Electronic Supplementary Information

The hybrid compounds of cationic pillararene and efflux pump inhibitor show synergistic effect against Gram-negative bacteria

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[c] Department of Clinical Laboratory, Affiliated Hospital of North Sichuan Medical College, Nanchong 637000, Sichuan, P. R. China.

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Characterization data

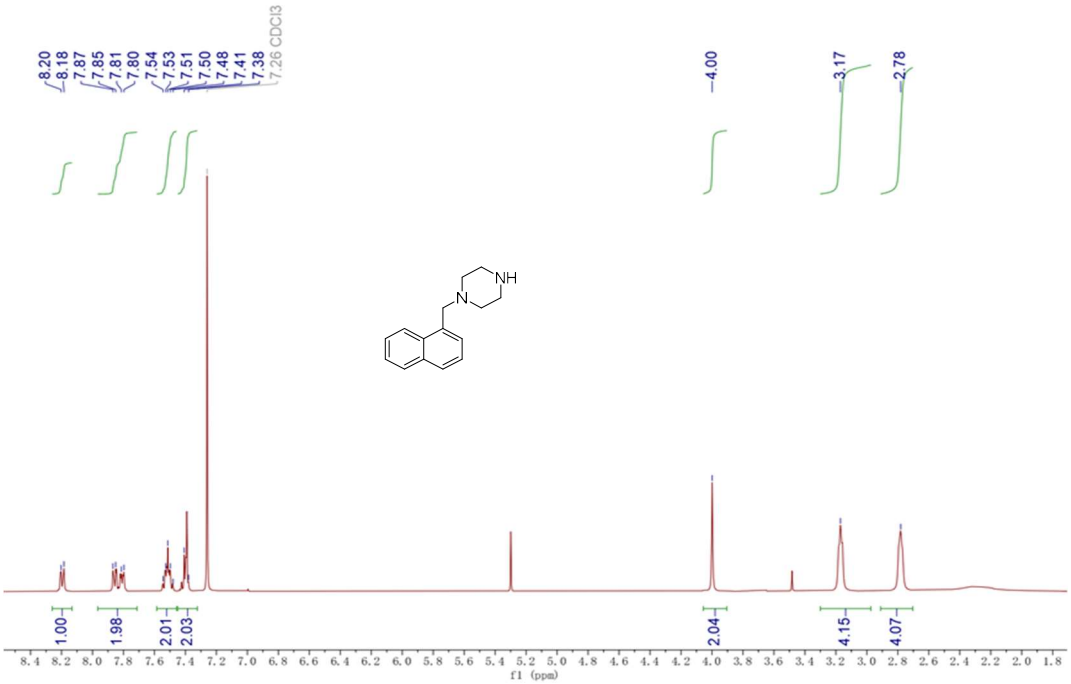


Fig. S1 ^{1}H NMR spectrum of NMP in $CDCl_3$.

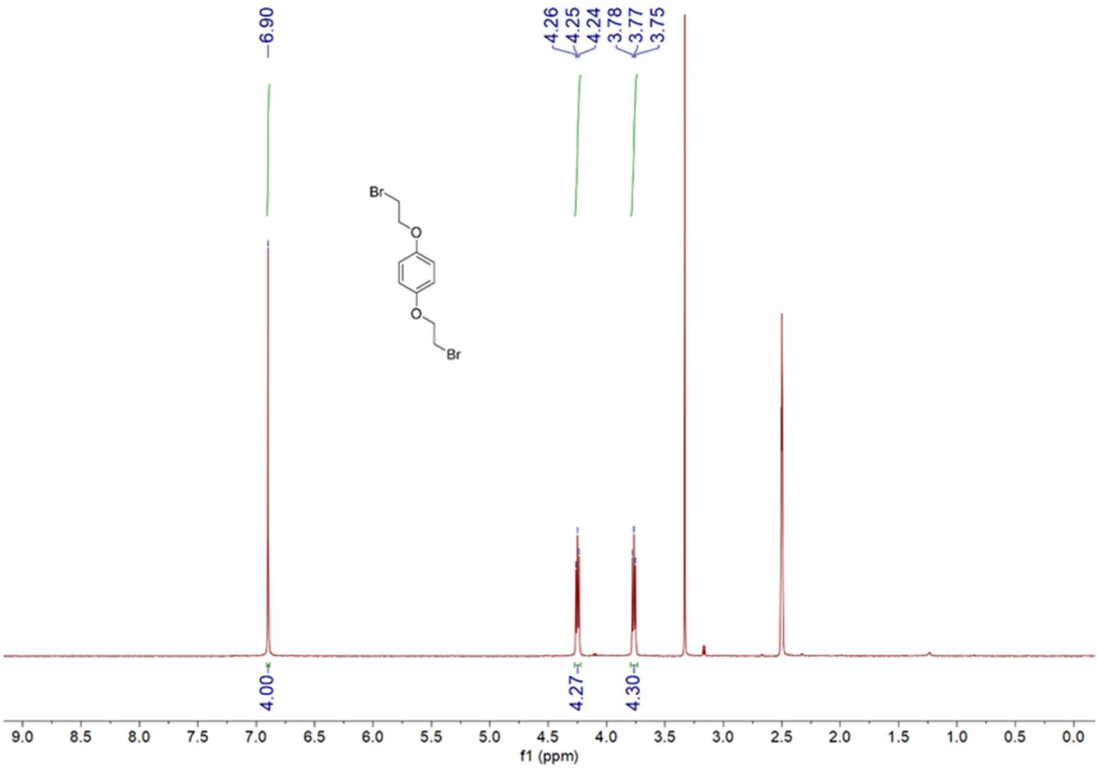


Fig. S2 ^{1}H NMR spectrum of P1B2 in DMSO.

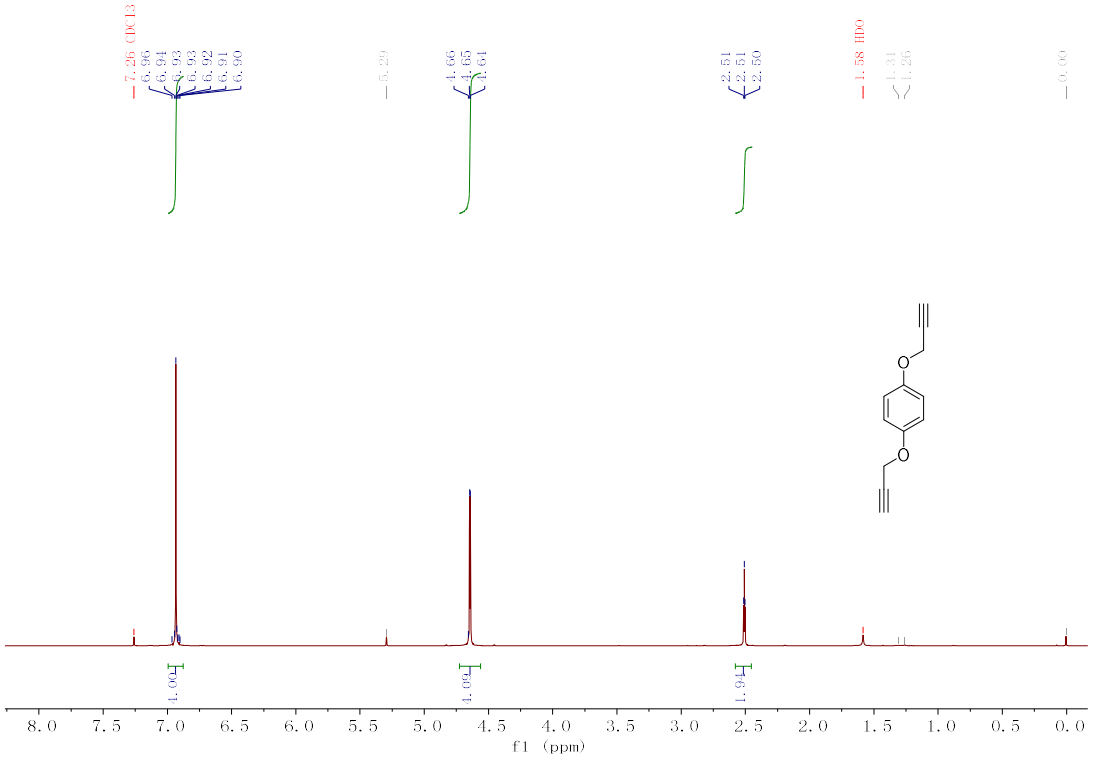


Fig. S3 ^{1}H NMR spectrum of P1Q2 in $CDCl_3$.

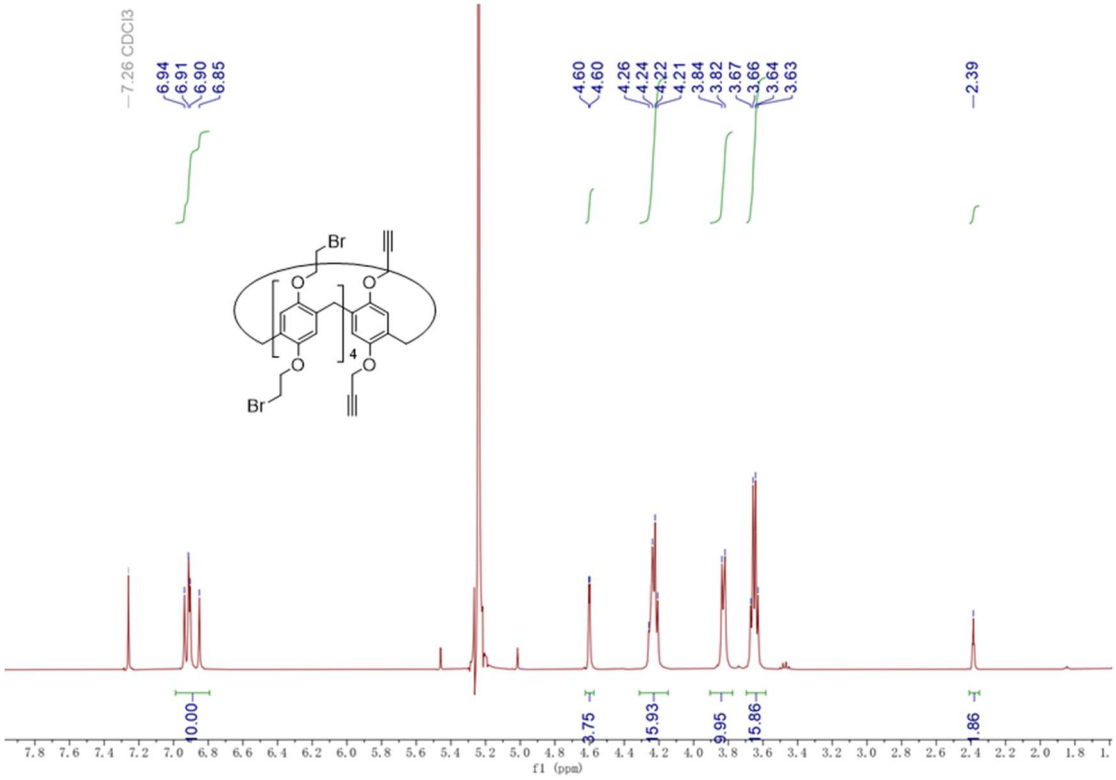


Fig. S4 ^{1}H NMR spectrum of PBQ in $CDCl_3$.

