Supplementary information

Enhancing the Antibacterial Efficacy of Vancomycin Analogues: Targeting Metallo-ß-lactamases and Cell Wall Biosynthesis

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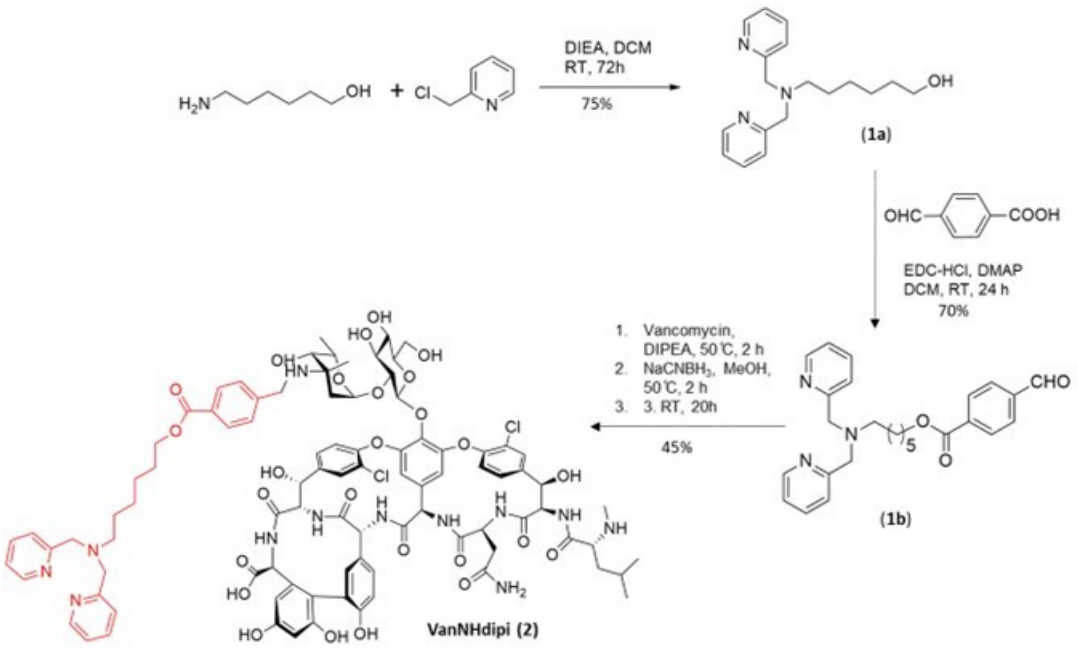
Supplementary figures



Scheme S1. Synthesis of dipicolyl-amine conjugated vancomycin derivative, VanNHdipi (**2**)

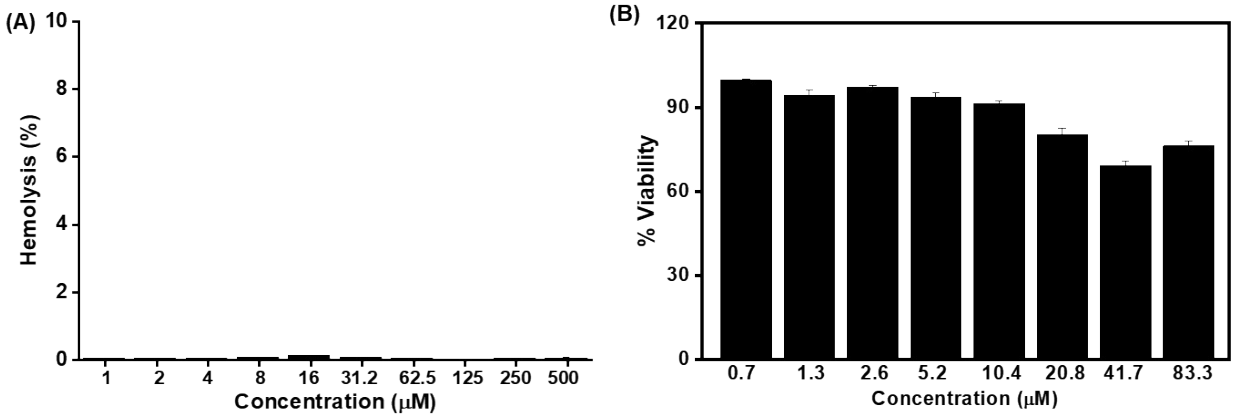


Figure S1. In vitro toxicity of VanNHdipi against (A) human red blood cells (RBCs) and (B) HEK 293 cells.

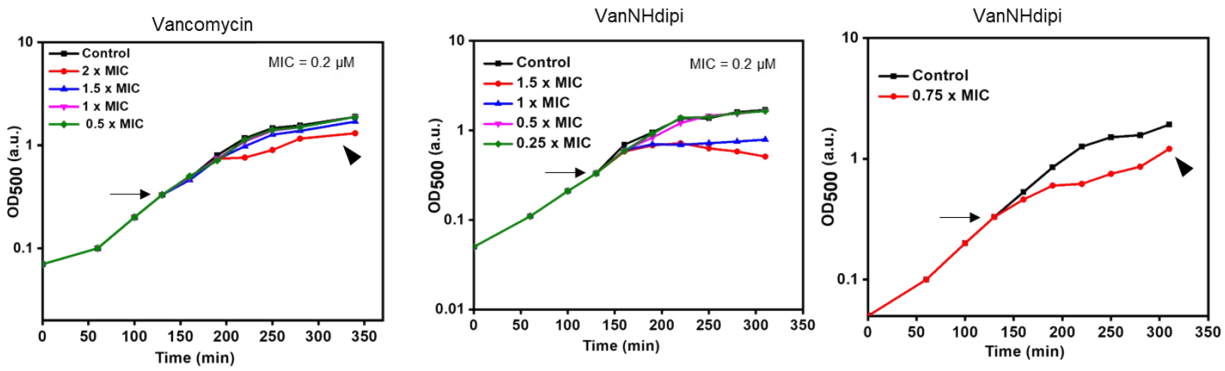


Figure S2. Study of acute growth retardation of cultures of *B. subtilis* upon treatment with varying concentrations of vancomycin and VanNHdipi (long arrow indicates timepoint of compound addition, bold arrow indicates physiologically effective concentration (PEC).

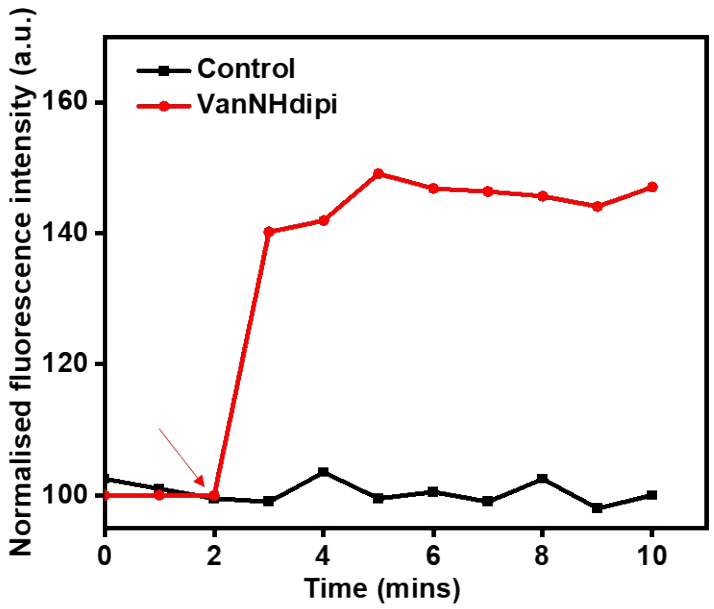


Figure S3. Outer membrane permeabilization upon treatment of *K. pneumoniae* R3934 with VanNHdipi at 40 µM. Red arrow indicates compound addition.

