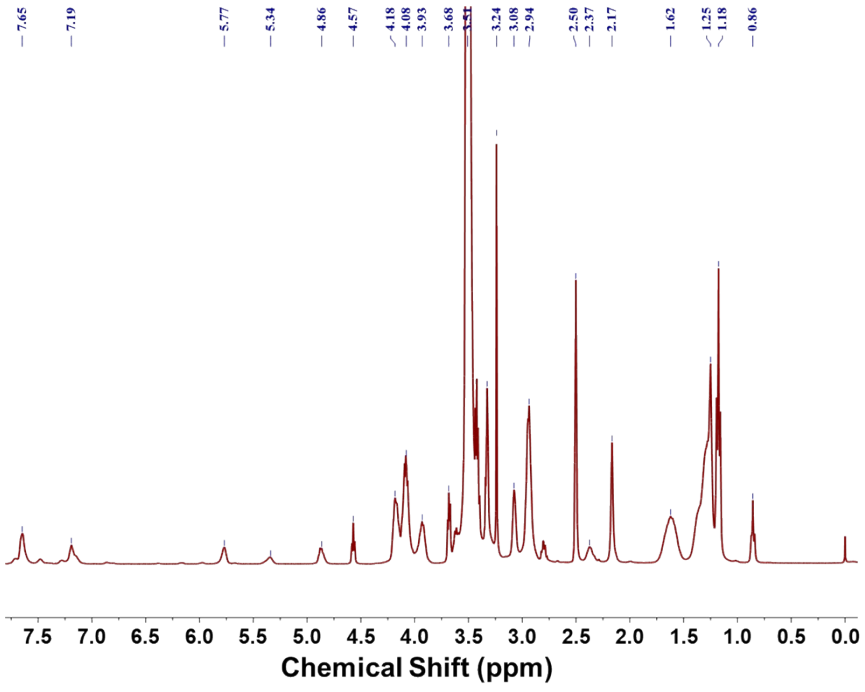

Figure S11 ^{1}H NMR spectrum of PQA-4 in dimethyl sulfoxide-d6

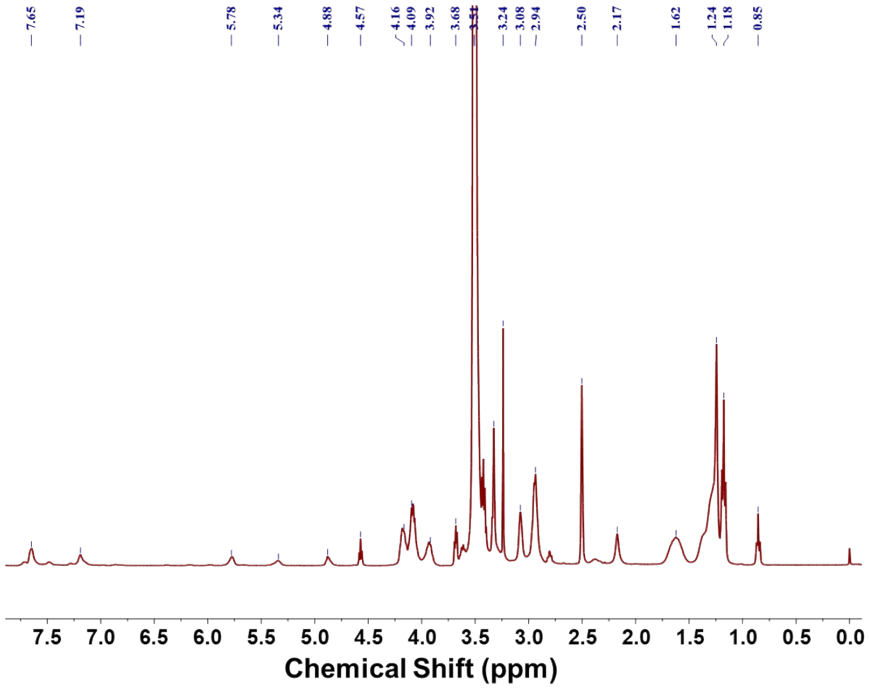

Figure S12 ^{1}H NMR spectrum of PQA-5 in dimethyl sulfoxide-d6

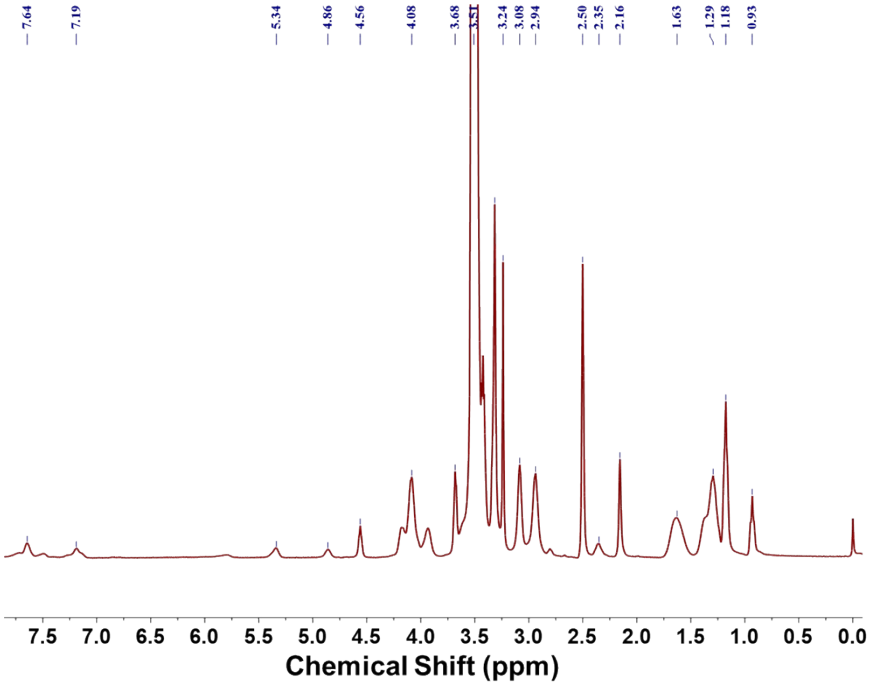