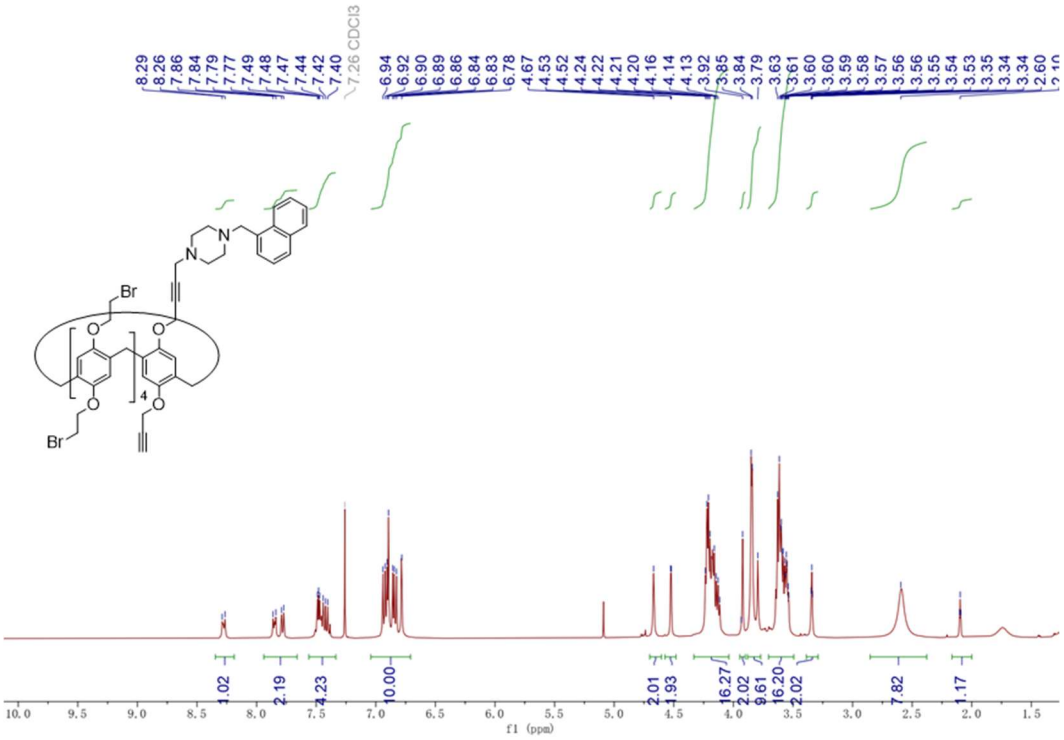


Fig. S5 ^{1}H NMR spectrum of PBNMPa in $CDCl_3$.

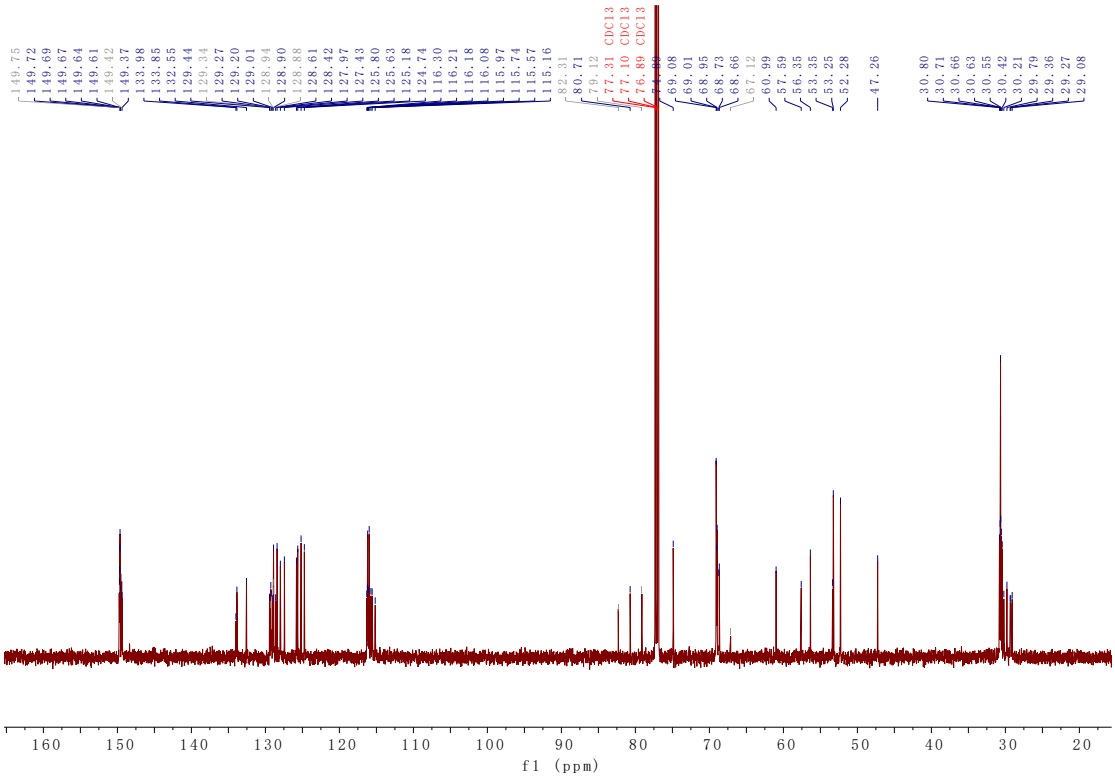


Fig. S6 ^{13}C NMR spectrum of PBNMPa in $CDCl_3$.

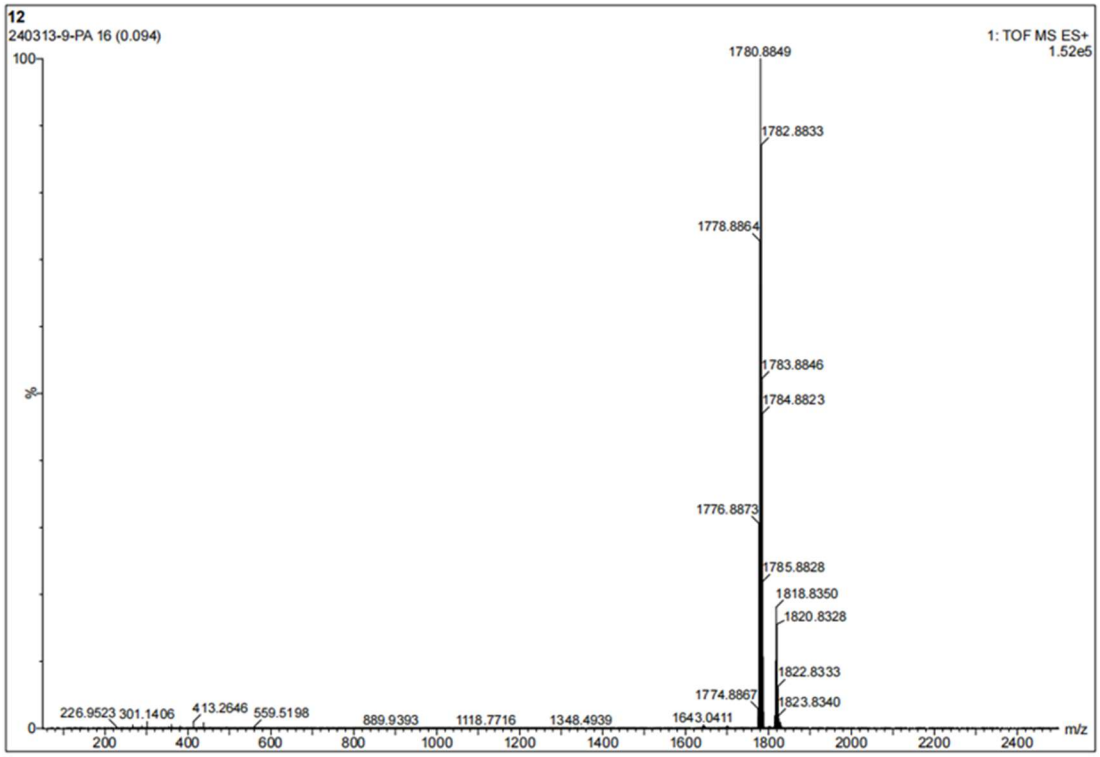


Fig. S7 HR-MS spectrum of PBNMPa.

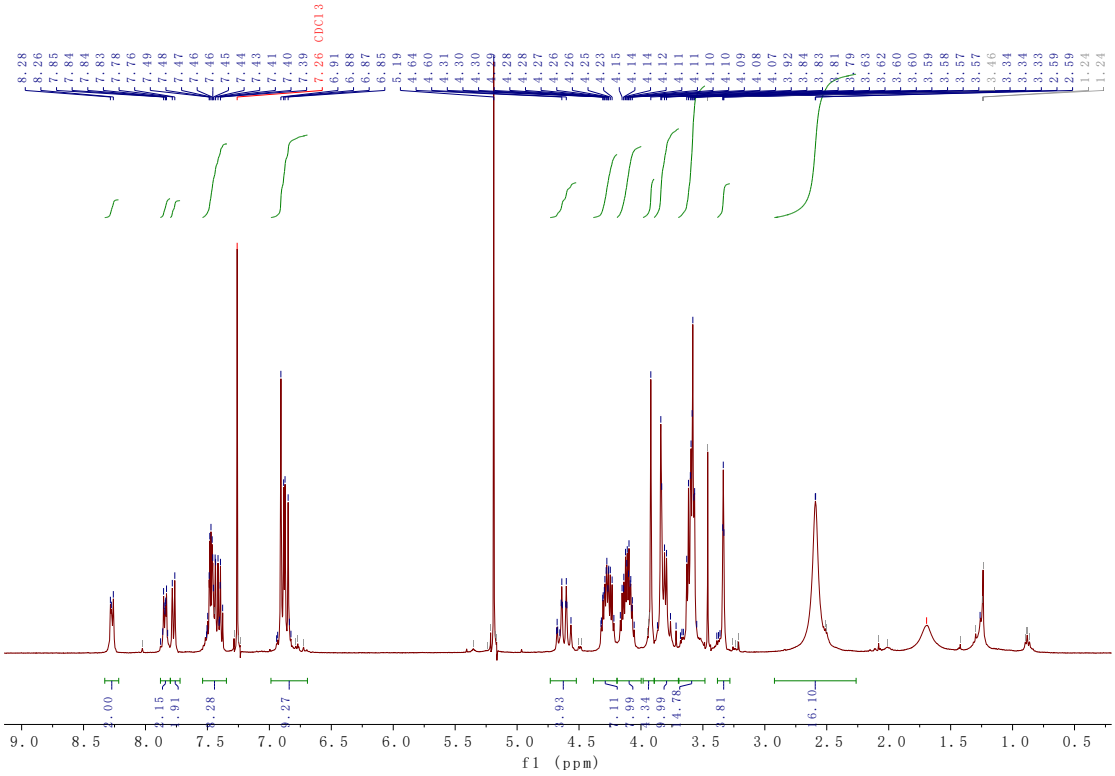


Fig. S8 ^{1}H NMR spectrum of PBNMPb in $CDCl_3$.