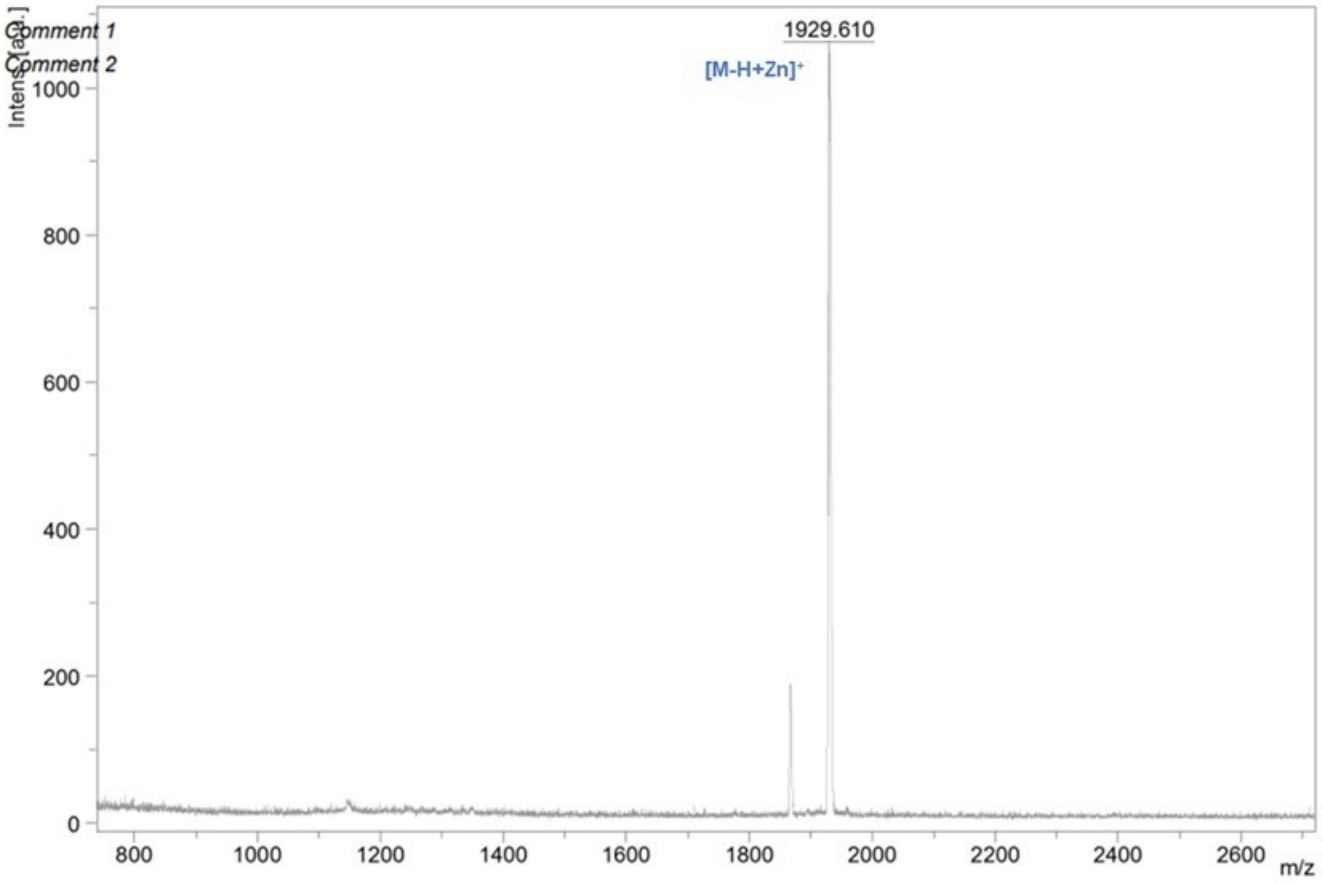


Figure S4. Mass spectrometry data indicating VanNHdipi-Zn^{2+} complex formation.

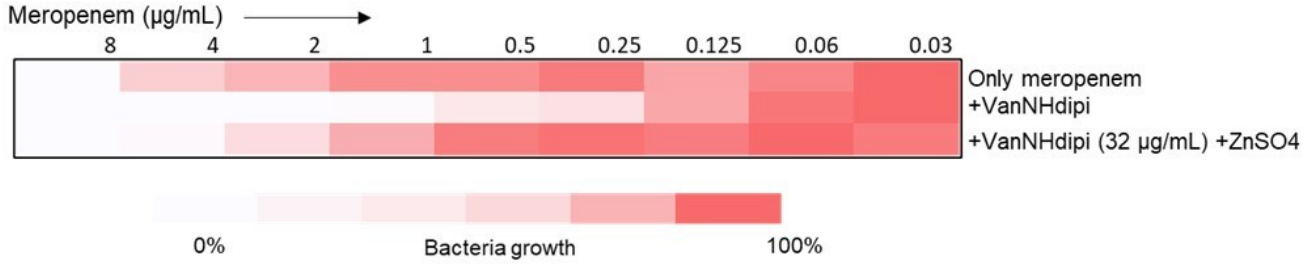


Figure S5. VanNHdipi loses its ability to resensitize *K. pneumoniae* BAA2146 to meropenem when pre-incubated with $ZnSO_4$ (16 µM).

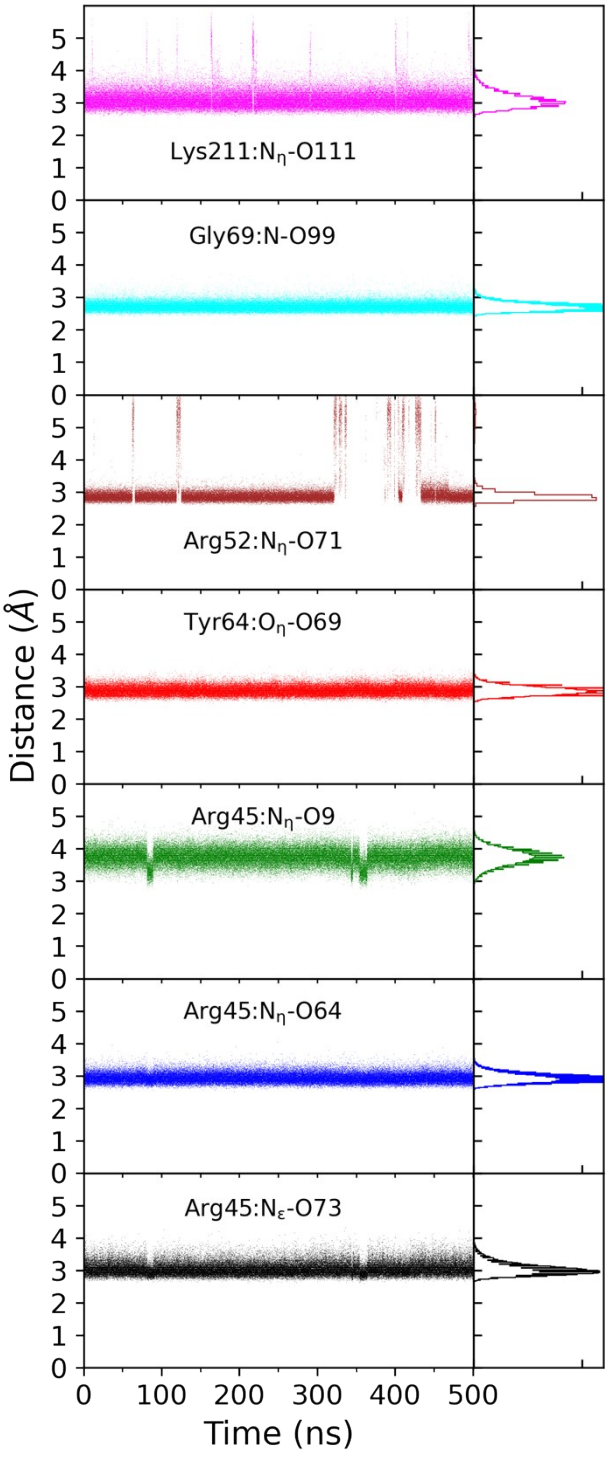


Figure S6. (Left) Time series and (Right) probability distribution (arb. units) plots of distances between the H-bond donor atom of Arg45, Tyr64, Arg52, Lys211, Gly69, and Lys211 residues and the corresponding H-bond acceptor atom of VanNHdipi during MD

simulation. These stable H-bonding interactions provide stability to the VanNHdipi on the surface of NDM-1.