Figure S13 ^{1}H NMR spectrum of PQA-6 in dimethyl sulfoxide-d6

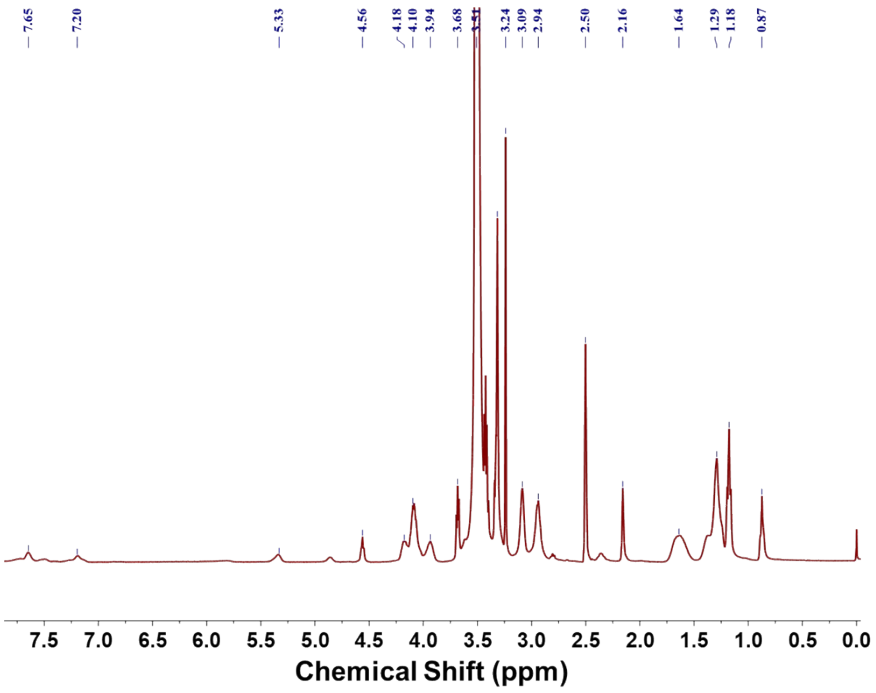

Figure S14 ^{1}H NMR spectrum of PQA-7 in dimethyl sulfoxide-d6

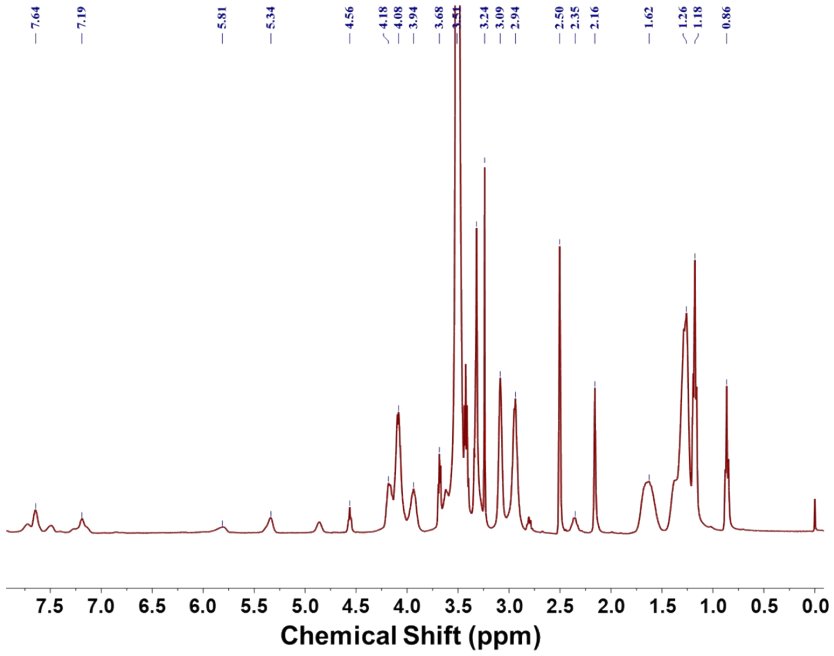

Figure S15 ^{1}H NMR spectrum of PQA-8 in dimethyl sulfoxide-d6