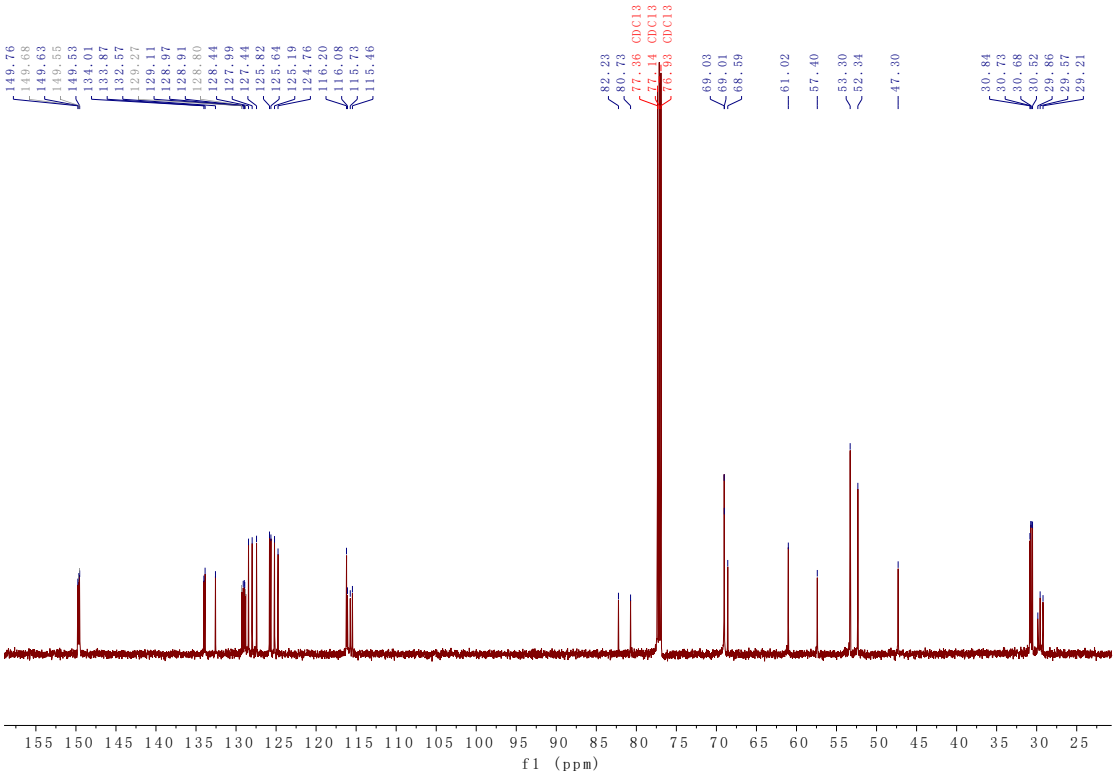


Fig. S9 ^{13}C NMR spectrum of PBNMPb in $CDCl_3$.

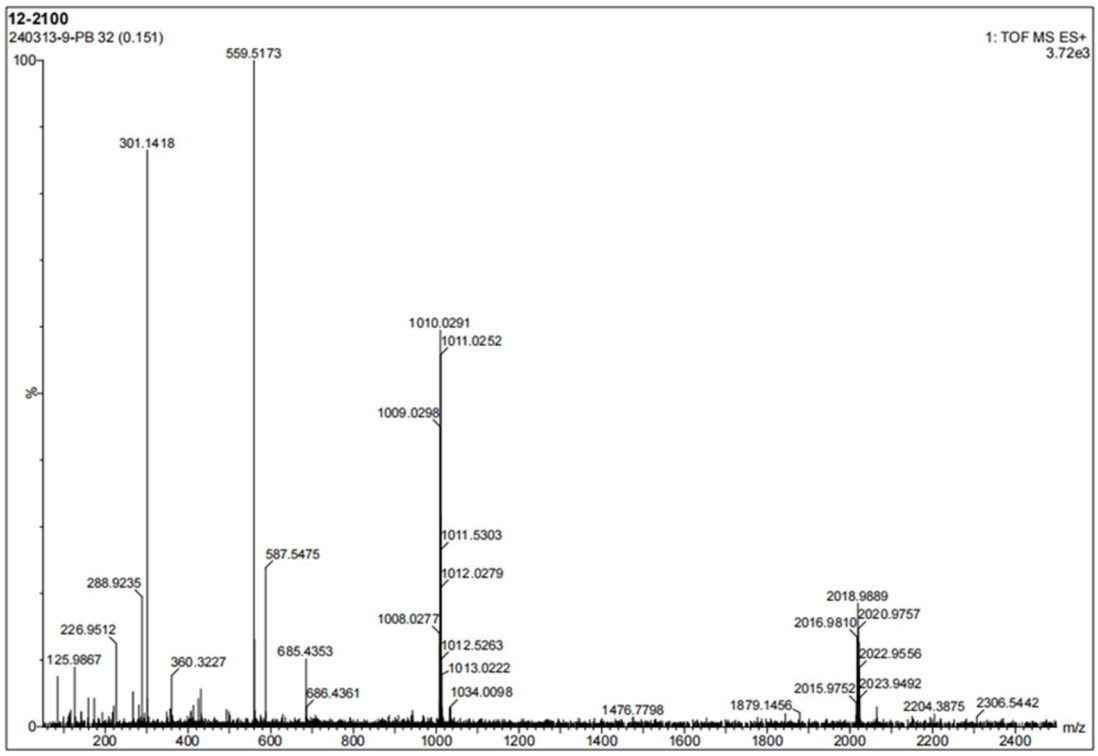


Fig. S10 HR-MS spectrum of PBNMPb.

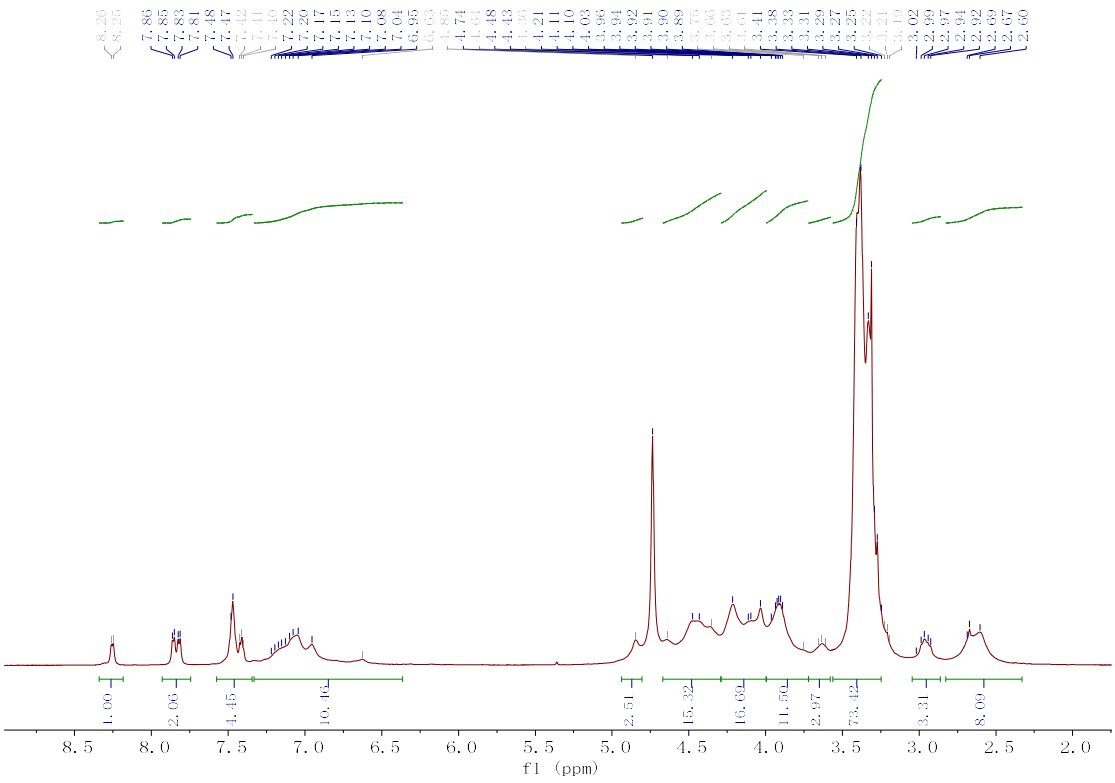


Fig. S11 ^{1}H NMR spectrum of PNNMPa in CD_3OD.