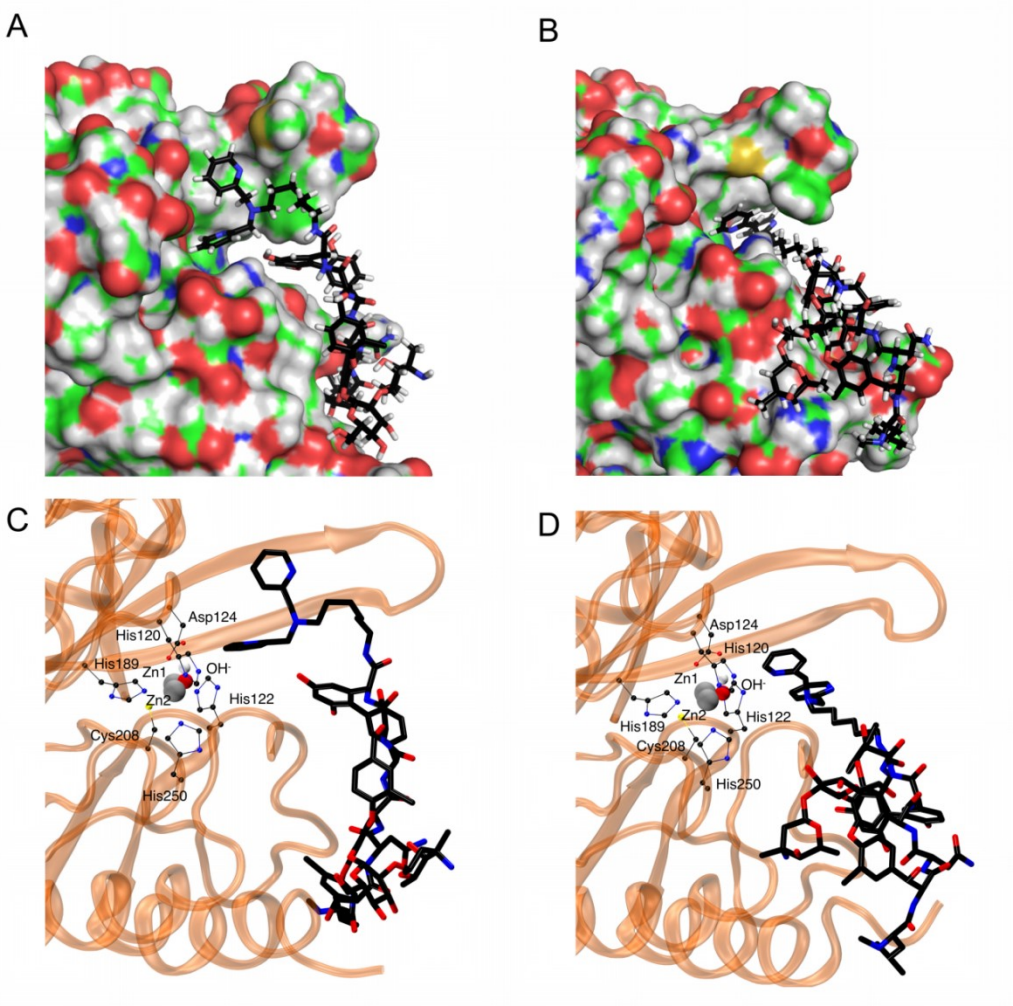


Figure S7. The surface representation of Dipi-Van (thick-stick representation) bound to NDM-1 is shown in (A) 250 ns and (B) 500 ns of MD simulation. Cartoon representation Dipi-Van bound to NDM-1 (in orange) is shown in (C) 250 ns and (D) 500 ns of MD simulation. The vancomycin group of Dipi-Van is not stable and changes its orientation on the surface of NDM-1. Hydrogen atoms of Dipi-Van are not shown for clarity. The important residues of the NDM active site are shown in CPK representations. Color code: C (black), O (red), N (blue), Zn (silver).

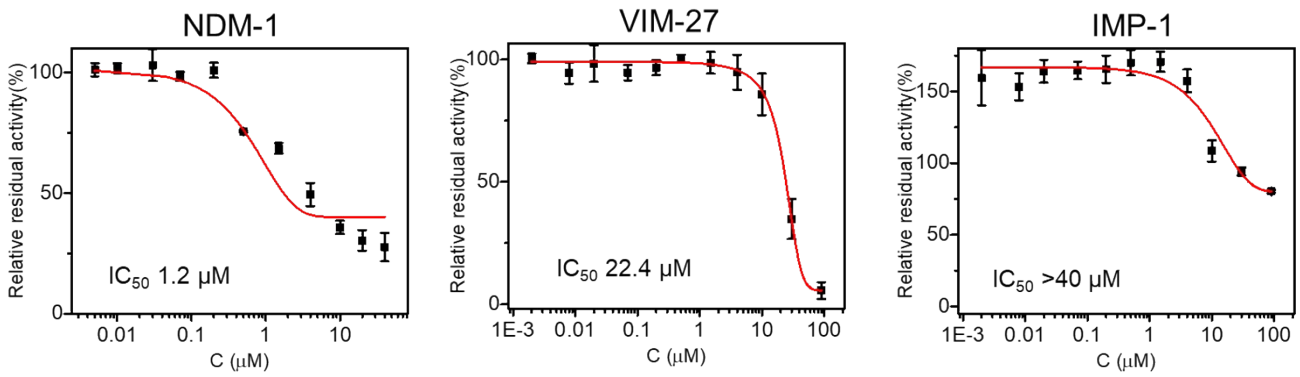


Figure S8. Inhibition of MBL enzyme activity by BPYA were determined using the absorption assay (nitrocefin).

Experimental section

Materials and Methods

All chemicals were purchased from Sigma-Aldrich, Spectrochem and SD Fine and used without further purification. Vancomycin was purchased from ChemImpex Pvt. Ltd. USA. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} (250 µm thickness). Visualization of TLC was done using UV light, ninhydrin and Iodine. Column chromatography was performed on neutral alumina gel (60-120 Å pore size). Final vancomycin derivatives were purified by reverse phase HPLC using 0.1 % trifluoroacetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase to more than 95 % purity. HPLC analysis was performed on a Shimadzu-LC 8Å Liquid Chromatography instrument (C_{18} column, 10 mm diameter, 250 mm length) with UV detector monitoring at 254 nm and 270 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. High resolution mass spectra (HR-MS) were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument. Staphylococcal strains were grown in nutrient broth, Enterococcal strains were grown in brain heart infusion broth, *Bacillus subtilis* was grown in chemically defined Belitzki minimal medium (10). Eppendorf 5810R centrifuge was used. TECAN (Infinite series, M200 pro) Plate Reader was used to measure absorbance and fluorescence of 96-well plate. Human RBCs were isolated from fresh blood and used for hemolytic assay. HEK cell line was purchased from ATCC.