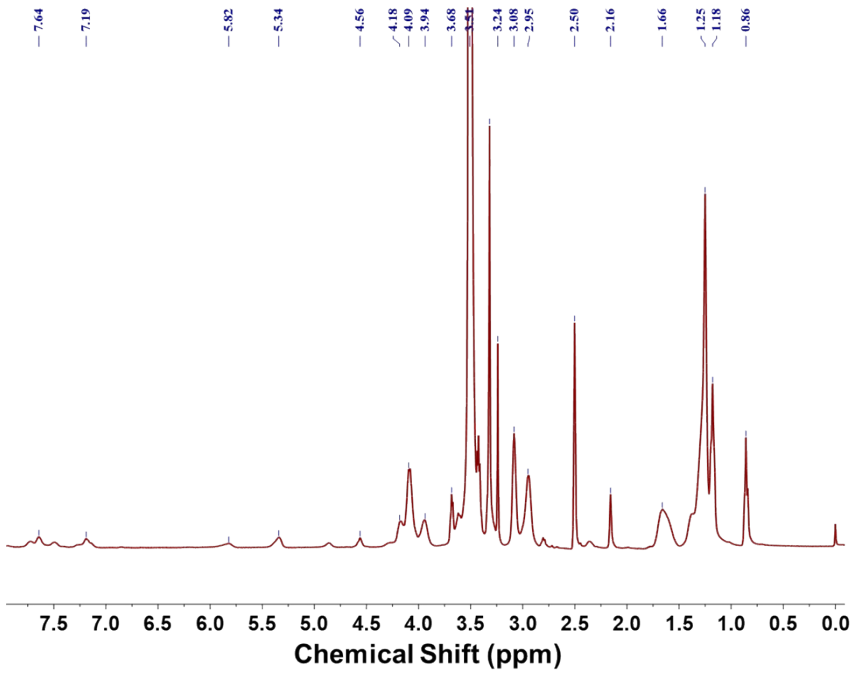

Figure S16 ^{1}H NMR spectrum of PQA-9 in dimethyl sulfoxide-d6

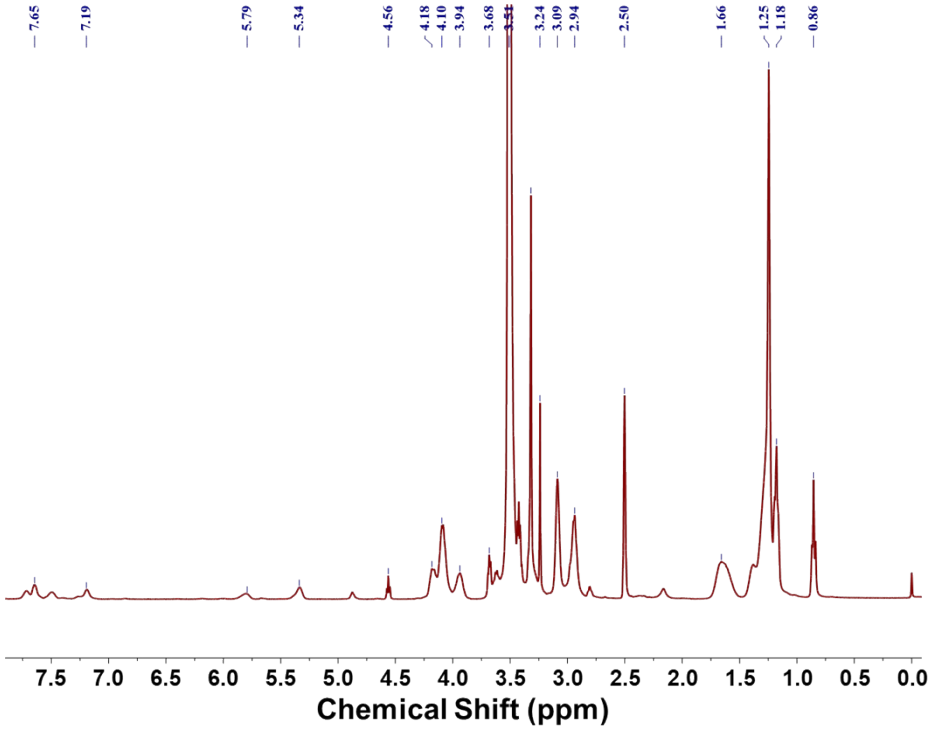

Figure S17 ^{1}H NMR spectrum of PQA-10 in dimethyl sulfoxide-d6

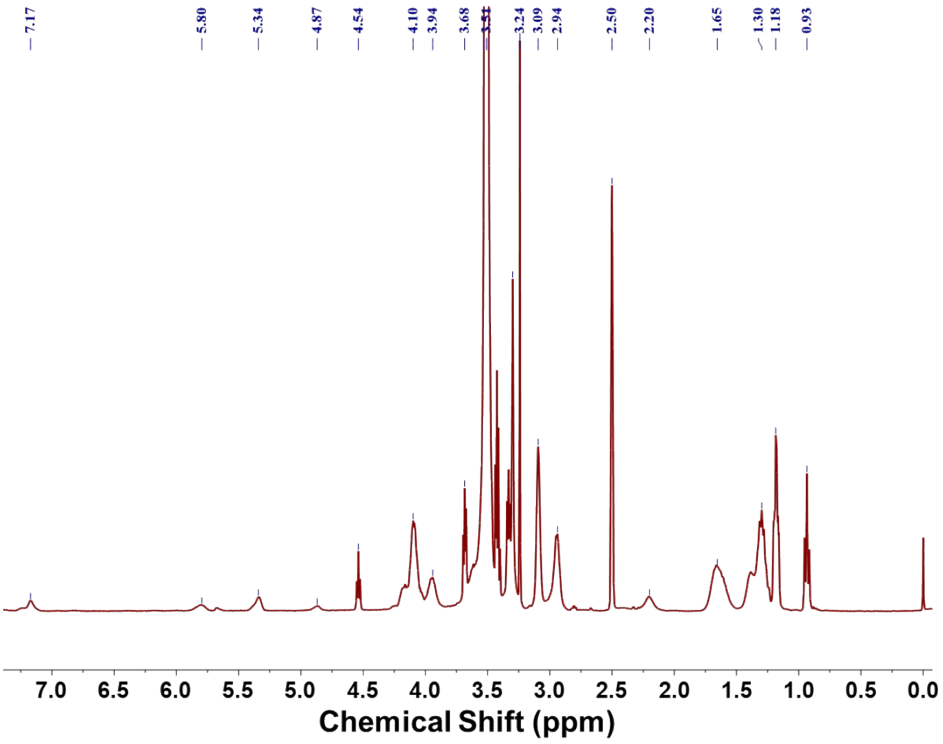