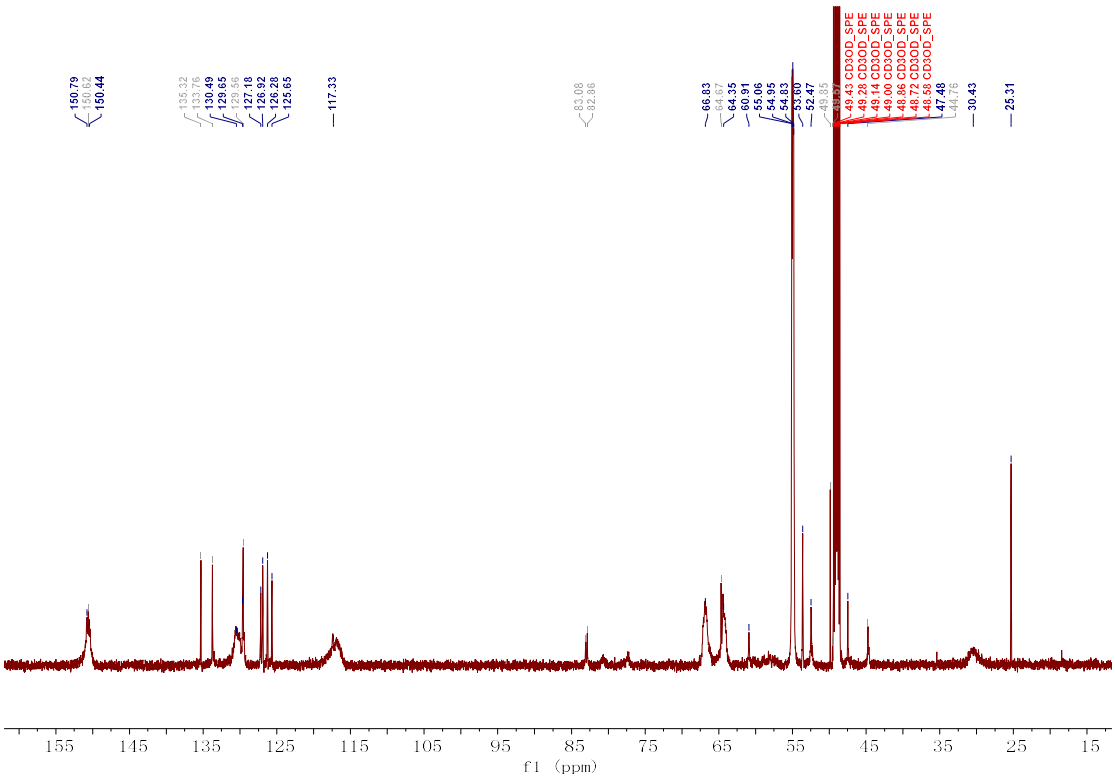
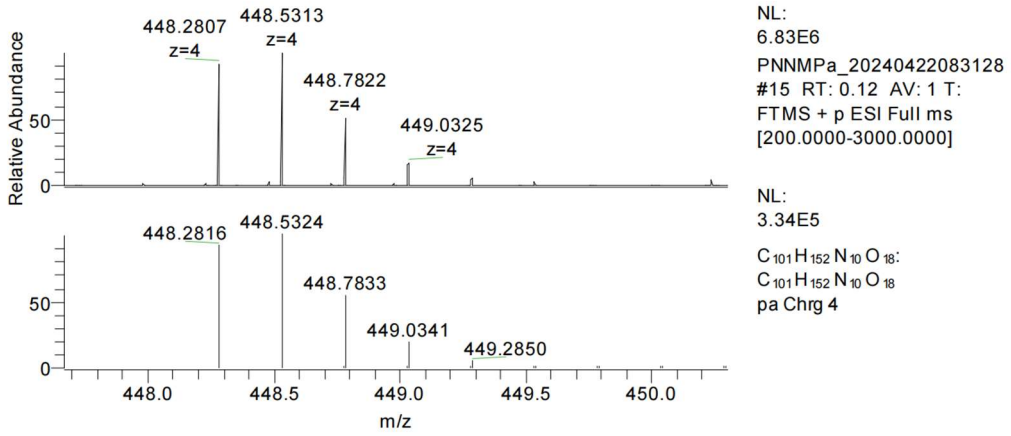


Fig. S12 ^{13}C NMR spectrum of PNNMPa in CD_3OD.



$[M^{8+}+4HCOO^-]^{4+}$

Elemental composition search on mass 448.2807

m/z= 443.2807-453.2807

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
448.2807	448.2816	-2.02	31.0	$C_{101}H_{152}O_{18}N_{10}$

Fig. S13 HR-MS spectrum of PNNMPa.

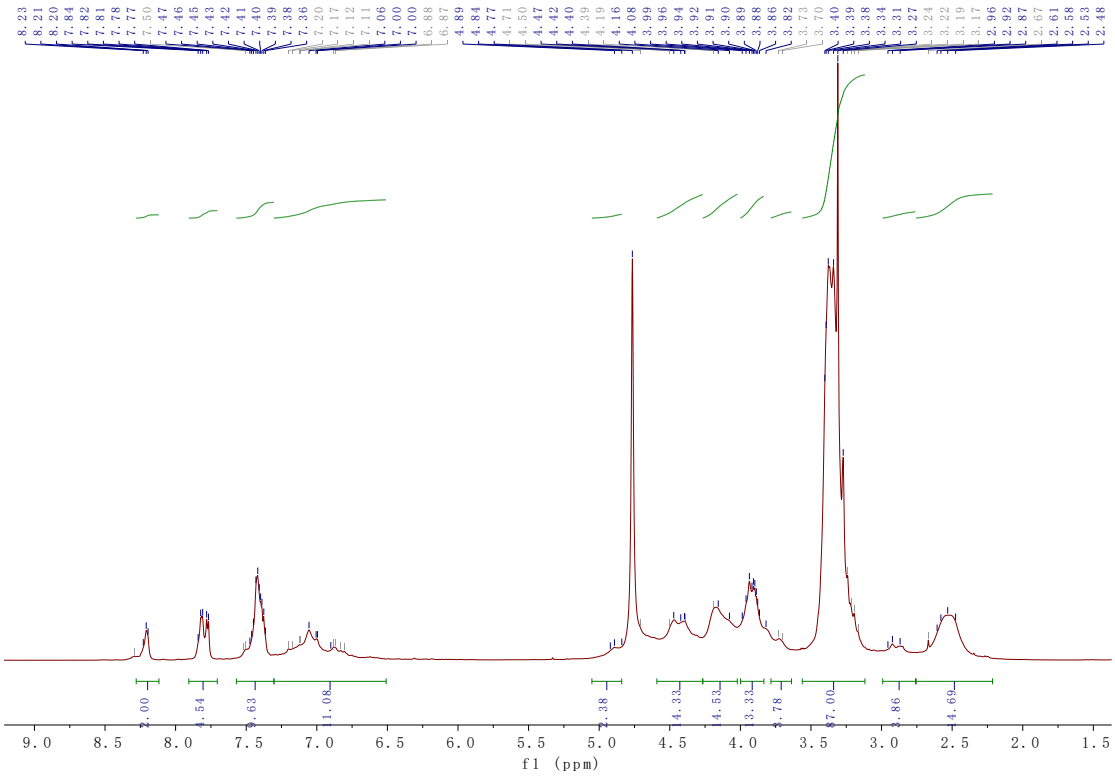


Fig. S14 ^1H NMR spectrum of PNNMPb in CD_3OD.

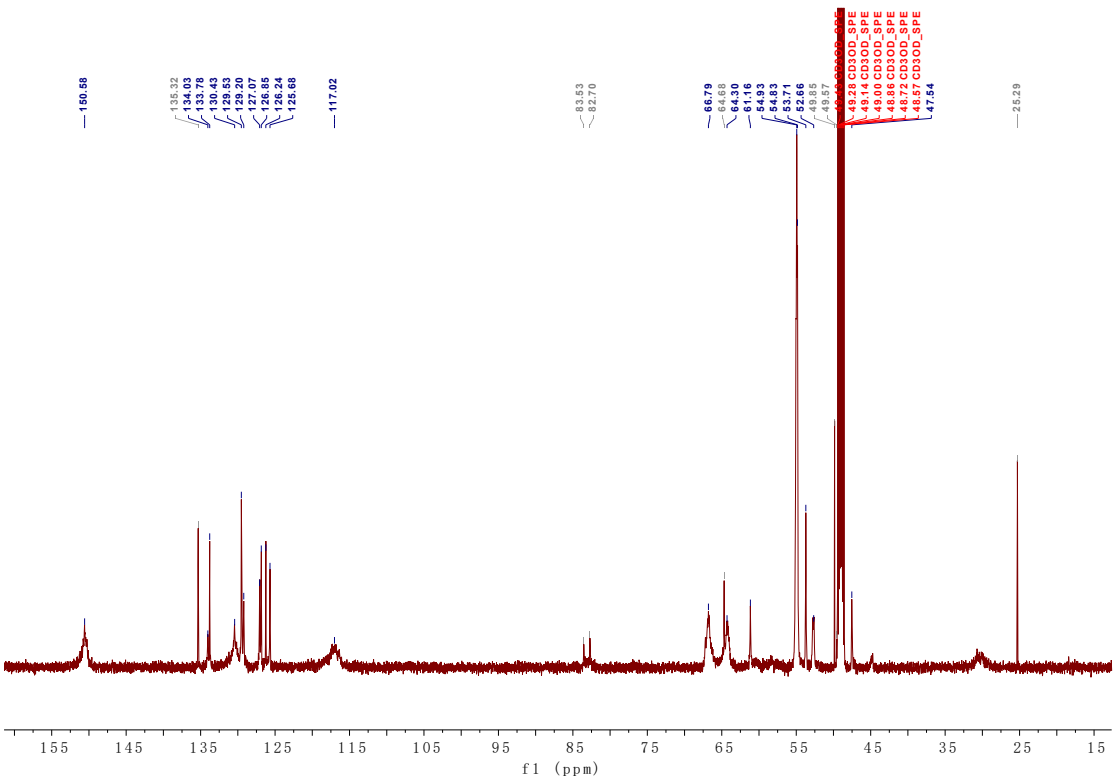
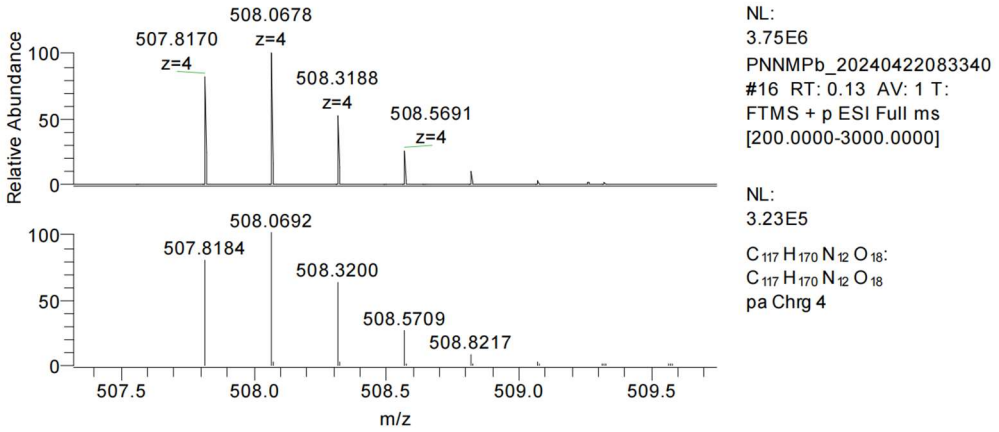


Fig. S15 ^{13}C NMR spectrum of PNNMPb in CD_3OD.



$[M^{8+}+4HCOO^-]^{4+}$

Elemental composition search on mass 507.8170

m/z= 502.8170-512.8170

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
507.8170	507.8184	-2.57	39.0	$C_{117}H_{170}O_{18}N_{12}$

Fig. S16 HR-MS spectrum of PNNMPb.