Bacterial Strains: Clinical isolate *K. pneumoniae* R3934 was obtained from the Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences (NIMHANS), Hosur Road, Bangalore 560029, India. Bacterial identification was performed by the Vitek 2 Compact 60 system, bioMerieux, France, and Gram-negative bacteria were screened for carbapenem resistance using Kirby-Bauer disc diffusion method (data not shown). Methicillin-resistant *S. aureus* (MRSA) ATCC 33591, vancomycin-resistant *E. faecium* (VRE, VanA) ATCC 51559, vancomycin-resistant *E. faecalis* (VRE VanB) ATCC 51575, *K. pneumoniae* BAA2146 were obtained from the American Type Culture Collection (ATCC). Clinical isolate KP EN 5141, EN 5136 were obtained from Prof. Sulagna Basu, NICED, Kolkata, India. VRE903 and VRE 909 were obtained as a gift from Dr. Sidharth Chopra, CDRI, Lucknow. *B. subtilis* 168 (trpC2) was obtained as a gift from Prof. Dr. Leendert Hamoen, University of Amsterdam.

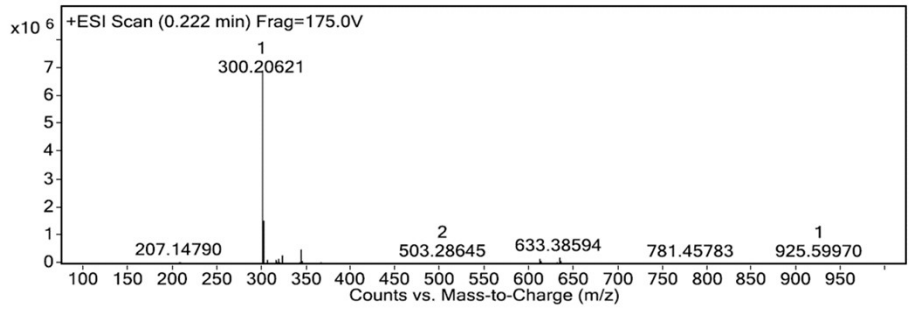
Animals: The animal experiments were performed following appropriate protocols as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi and approved by the Institutional Animal Ethics Committee (IAEC) of JNCASR (201/Go/ReBi/S/2000/CPCSEA).

Synthesis and characterization

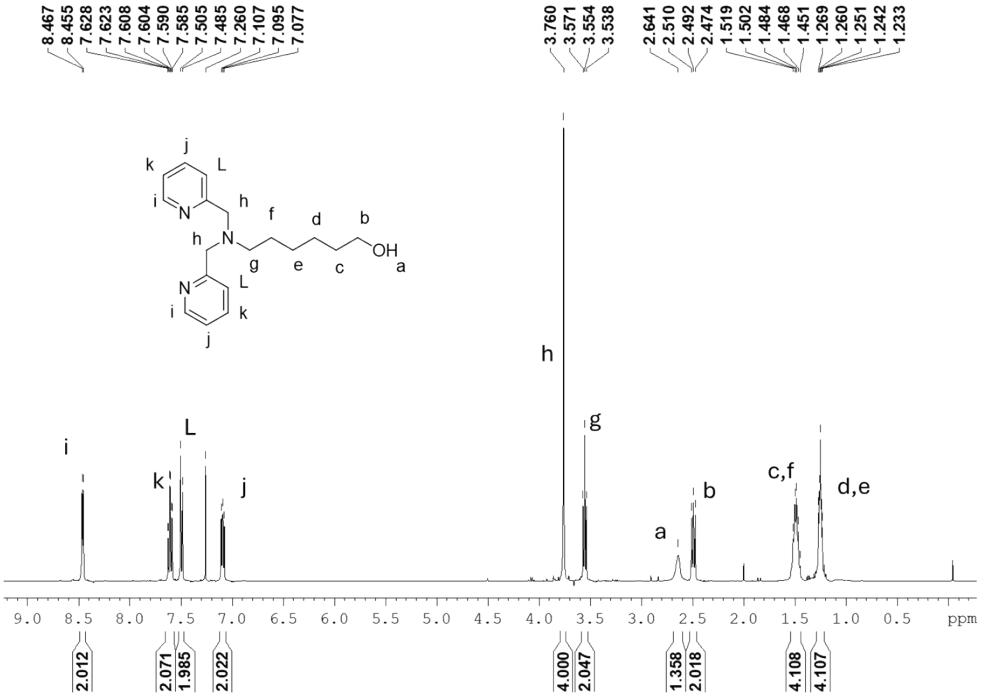
Synthesis of *N,N*-Dipicolyl hexanol (1a): To a solution of 6-amino-1-hexanol (0.5 g, 1 equiv) and DIPEA (5.0 equiv) in DCM (5 mL) and DMF (2mL) mixture, 2-(chloromethyl) pyridine hydrochloride (2.2 equiv) was added. The reaction mixture was allowed to stir for 72 h, following which it was diluted with DCM (30 mL) and the solution was washed sequentially with water (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. Purification using flash column chromatography alumina a stationary phase (gradient: 2% - 5% methanol/chloroform) afforded *N,N*-Dipicolyl hexanol (**2a**) as a pale yellow liquid with a yield of 75%.

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 8.47- 8.45 (m, 2H; H_{Ar}), 7.63- 7.59 (m, 2H; H_{Ar}), 7.49 (d, J = 7.6 Hz, 2H; H_{Ar}), 7.11-7.08 (m, 2H; H_{Ar}), 3.76 (s, 4H; NC$H_{2(Pyr)}$), 3.55 (t, J = 6.4 Hz, 2H; OCH_2), 2.64 (1H; OH), 2.49 (t, J = 7.6 Hz, 2H; NCH_2), 1.52- 1.45 (m, 4H; CH_2), 1.27- 1.23 (m, 4H; CH_2); HR-MS (ESI^+) *m/z* Observed: 300.2062 $(M+H)^+$, Calculated for $C_{18}H_{26}N_3O^+$: 328.2047 $(M+H)^+$

HRMS

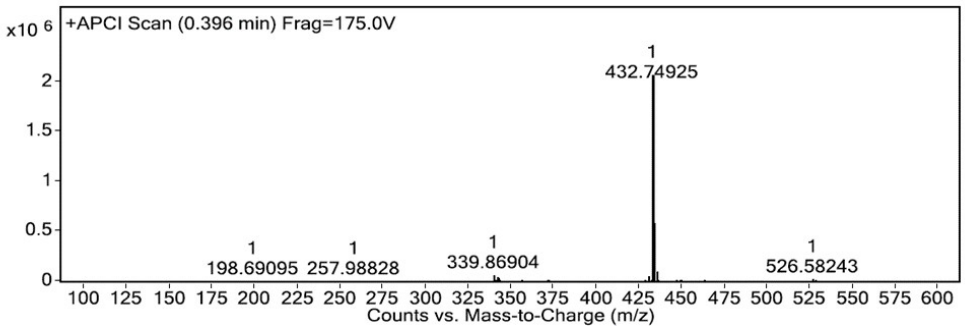


[1]HNMR



Synthesis of *N,N*-Dipicolyl aldehyde (1b)*:* 4-formyl benzoic acid (0.5 g, 1 equiv) and DMAP (0.026 g, 0.21 mmol, 0.25 equiv) were dissolved in DCM (5 mL) and DMF (2mL). EDC hydrochloride (0.156 g, 1.0 mmol, 1.2 equiv) was then added to the reaction mixture in an ice bath. *N*,*N*-dipicolyl hexanol (**2a**, 0.25 g, 0.84 mmol, 1.0 equiv) was added and the reaction mixture was allowed to stir for 24 h. Following this, organic solvent was removed in vacuo and diluted with ethyl acetate (30 mL) and the solution was washed sequentially with water (3 × 20 mL). The organic layer was dried over anhydrous sodium sulfate, filtered and concentrated in vacuo. Purification using flash column chromatography using alumina a stationary phase (gradient: 20% - 30% ethyl acetate/hexane) to obtain *N,N*-Dipicolyl-aldehyde (**2b**) as a pale yellow sticky liquid with yield of 70%.