Figure S18 ^{1}H NMR spectrum of PQA-11 in dimethyl sulfoxide-d6

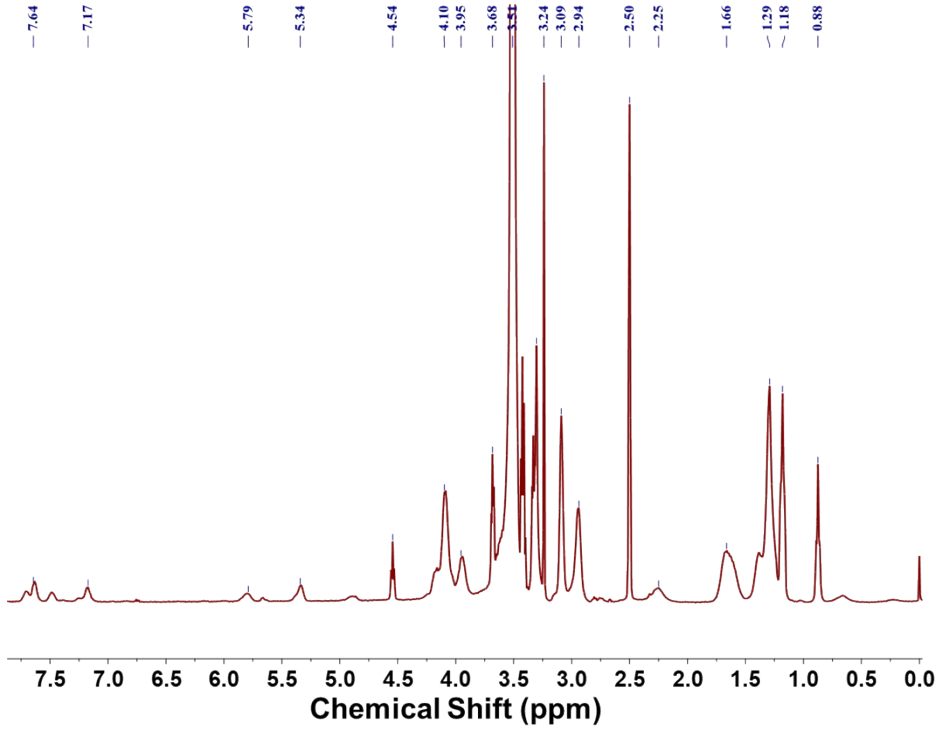

Figure S19 ^{1}H NMR spectrum of PQA-12 in dimethyl sulfoxide-d6

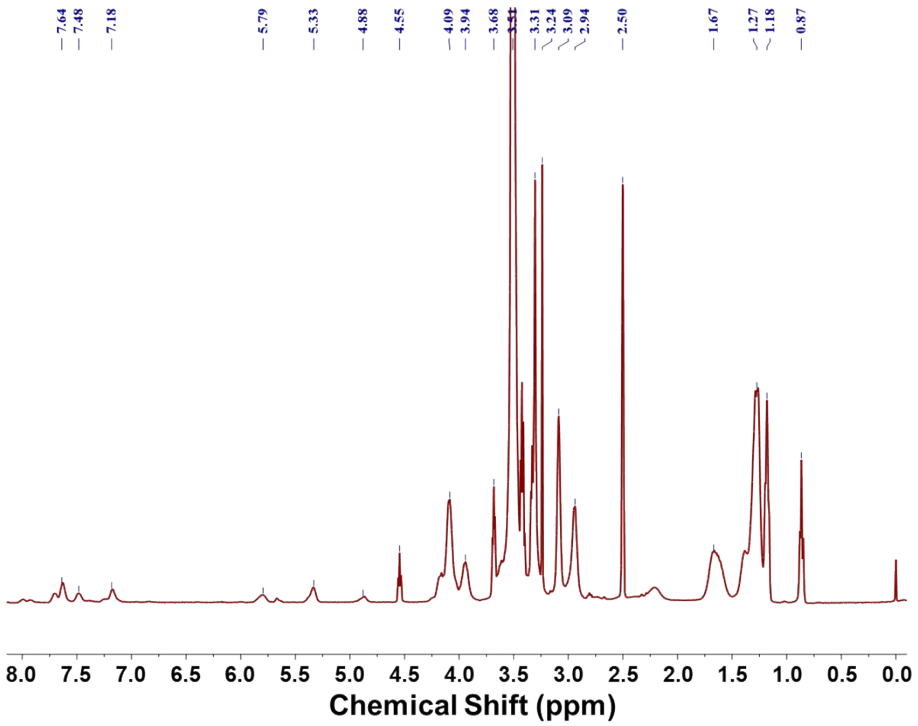