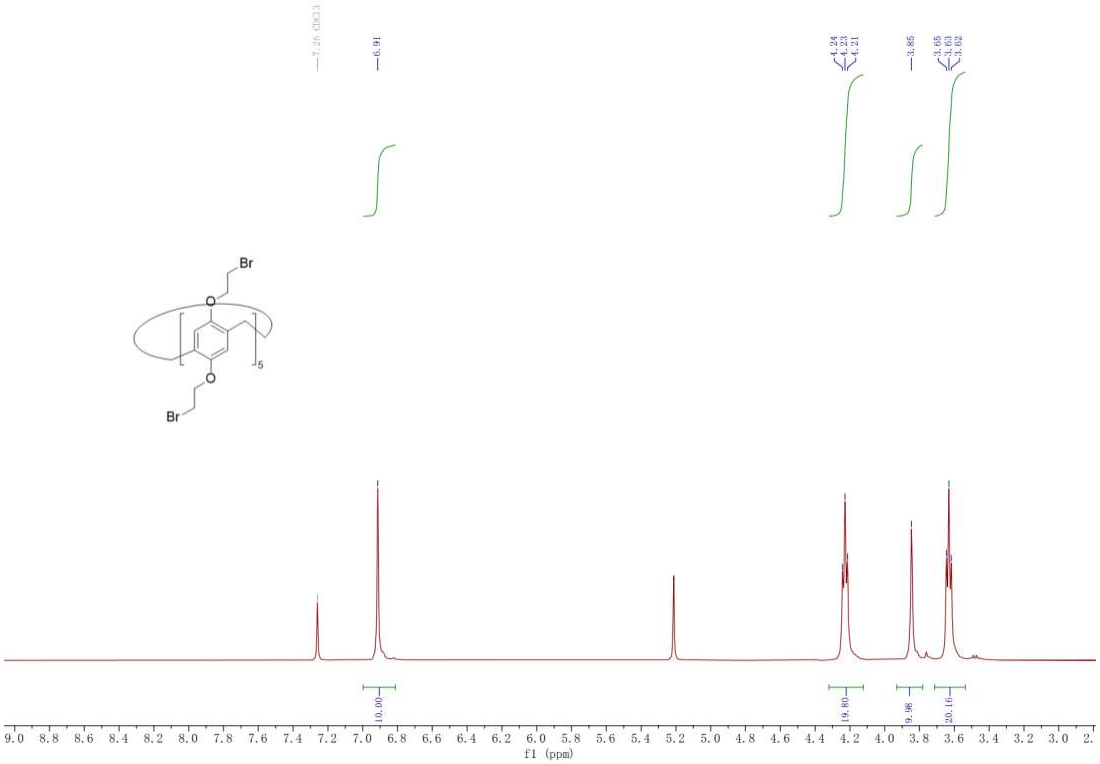


Fig. S17 ^{1}H NMR spectrum of PB5 in CD_3Cl_3.

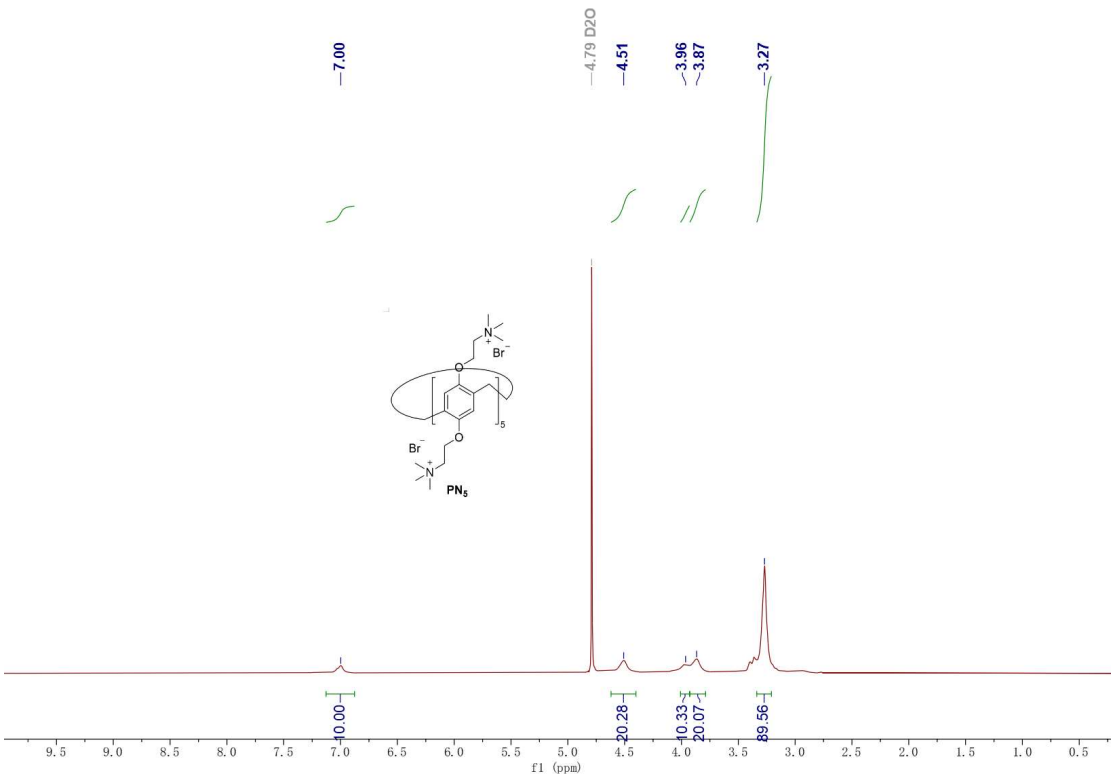


Fig. S18 ^{1}H NMR spectrum of PN5 in D_2O.

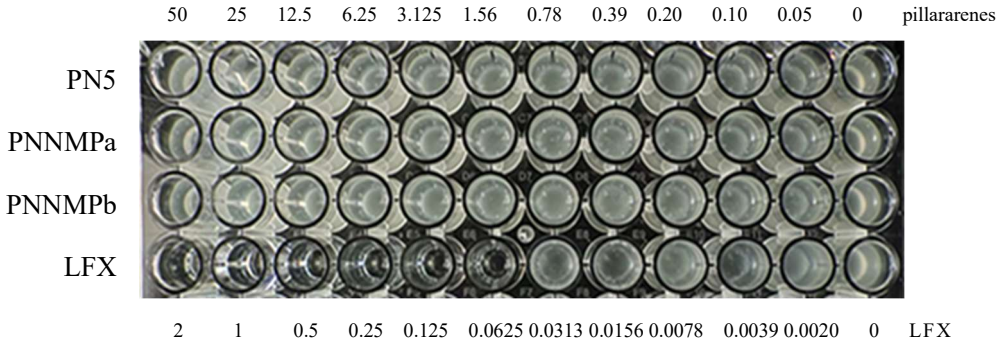
Biological assays

The bacterial strains used for biological activities assays were *S. aureus* ATCC 25923, *E. coli* ATCC 11775, and *P. aeruginosa* ATCC 15442. The bacterial concentration was determined using a microplate reader at optical density (OD) of 600 nm (OD_{600} of 0.1 corresponded to a concentration of 10^8 colony-forming units per milliliter (CFU/mL)).[2] Wells without bacteria were prepared as negative control. Wells without compound were prepared as positive control.

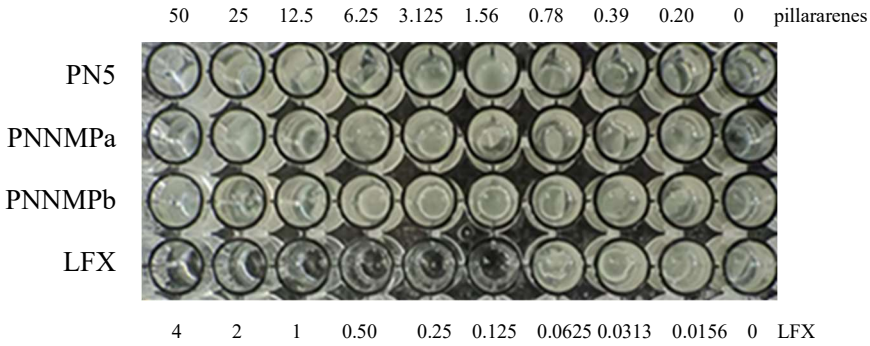
1. Minimum inhibition concentration (MIC)

MIC was determined by using the double microdilution method (CLSI 2013), and all determinations were performed in triplicate. Bacteria were cultured in a Mueller-Hinton II (MH) broth at 37 °C for 24 h, and the bacterial suspension was diluted to a concentration of about 3×10^5 CFU/mL. Then 100 μl of serial 1:2 dilutions of compounds were prepared in a 96-well microplate. 100 μl of the diluted suspension was added to each well. After a 24 h incubation at 37 °C, the MIC values of compounds were recorded. MIC was defined as the lowest concentration of testing compound that inhibited visible growth of bacteria as observed with the naked eye.[3]

a) *E. coli*



b) *S. aureus*



c) *P. aeruginosa*

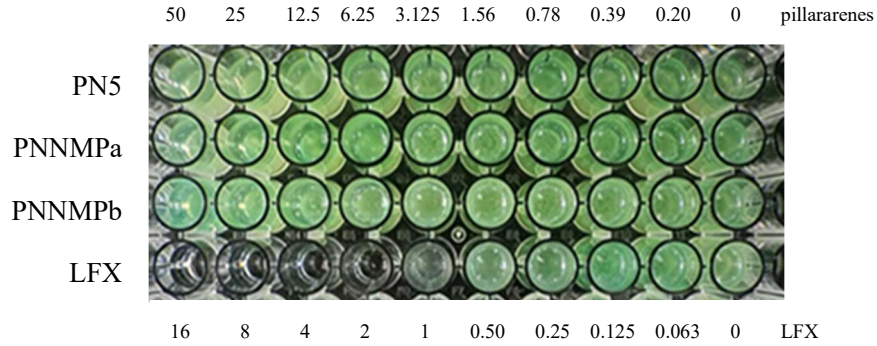
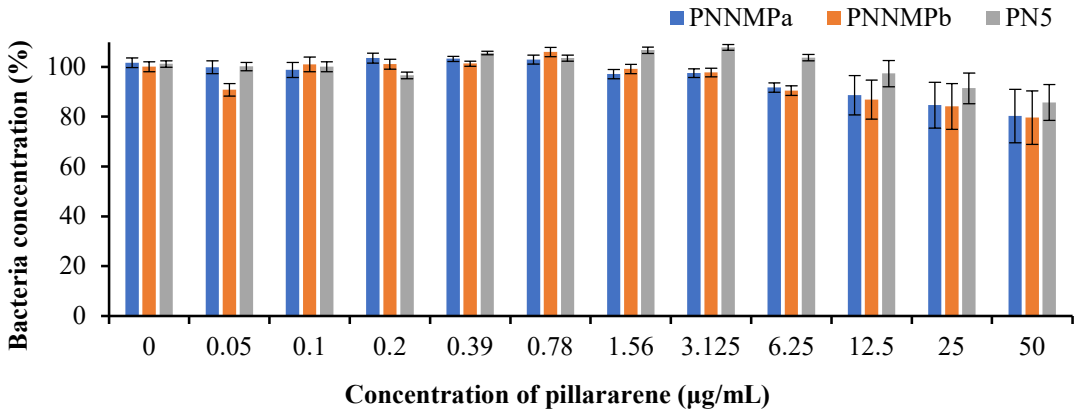
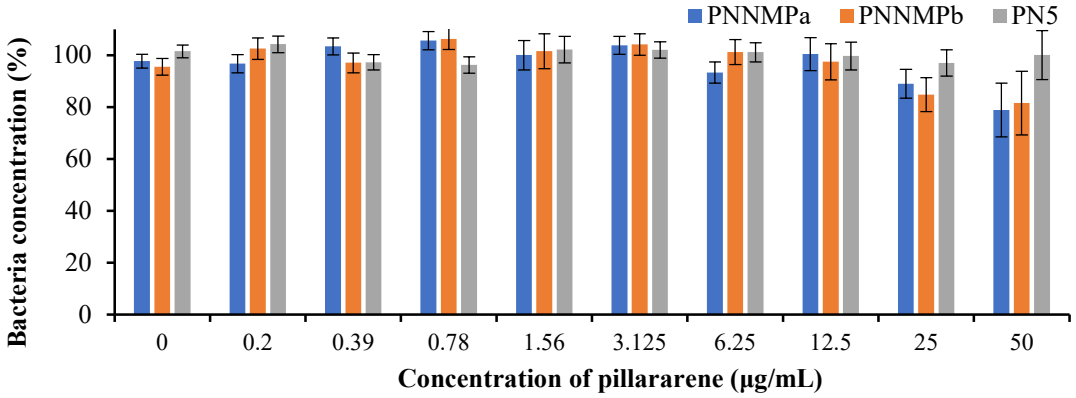