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 10.08 (s, 1H; C*H*O), 8.49 (d, *J* = 4.4 Hz, 2H; H_{Ar}), 8.16 (d, *J* = 8.4 Hz, 2H; H_{Ar}), 7.93 (d, *J* = 8.4 Hz, 2H; H_{Ar}), 7.65-7.61 (m, 2H; H_{Ar}), 7.51 (d, *J* = 7.6 Hz, 2H; H_{Ar}), 7.12 (t, *J* = 6.4 Hz, 2H; H_{Ar}), 4.29 (t, *J* = 6.4 Hz, 2H; OCH_2), 3.80 (s, 4H; N$CH_{2(Pyr)}$), 2.54 (t, *J* = 7.2 Hz, 2H; NCH_2), 1.76- 1.69 (m, 2H; CH_2), 1.59- 1.52 (m, 2H; CH_2), 1.37- 1.32 (m, 4H; CH_2); **^{13}C-NMR** (100 MHz, $CDCl_3$) δ 192.8, 170.2, 164.9, 162.2, 159.3, 148.6, 139.1, 136.3, 134.5, 129.7, 129.6, 122.5, 122.0, 65.1, 59.7, 59.7, 53.4, 35.7, 30.7, 28.0, 26.3, 26.2, 25.2, 20.7, 14.0. **HRMS** *m/z:* Observed: 432.74925, Calculated for $C_{26}H_{30}N_3O_3^+$: 432.5435 $(M+H)^{+\cdot}$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1
432.74925
1
198.69095
1
257.98828
1
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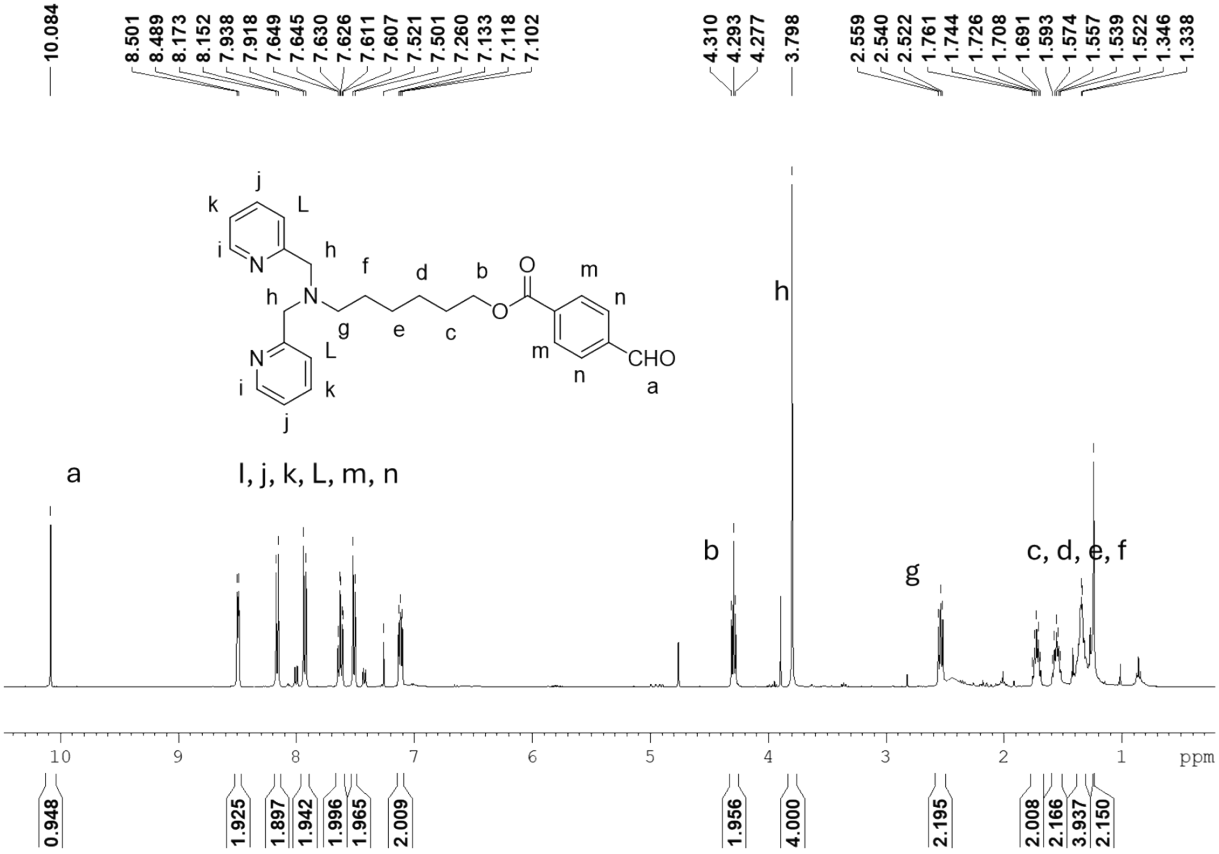
1
526.58243
Counts vs. Mass-to-Charge (m/z)

^{1}H-NMR


10.084
8.501
8.489
8.173
8.152
7.938
7.918
7.649
7.645
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7.521
7.501
7.260
7.133
7.118
7.102
4.310
4.293
4.277
3.798
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2.540
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1.761
1.744
1.726
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1.557
1.539
1.522
1.346
1.338
a
l, j, k, L, m, n
b
h
g
c, d, e, f
ppm
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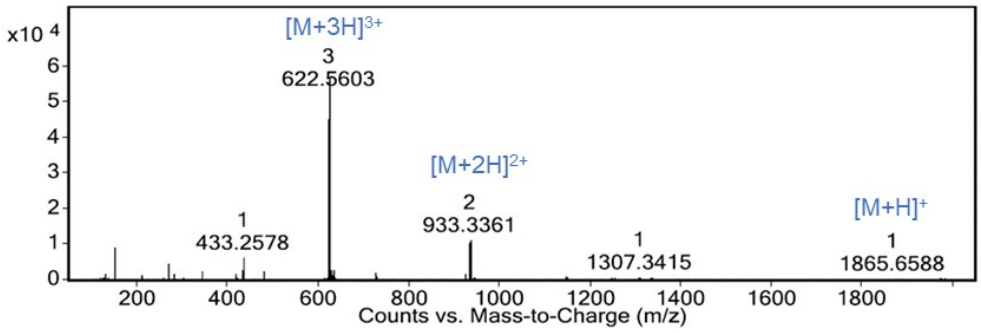
1.925
1.897
1.942
1.996
1.965
2.009
1.956
4.000
2.195
2.008
2.166
3.937
2.150