Figure S20 ^{1}H NMR spectrum of PQA-13 in dimethyl sulfoxide-d6

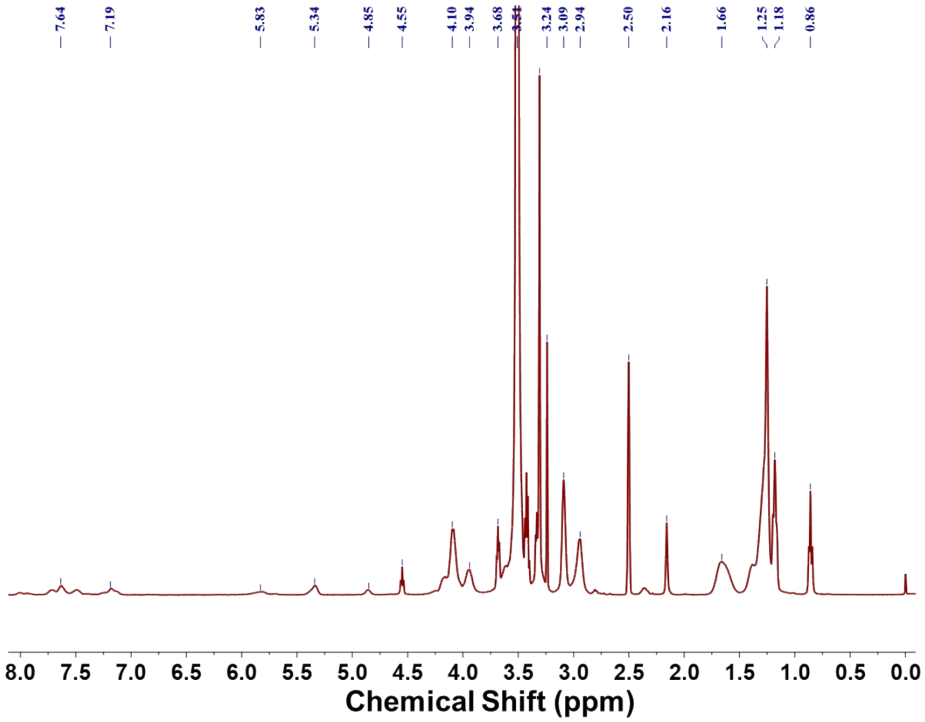

Figure S21 ^{1}H NMR spectrum of PQA-14 in dimethyl sulfoxide-d6

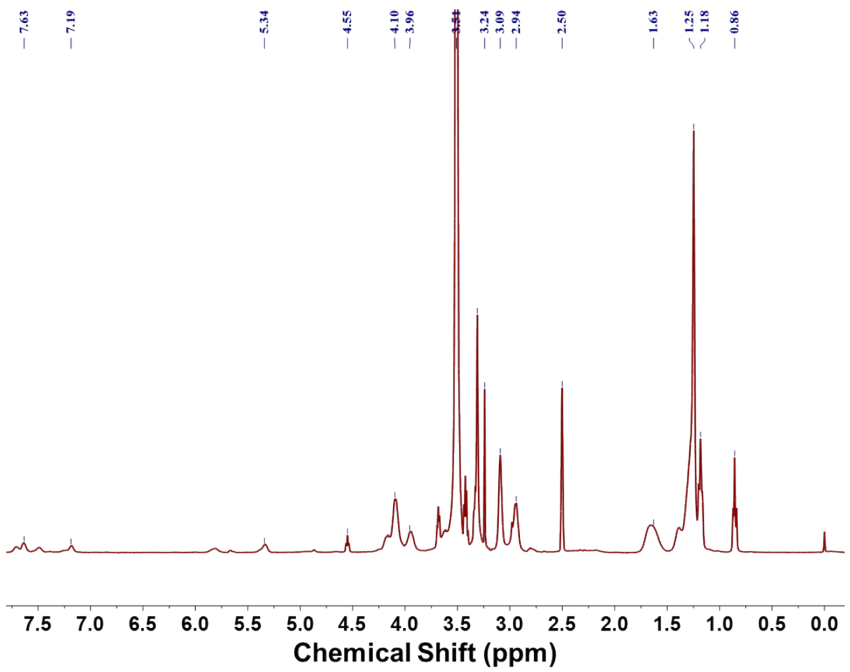

Figure S22 ^{1}H NMR spectrum of PQA-15 in dimethyl sulfoxide-d6