Fig. S19 Schematic diagram of the double microdilution assay with (a) *E. coli* ATCC 11775, (b) *S. aureus* ATCC 25923 and (c) *P. aeruginosa* ATCC 15442.

a) E. coli



b) *S. aureus*



c) *P. aeruginosa*

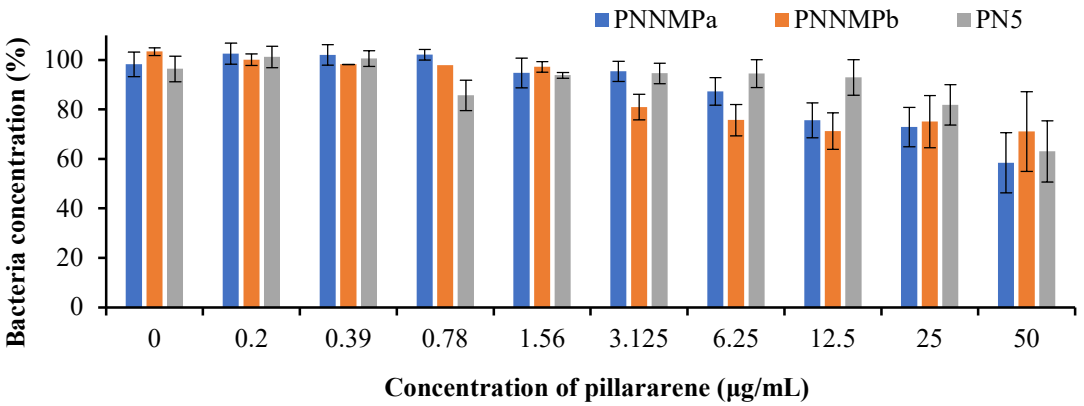


Fig. S20 Antibacterial activities of the hybrid compounds towards (a) *E. coli* ATCC 11775, (b) *S. aureus* ATCC 25923 and (c) *P. aeruginosa* ATCC 15442 were determined using the double microdilution method. Each concentration was tested in triplicate, and the results are expressed as means ± standard error from two independent experiments.

Table S1. Antibacterial activities of compounds.

Compound	MIC[a] in μg/mL (μM)		
	E. coli ATCC 11775	*S. aureus* ATCC 25923	*P. aeruginosa* ATCC 15442
PNNMPa	> 50 (22.2)	> 50 (22.2)	> 50 (22.2)
PNNMPb	> 50 (20.1)	> 50 (20.1)	> 50 (20.1)
PN5	> 50 (22.0)	> 50 (22.0)	> 50 (22.0)
LFX[b]	0.0625 (0.173)	0.125 (0.346)	2.0 (5.54)

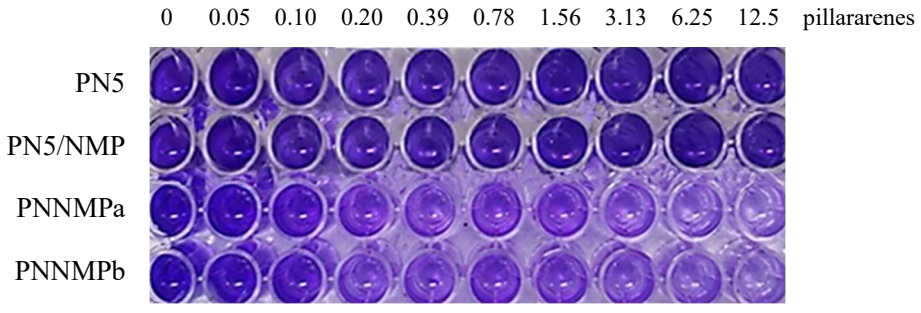
[a] Minimum inhibitory concentration.

2. Biofilm inhibition concentration ($MBIC_{50}$)

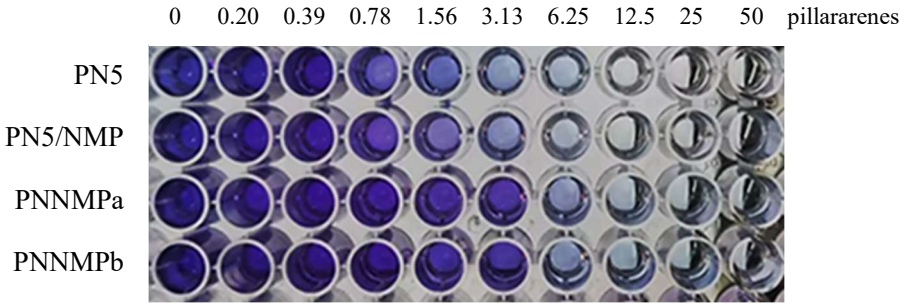
$MBIC_{50}$ was determined by crystal violet staining,[4] and all determinations were performed in triplicate. Bacteria were cultured in a Luria-Bertani broth (LB) at 37 °C for 24 h. The bacterial suspension was diluted to an OD_{600} value of about 0.1. Next, 100 µl of serial 1:2 dilutions of compounds were prepared in a 96-well microplate. 100 µl of the diluted suspension was added to each well. After a 24 h incubation at 37 °C, free-floating bacteria and medium were removed by turning over the plate. The wells were rinsed vigorously four times with doubly distilled water (DDW). 1% crystal violet (250 µl) was added to each well. After 15 min, wells were rinsed vigorously three times with DDW to remove unbound dye. After adding 200 µl of 33% acetic acid to each well, the microplate was shaken for 15 min to release the dye. The formation of biofilm was quantified by measuring the difference between the absorbance of untreated and treated bacterial samples for each tested concentration of the compounds and the absorbance of appropriate blank well at 600 nm (A_{600}) using microplate reader. The $MBIC_{50}$ was defined as the lowest concentration at which at least 50% reduction in biofilm formation was measured compared to untreated cells.

Biofilm formation % = $\frac{OD_{sample} - OD_{negative\ control}}{OD_{control} - OD_{negative\ control}} \times 100\%$

a) E. coli



b) S. aureus



c) *P. aeruginosa*

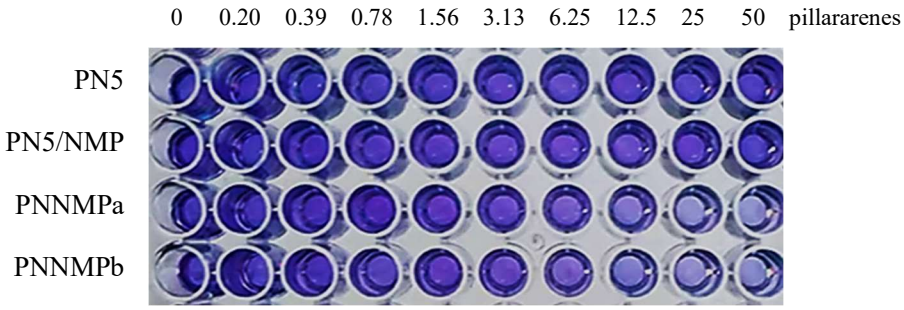
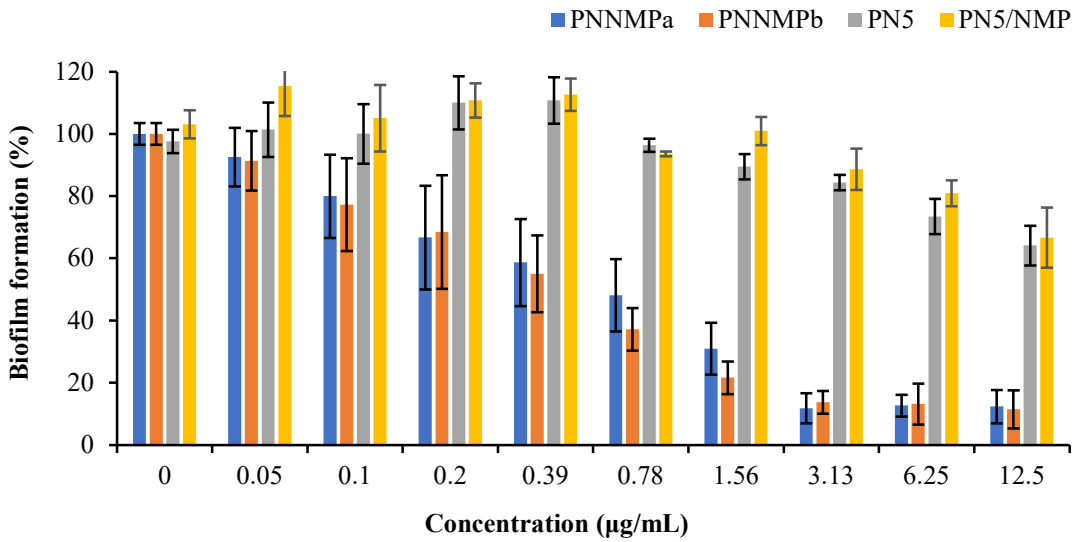
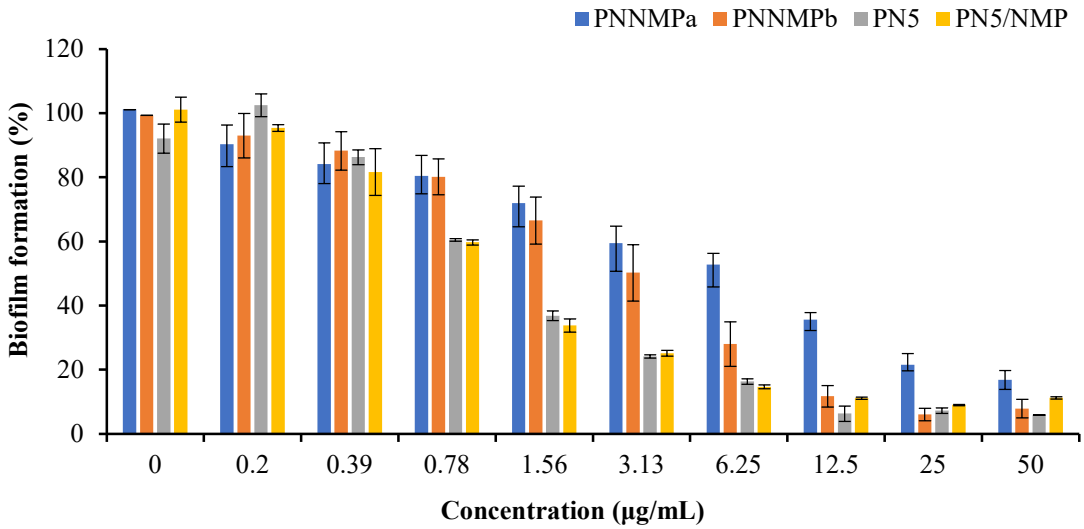