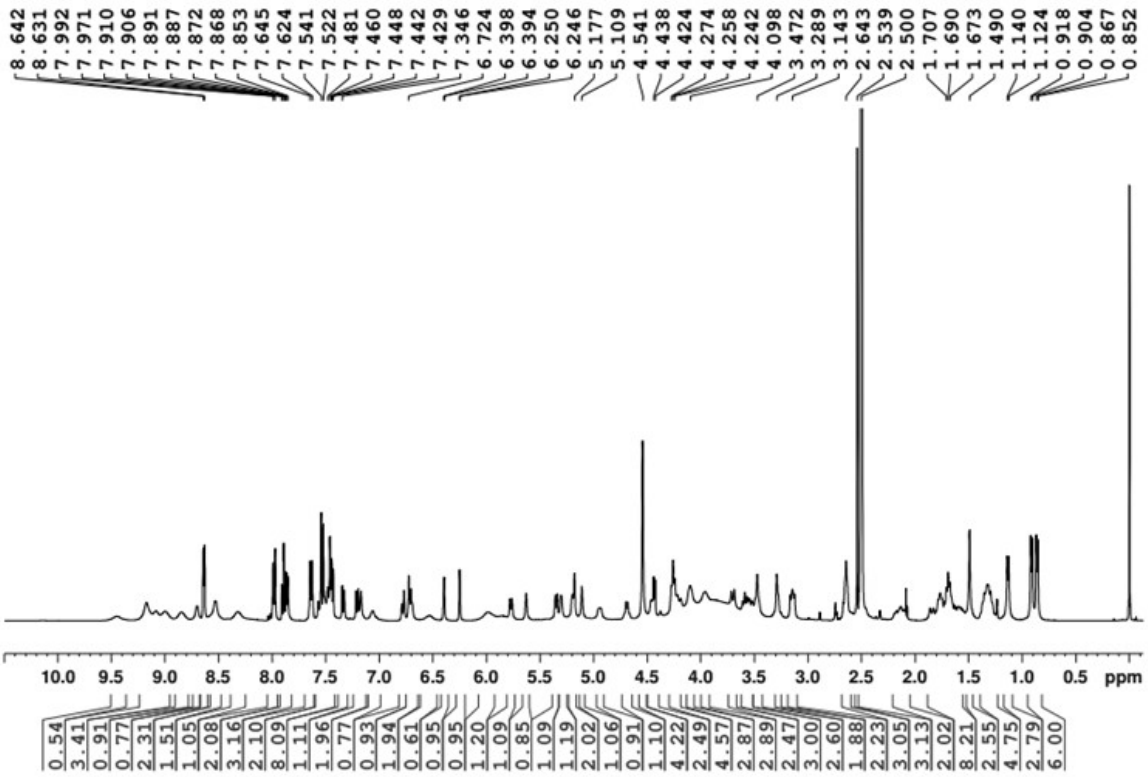
Synthesis of VanNHdipi (2): Vancomycin (50 mg, 1 equiv) was dissolved in a 1:1 solution of DMF and DMSO (1 mL each). DIPEA (5.0 equiv) and *N*,*N*-Dipicolyl aldehyde (**2b**) were then added (1.2 equiv) keeping reaction in an oil bath. Reaction was continued in 50 °C for 2 hours. After cooling Sodium cyanoborohydride $NaCNBH_3$ (2.0 equiv) and MeOH (2 mL) were added and the reaction mixture was allowed to stir in 50 °C for 2 hours and then stir in RT for 20 hours. Purification using rp-HPLC was done (gradient: 5% - 95% acetonitrile/water, 20 minutes). Then product was then lyophilized to obtain a white fluffy powder of trifluoroactate salt of VanNHdipi with a yield of 35-45%.

^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 9.24-8.96 (m, 3H), 8.84 (s, 1H), 8.69 (s, 2H), 8.63 (d, 1H), 8.53 (d, 2H), 8.32 (s, 1H), 8.04-7.96 (m, 2H), 7.93-7.84(m, 3H), 7.68-7.61 (d, 2H), 7.59-7.42 (m, 8H), 7.36-7.31 (d, 2H), 7.2 (t, 2H), 7.06 (s,1H), 6.79 (s, 1H), 6.79 (s, 1H), 6.7 (d, 2H),6.53 (s, 1H), 6.4 (d, 1H), 6.25 (d, 1H), 5.98 (s, 1H), 5.76 (d, 1H), 5.63 (s, 1H), 5.35 (d, 1H), 5.3 (s, 1H), 5.18 (d, 2H), 4.94 (s, 1H), 4.69 (d, 1H), 4.54 (s, 4H), 4.44 (d, 2H), 4.29(m, 6H), 4.1 (s, 3H), 3.96 (s, 2H), 3.62-3.52 (m, 3H),3.47 (s, 2H), 3.29 (s, 2H), 3.14 (t, 2H), 2.64 (s, 3H), 2.54 (s, 3H), 2.2-2.06 (m, 2H), 1.88-1.55 (m, 6H), 1.49 (s, 3H), 1.39-1.23 (m, 5H), 1.14 (d, 3H), 0.94-0.84 (q, 6H). **HRMS** *m/z:* Observed: 1865.6588 $(M+H)^+$, 933.3361 $(M+2H)^{2+}$, 622.5603 $(M+3H)^{3+}$; Calculated for $C_{92}H_{105}Cl_2N_{12}O_{26}^+$: 1865.6606 $(M+H)^+$, 933.3339 $(M+2H)^{2+}$, 622.5575 $(M+3H)^{3+}$

HRMS



^{1}H-NMR



Biological assays

Minimum Inhibitory Concentration (MIC) determination[1]

Test compound was assayed in a micro-dilution broth format as per the CLSI guideline. The bacterial stock samples were stored at -80 ºC in 30 % glycerol. About 5 µL of these stocks were plated onto nutrient agar and grown for 24 h at 37 °C. Single colonies of bacteria were then picked and inoculated into 3 mL of the respective culture broths and the culture was grown for 6 h at 37 °C before the experiments. This 6 h mid-log phase culture was diluted to give an effective cell concentration of ~5×10^5 CFU/mL in Muller Hinton medium, which was then used for the MIC assay. Compounds were respectively serially diluted by 2-fold in sterile millipore water. 20 µL of these serial dilutions were then added into the wells of a 96-well plate followed by the addition of 180 µL of bacterial suspension. The plates were then incubated for 18-24 h at 37 ºC. The OD value at 600 nm was recorded using a TECAN (Infinite series, M200 pro) Plate Reader. One row containing only serial dilutions of compound and culture medium was kept as negative control; three wells containing only vehicle control (water) and bacteria were kept as positive growth

control. Each concentration was tested in triplicate and the whole experiment was done at least twice. The MIC value was determined by identifying the well with the lowest antibiotic concentration with a recorded OD value comparable to the media control well.

Hemolysis assay

A standardised protocol as previously published was followed.[2] VanNHdipi was serially diluted 2-fold with varying final plate concentrations of 500 μM to 2 μM. 50 μL of the serial dilutions of the compound were added into 96-well microtiter plates. To obtain human erythrocytes, freshly drawn heparinised human blood was centrifuged down at 3000 rpm for 5 mins and re-suspended to 5 vol % in PBS (pH 7.4). 150 μL of the erythrocyte suspension were added to the serially diluted compounds (from 500 μM to 2 μM). One set without compound and another with 50 μL of 1 vol % solution of Triton X-100 were kept as controls. The plates were incubated at 37 °C for 1 h followed by centrifugation at 3500 rpm for 5 min. 100 μL of the supernatant from each well were transferred into fresh microtiter plates, and A_{540} was measured.

$\%\ hemolysis = \frac{A - A0}{A(total) - A0} \times 100$; A is the absorbance of the test well, A0, the absorbance of the negative controls (without compound), and A(total) the absorbance of 100% hemolysis wells (with Triton X-100), all at 540 nm.