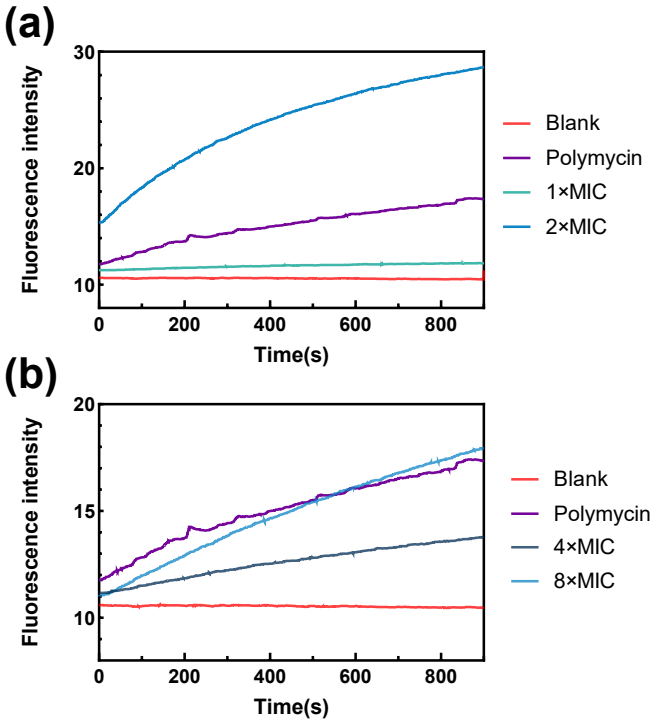

Figure S23 Cytoplasmic membrane permeabilization of E.coli after treatment with PQA-12 (a), PTA-6 (b) and polymyxin B at 8 × MIC.

The results showed that polymyxin B at 8 × MIC is between PQA-12 at MIC and at 2 × MIC in cytoplasmic membrane permeability. But polymyxin B at 8 × MIC is equivalent to PTA-6 at 8 × MIC in cytoplasmic membrane permeability.

References

[1] Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006;1(3):1559-82. doi: 10.1038/nprot.2006.236. PMID: 17406449.