Fig. S21 Schematic diagram of crystal violet staining experiments with (a) *E. coli* ATCC 11775, (b) *S. aureus* ATCC 25923 and (c) *P. aeruginosa* ATCC 15442.

a) E. coli



b) S. aureus



c) *P. aeruginosa*

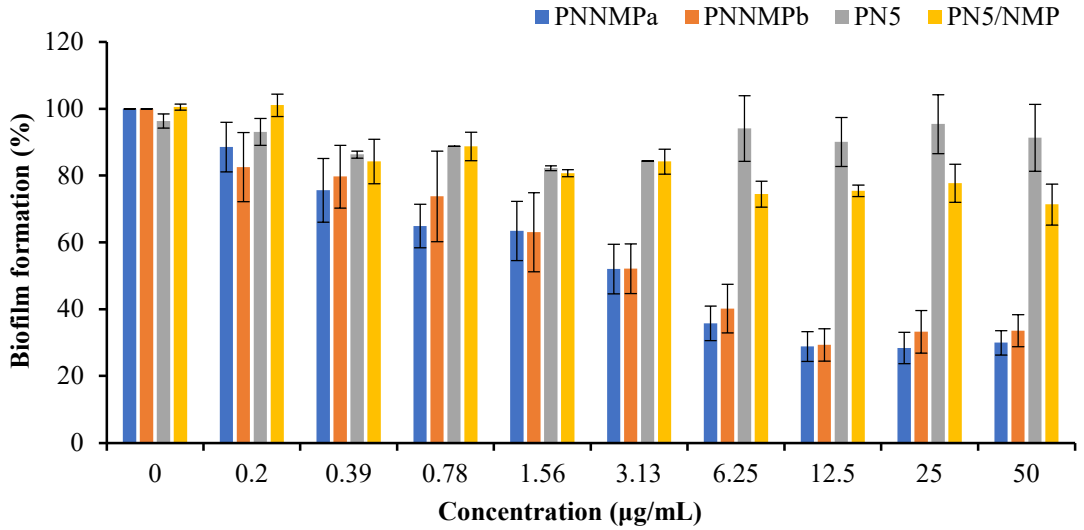
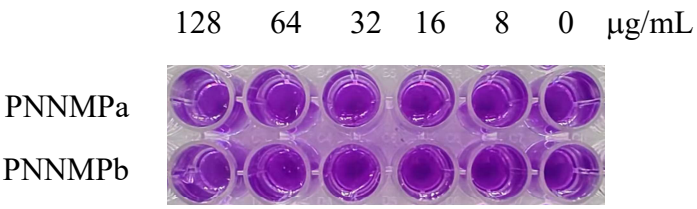


Fig. S22 Biofilm formation by (a) *E. coli* ATCC 11775, (b) *S. aureus* ATCC 25923 and (c) *P. aeruginosa* ATCC 15442 was evaluated using the double microdilution method. Each concentration was tested in triplicate, and the results are expressed as means ± standard error from two independent experiments.

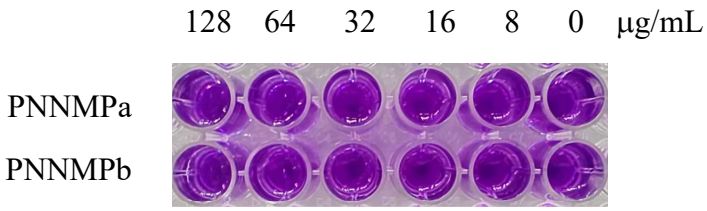
3. Biofilm eradication concentration ($MBEC_{50}$)

$MBEC_{50}$ was determined against mature 24-h-old biofilms, and all determinations were performed in triplicate. Briefly, bacterial species were allowed to form biofilms in TSB broth in a 96-well microplate by incubation for 24 h at 37 °C. Biofilm samples were washed twice with DDW, then were exposed to 200 µl of the tested compounds prepared in TSB. After incubation at 37 °C for 24 h, the plates were rinsed three times with DDW, and biofilm samples were stained with crystal violet as described for biofilm inhibition assay. Untreated biofilm samples were used as control. Biofilms were quantified by measuring the absorbance at 600 nm. The mean IC_{50} value for biofilm eradication ($MBEC_{50}$) was defined as the lowest concentration at which at least 50% reduction in biomass of preformed biofilms was measured compared to untreated biofilm samples.

a) E. coli



b) S. aureus



c) *P. aeruginosa*

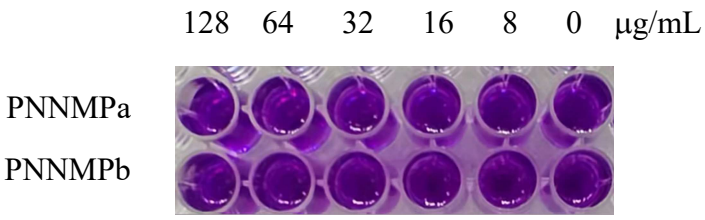


Fig. S23 Preformed biofilm of (a) E. coli ATCC 11775, (b) S. aureus ATCC 25923 and (c) P. aeruginosa ATCC 15442 was treated with the hybrid compounds ranging from 0 - 128 μg/mL.

4. Antibacterial activity of Levofloxacin (LFX) in combination with the hybrid drugs.

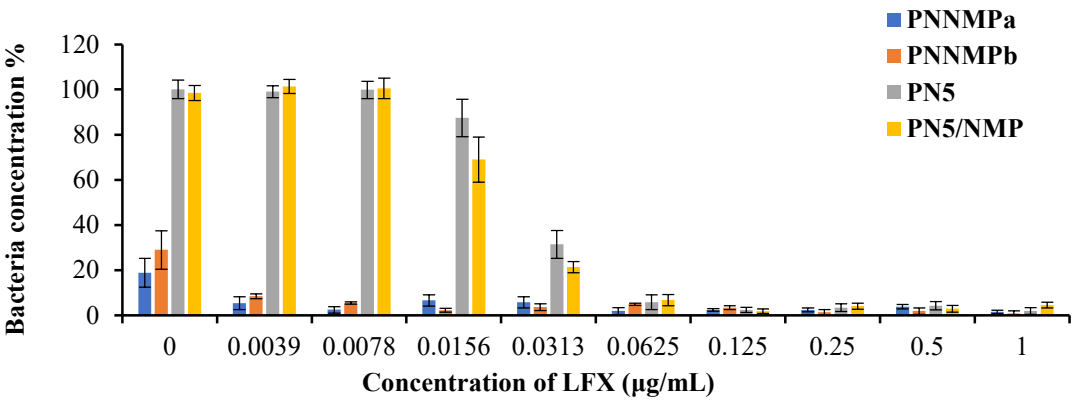
a) E. coli

b) S. aureus

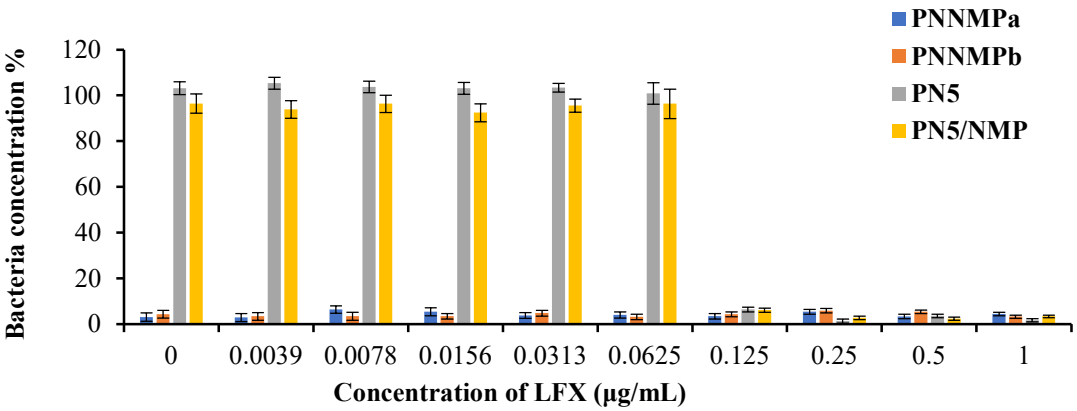
c) P. aeruginosa

Fig. S24 Schematic diagram of combination studies of levofloxacin with pillararene derivatives in .

a) E. coli



b) S. aureus



c) P. aeruginosa

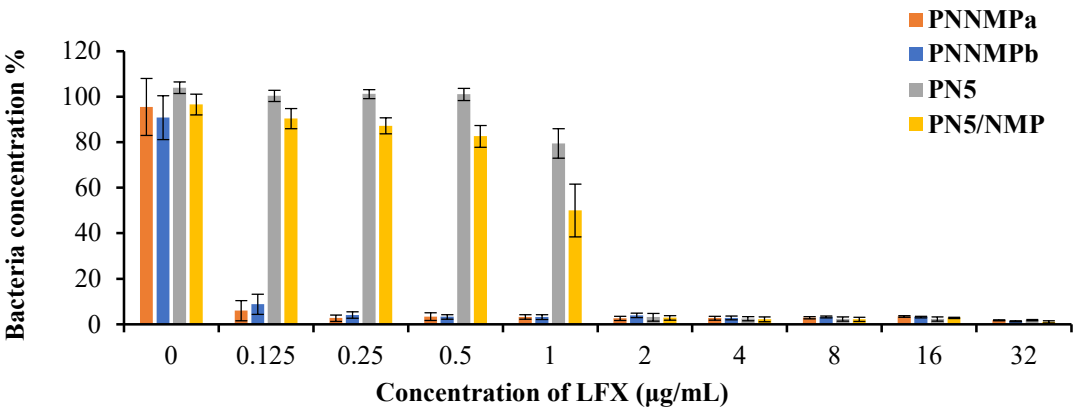


Fig. S25 Antibacterial activity of Levofloxacin (LFX) in the presence of various compounds (0.1 mM) towards (a) *E. coli* ATCC 11775, (b) *S. aureus* ATCC 25923 and (c) *P. aeruginosa* ATCC 15442.