Cytotoxicity assay (Alamar blue assay)

The standard protocol as mentioned in the kit was followed. Briefly, 2 × 10^4 HEK 293 cells were seeded per well in 100 μL of DMEM media in a 96-well plate and incubated for 24 h. Cells were treated at various concentrations (serially diluted 2-fold) of VanNHdipi and incubated for 24 h after which 10 μL of 10× Alamar blue dye was added. 2 h post incubation with dye, the absorbance was measured at 570 nm using 600 nm as reference wavelength.

Time-kill kinetics assay[3]

VanNHdipi and vancomycin was added to the bacterial solution (~1.8×10^5 CFU/mL) at the concentration corresponding to MIC, 2x MIC, 4X MIC (MIC of vancomycin = 0.6 μM and MIC of VanNHdipi = 0.8 μM). The inoculum was incubated at 37°C. Bacterial titre was determined at different time intervals of 0, 6, 12 and 24 h.

Antagonization assay

Antagonization of antibacterial activity of VanNHdipi was examined by addition of 500 μM and 250 μM of *N*,*N*′-diacetyl–L-Lys–D-Ala–D-Ala to serial dilutions of the test compound and pre-incubating for 10 minutes in a 96-well plate. A bacterial suspension of 5×10^5 CFU/mL was then added to make a final volume of 200 μL. The MIC was then determined against MRSA by measuring the OD_{600} 18-24 h post-incubation.

To test the loss of potentiation of meropenem against *K. pneumoniae* (strain ATCC BAA2146), VanNHdipi (32 μg/mL) was pre-incubated with 16 μM $ZnSO_4$ solution for 10 minutes and then subjected to MIC determination in combination with serial dilutions of meropenem (starting concentration of 8 μg/mL).

Activity against mature biofilms of MRSA

Biofilms were grown as per previously reported protocol.[4] A mid log phase culture of MRSA ATCC 33591 was diluted to a concentration of approximately 10^5 CFU/mL in a nutrient broth supplemented with 1% w/v glucose and 1% w/v NaCl to make the bacterial stock solution. Sterile glass cover slips of 18 mm diameter were placed in 6-well plates individually. Biofilms of MRSA were then allowed to form on the glass cover-slip by incubating the bacterial solution at 37 C for 24 h. The cover slips containing mature biofilms were removed and washed with 0.9% saline to remove planktonic bacteria. The biofilm containing cover-slips were then taken into a fresh well in a 6-well plate and treated with 2 mL complete nutrient media containing the test compounds (vancomycin, VanNHdipi) at the concentration of 20 μM each. 24 h post-treatment with the test compounds at the mentioned concentrations, the glass cover slips were carefully removed from the well, washed with 0.9 % saline. The biofilms were then stained with 10 μL of SYTO9 (60μM) and imaged using a Zeiss 510 Meta confocal laser-scanning microscope. The orthogonal projections of the images were processed with LSM 5 Image examiner. The images are representative of two independent experiments.

The % reduction in biofilm thickness was calculated as:

$$\%\ change\ in\ thickness = \frac{Thickness(control)\ -\ Thickness(sample)}{Thickness(control)}$$

Determination of Physiologically Effective Concentrations

Cultures of *B. subtilis* 168 were grown overnight in 50 mL flasks with 10 mL of Belitzki minimal medium. Log-phase bacteria (OD_{500} between 0.5 and 1) were used to inoculate 100 mL of fresh BMM in a 500 ml flask to an OD_{500} of 0.05. When the cultures reached an OD_{500} of 0.35, 5 ml aliquots were placed into separate flasks (50 mL) and compounds were added. The OD of the solutions were recorded every 30 minutes. The concentrations which retarded growth by approx. 50% were selected as the PEC. The experiments were performed two times independently and the graphs are representative of the results from the two experiments. The experiment was preformed together with the compound published in (*J. Med. Chem.* 64, **2021,** 10185) and therefore the graph for vancomycin is the same.

Proteome analysis

Bacillus subtilis 168 was cultured in Belitzky minimal medium (BMM) as reported previously [5]. Pulse-labeling with L-[35S]methionine and the 2D-PAGE-based proteome analysis were performed as described previously by Senges *et al*.[5] Briefly, for radioactive labeling with L-[^{35}S]methionine, 5 ml of the cultures at an OD_{500} of 0.35 were exposed to vancomycin at 0.4 µM or VanNHdipi at 0.16 µM for 10 min followed by addition of 1.8 MBq radioactive methionine (Hartmann Analytic, Braunschweig, Germany). Incorporation was stopped after 5 min by the addition of 1 mg/ml chloramphenicol and an excess of nonradioactive L-methionine and cooling of the cells on ice. The cells were then harvested and washed before disruption with the VialTweeter sonicator (Hielscher, Teltow, Germany). Cell debris was removed by centrifugation, and protein concentrations were estimated using the Roti NanoQuant (Roth, Karlsruhe, Germany). For the radioactive gels, 50 µg of protein (300 µg for nonradioactive gels) was loaded onto 24-cm immobilized pH gradient strips pH 4 to 7 (GE Healthcare, Chicago, USA) by passive rehydration for 18 h. Proteins were separated by isoelectric focusing in the first dimension using a Multiphore II electrophoresis system (GE Healthcare). After equilibration with first iodoacetamide and then dithiothreitol (DTT), in the second dimension, proteins were separated according to molecular size by SDS-PAGE using the Ettan DaltTwelve system (GE Healthcare). Radioactive gels were dried on Whatman paper and exposed to storage phosphor screens (GE Healthcare). Screens were scanned with a Typhoon Trio instrument (GE Healthcare) with a 633-nm excitation wavelength and a 390-nm emission filter. Nonradioactive gels were stained with 0.003% ruthenium (II)-tris(4,7)diphenyl-1,10-phenanthroline disulfonate and scanned on the Typhoon Trio instrument with excitation at 532 nm and a 610-nm emission filter. Image analysis was performed as described previously by Bandow *et al*. using Decodon Delta 2D 4.2.1

(Decodon, Greifswald, Germany). The signal intensities of protein spots were normalized to the total signal on the autoradiograph after background subtraction and set in relation to the synthesis rate in the respective control to obtain relative synthesis rates (regulation factors) (RF= relative signal intensity$_{\text{antibiotic treated}}$ /relative signal intensity$_{\text{untreated control}}$) for each individual protein spots. "Marker proteins," were designated using the relative synthesis rates and had to be at least 2 in each biological replicate, as well as the protein had to accumulate in sufficient amounts to allow protein identification from a preparative gel. Proteins were identified from preparative 2D gels after tryptic in-gel digestion by nano ultraperformance liquid chromatography coupled tandem mass spectrometry with electrospray ionization (nUPLC-ESI-MS/MS) a Synapt XS mass spectrometer (Waters) as described previously.[6, 7] Proteomic responses were compared to a library of responses and the similarity of responses was visualized in a tSNE plot as described previously.

Cell wall biosynthesis inhibition assay

Overnight cultures of *B. subtilis* 168 were inoculated in BMM and allowed to grow to an OD_{500} of 0.35. 200 µL of the bacterial culture were incubated with compounds at 37 ℃ for 15 minutes. Treatment of vancomycin and VanNHdipi was at their PECs of 0.4 µM and 0.16 µM respectively. Post-treatment, the cells were fixed with 1 mL of 1:3 acetate/methanol.[8] The morphology of the cells were then examined through microscopy. The images are representative of results from two independent experiments.