a)
b)
c)
d)
e)
f)

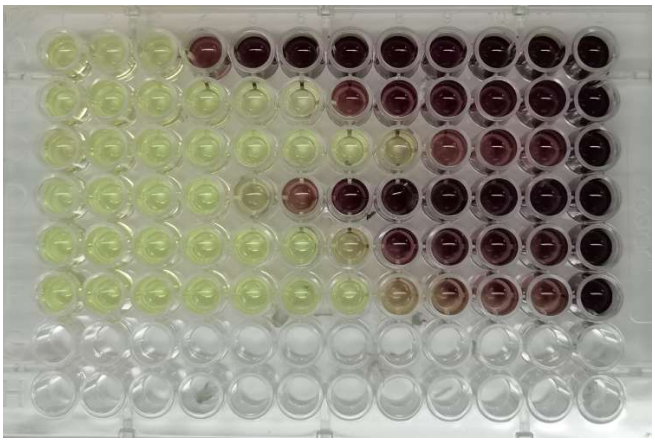


Fig. S26 Antibacterial activity of Tetracycline (TC, 128, 64, 32, …, 0 μg/mL) and Levofloxacin (LFX, 64, 32, 16, …, 0 μg/mL) in the presence of hybrid compounds (250 μg/mL) towards MDR *P. aeruginosa* isolate. (a) TC; (b) TC + PNNMPa; (c) TC + PNNMPb; (d) LFX; (e) LFX + PNNMPa; (f) LFX + PNNMPb.

5. Checkerboard method and fractional inhibitory concentration index determination.

The checkerboard consists of column in which each well contains the same amount of the antibiotic being 2-fold diluted along the x axis of 96-well plate. Rows in which each well contains the same amount of the hybrid compounds were 2-fold diluted on the y axis. The result is that each well in the checkerboard contains a unique combination of the two agents being tested. Overnight bacterial culture was standardized in saline using the 0.5 McFarland turbidity standard and diluted 1:500 in MH broth. An amount of 50 μL of standardized culture was added to each of the wells, and the plate was incubated at 37 °C for 24 h.

FICs were determined by checkerboard method. The MIC for each drug was the lowest concentration showing no bacterial growth. The FIC values for the compounds and antibiotics were calculated as the [MIC of agents in combination] /[MIC of agent alone]. The FICi is the sum of the FIC of the hybrid compound and the antibiotic. The combination is considered synergistic when the FICI is ≤ 0.5, no interaction when the FICI is > 0.5 to < 4.0, and antagonistic when the FICI is ≥ 4.0.

6. Outer membrane disruption against *P. aeruginosa*

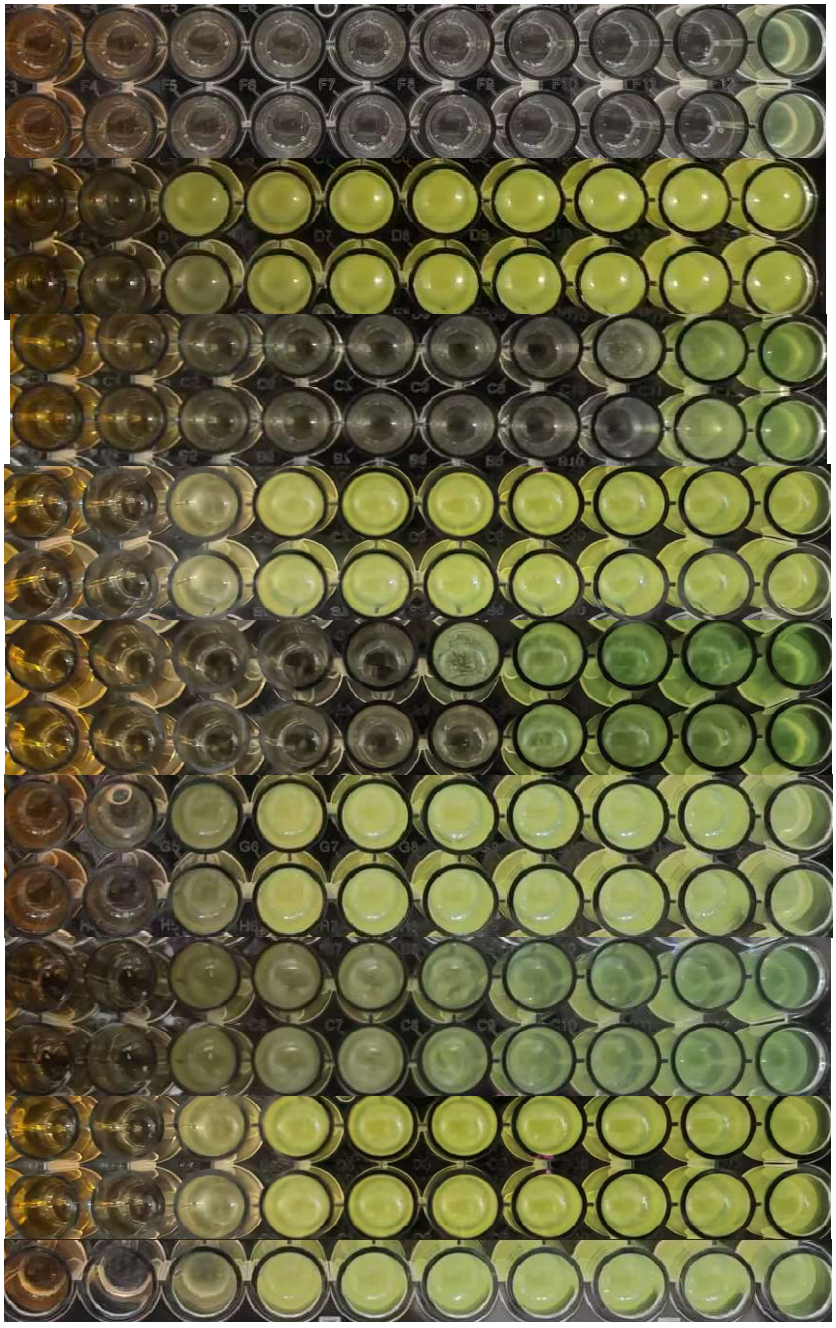


Fig. S27 Schematic diagram of combination studies of hybrid compounds with rifampicin (64, 32, 16, 8, …, 0 μg/mL) on the disruption of outer membrane in *P. aeruginosa.* (a) 250 μg/mL PNNMPa; (b) 250 μg/mL PNNMPb; (c) 250 μg/mL PNNMPa + 20 mM $MgCl_2$; (d) 250 μg/mL PNNMPb + 20 mM $MgCl_2$; (e) 125 μg/mL PNNMPa; (f) 125 μg/mL PNNMPb; (g) 125 μg/mL PNNMPa + 20 mM $MgCl_2$; (h) 125

μg/mL PNNMPb + 20 mM $MgCl_2$; (i) 62.5 μg/mL PNNMPa; (j) 62.5 μg/mL PNNMPb; (k) 62.5 μg/mL PNNMPa + 20 mM $MgCl_2$; (l) 62.5 μg/mL PNNMPb + 20 mM $MgCl_2$; (m) 250 μg/mL PN5 + 25 μg/mL NMP; (n) 250 μg/mL PN5 + 25 μg/mL NMP + 20 mM $MgCl_2$; (o) 25 μg/mL NMP; (p) 25 μg/mL NMP + 20 mM $MgCl_2$; (q) control.

7. Red blood cell hemolysis assay

A sample of red blood cells (2% w/w) were incubated with each of the tested compounds for 1 h at 37 °C using the double dilution method starting at a concentration of 1600 μg/mL. The negative control was PBS, and the positive control was 1% w/v solution of Triton X-100 (which induced 100% hemolysis). Following 10 min of centrifugation (2000 rpm), the supernatant was removed and the corresponding absorbance was measured using a microplate reader. The results are expressed as percentage of hemoglobin released relative to the positive control (Triton X-100). Experiments were performed in triplicate, and the results are an average of experiments in blood samples.

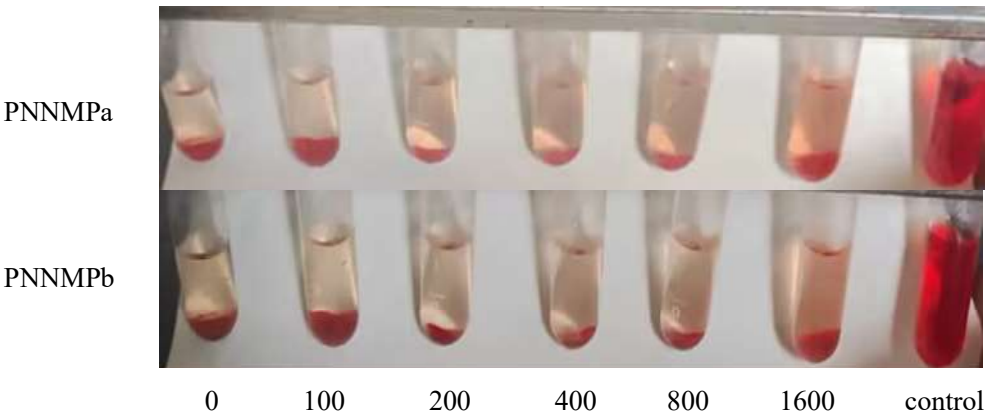


Fig. S28 Schematic diagram of red blood cell hemolysis assay. The concentration of tested compounds ranges from 0 to 1600 μg/mL.

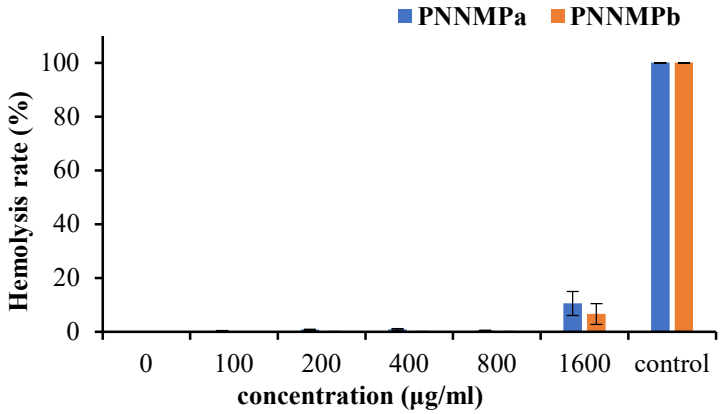


Fig. S29 Hemolytic rates of the hybrid compounds. Each concentration was tested in triplicate, and the results are expressed as means ± standard error from two independent experiments.

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