GFP-MinD localization

B. subtilis 1981 expressing GFP-MinD was cultured overnight in BMM.[9] Cells were then inoculated in xylose containing BMM instead of glucose to an OD_{500} of 0.1 to induce expression of the GFP-MinD fusion protein. Upon reaching an OD_{500} of 0.35, the cells were treated with test compounds at the PEC (treatment of vancomycin was at 0.4 µM while VanNHdipi was at 0.16 µM and 0.4 µM.) for 15 min. 0.5 µl of nonfixed, non-immobilized samples of the culture were imaged immediately in fluorescent mode (Olympus microscope with a U-LH100HGAPO burner and a U-RFL-T power supply). The images are representative of results from two independent experiments.

Membrane permeabilization assay

The 6 h grown culture (mid log phase) of MRSA were harvested (5000 rpm, 5 min), washed, and resuspended in 1:1 solution of 5 mM glucose and 5 mM HEPES buffer at pH 7.2. 10 µM of propidium iodide (PI) was added to the bacterial suspension and 200 µL of this mixture was put

into 96-well flat clear bottomed black well plates. 20 µL of test compounds, vancomycin and VanNHdipi were treated at final concentrations of 16 µM were added to the bacterial suspension. Fluorescence has monitored at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). As a measure of inner membrane permeabilization the uptake of PI was monitor by the increase in fluorescence for 30-50 min. The graph is representative of the results from two independent experiments.

Cytoplasmic membrane depolarization assay

Mid log phase MRSA were harvested (3500 rpm, 5 min), washed in 1:1 solution of 5 mM glucose and 5 mM HEPES buffer (pH 7.2). It was then resuspended in 1:1:1 solution of 5 mM HEPES buffer, 5 mM glucose and 100 mM KCl solution. The culture was then diluted to an OD_{600} of 0.2 and the depolarisation assays was performed as described. Then 2 µM of 3, 3′-Dipropylthiadicarbocyanine iodide (DiSC3(5)) was added to bacterial suspension and pre-incubated for 45 min. The fluorescence was monitored at excitation wavelength of 622 nm and emission wavelength of 670 nm. Then 20 µL of test compounds, VanNHdipi were treated at final concentrations of 28, 14, 7 µM and vancomycin at 45 µM were added 2-4 min after stabilization of DiSC3(5) fluorescence. Fluorescence was monitored for another 35 min to measure membrane depolarization. The graph is representative of results from two independent experiments done in duplicates.

Outer membrane permeabilization assay

K. pneumoniae R3934 was grown to mid-log phase, washed with a 1:1 solution of 5 mM HEPES and 5 mM glucose and resuspended (10^8 CFU/mL). To this solution N-phenylnaphthylamine dye was added to a final concentration of 5 µM and 200 µL of the mixture was put into flat clear bottomed black well plates. Fluorescence readings were taken for 4 minutes before the addition of compounds. 20 µL of test compounds (vancomycin, VanNHdipi added to a final concentration of 40 µM) were added to the bacterial suspension at the required concentration. CTAB was used as the positive control (data not shown). After addition, the fluorescence intensity (excitation wavelength: 350 nm; emission wavelength: 420 nm) was measured for 10 minutes. The experiment was performed in triplicate. Normalized fluorescence intensity was calculated as Fl (sample) / Fluorescence (control)*100.

In-vivo activity in murine thigh infection model

Groups of four 6 to 8 week-old Balb/c specific-pathogen-free female mice were used (weight ~24 g) for the experiment. The mice were rendered neutropenic by injecting two intraperitoneal doses

of cyclophosphamide, 4 days (150 mg kg^{-1}) and 1 day (100 mg kg^{-1}) before the infection experiment. 50 µL of ~10^6 CFU/mL bacterial inoculum (MRSA ATCC33591) was injected into the thigh. Mice were divided into three groups. 1 h post-infection, animals were treated intraperitoneally twice with 12 h intervals with saline, vancomycin (12 mg kg^{-1}), and VanNHdipi (12 mg kg^{-1}). 24 h post the first treatment, the animals were euthanized (using ether) and the thighs were collected aseptically. The thigh tissue was weighed and homogenized. The dilutions of the homogenate were plated onto agar plates, which were incubated overnight at about 37 °C. The bacterial titer was expressed as Log CFU/g of thigh weight and plotted in GraphPad Prism software.

Checkerboard assay

Microbroth dilutions were performed according to CLSI guidelines. Working stocks of test compounds corresponding to 10 × required plate concentrations were made and serially diluted by 2-fold. 20 µL of each of the dilutions of carbapenems (meropenem, doripenem) were added along the columns in a 96 well microplate while VanNHdipi was added along the rows in a 10×10 format. 160 µL of the mid-log phase bacterial culture was then added to the wells and incubated for 18 h, after which the OD of the wells at 600 nm was recorded. One row was kept growth control containing only 40 µL water and 160 µL bacterial culture.

MBL inhibition assay: Nitrocefin assay

The experiment was performed in collaboration with Prof. Xie, ECUST, China.[10, 11] 25 µL β-lactamase (8 nM of NDM-1, 3.2 nM for VIM-27, 6.4 nM for IMP-1 and L1) was mixed with 25 µL of serial dilutions of the compounds in HEPES buffer (0.1%triton, pH ~ 7.2) for 10 minutes at room temperature. Then, 50 µL of substrate (Nitrocefin, 40 µM) was added to the mixture. Assays were performed in 96-well microplate format, and the absorbance was recorded at 490 nm by SpectraMax I3 (Molecular Devices) at 37 °C. IC_{50} (Concentration that reduces the activity of enzyme by 50%) was calculated from a plot of percent loss of activity versus inhibitor concentration.

MBL inhibition assay: Fluorescence assay

The experiment was performed in collaboration with Prof. Xie, ECUST, China.[10, 11] Briefly, 25 µL of four times the concentration of β-lactamase (1 nM of NDM-1, NDM-12, 0.5 nM for VIM-1,VIM-27, IMP-1, CphA, L1) was mixed with 25 µL serial dilutions of the compounds in HEPES buffer (0.1%triton, pH ~ 7.2) for 10 min at room temperature. Then, 50 µL substrate (CDC-1 or CPC-1, 20 µM) was added. Assays were performed in 96-well microplate format, and the absorbance was

recorded at excitation wavelength 365 nm and emission wavelength of 460 nm by SpectraMax I3 (Molecular Devices) at 37 °C. IC50 was calculated from a plot of percent loss of activity versus inhibitor concentration.

Molecular Docking and Molecular Dynamic Simulation studies

We took the initial coordinates of the NDM protein from the meropenem bound structure using PDBID (4EYL).[12] We used the AutoDock package[13] with AutoDock4Zn force field parameters for Zn ions to dock the Vandipi and VanNHdipi molecule in the catalytic site of NDM.[14] We further performed molecular dynamics (MD) simulations of NDM-Vandipi and NDM-VanNHdipi docked complexes. We treated the protein molecule using AMBER99SB-ILDN force field[15] and Dipi-Van as well as VanNHdipi were treated using the general AMBER force field (GAFF).[16] Charges for ligand atoms were generated using RED after initial geometry optimization using Gaussian 09. The NDM active site histidine protonation states, Zn, and hydroxyl group charges were taken as reported in earlier studies.[17, 18] The two Zn metal ions in the catalytic site of NDM are bridged by a hydroxyl, and the Zn1 shows coordination with His120, His122, and His189 residues, whereas the Zn2 shows coordination with the Asp124, Cys208, and His250 residues.

We performed MD simulation using GROMACS package.[19] Initially, we equilibrated the system in the NPT ensemble (at 1 bar and 300 K) and then performed MD simulation in the NVT (at 300 K) ensemble for 500 ns.

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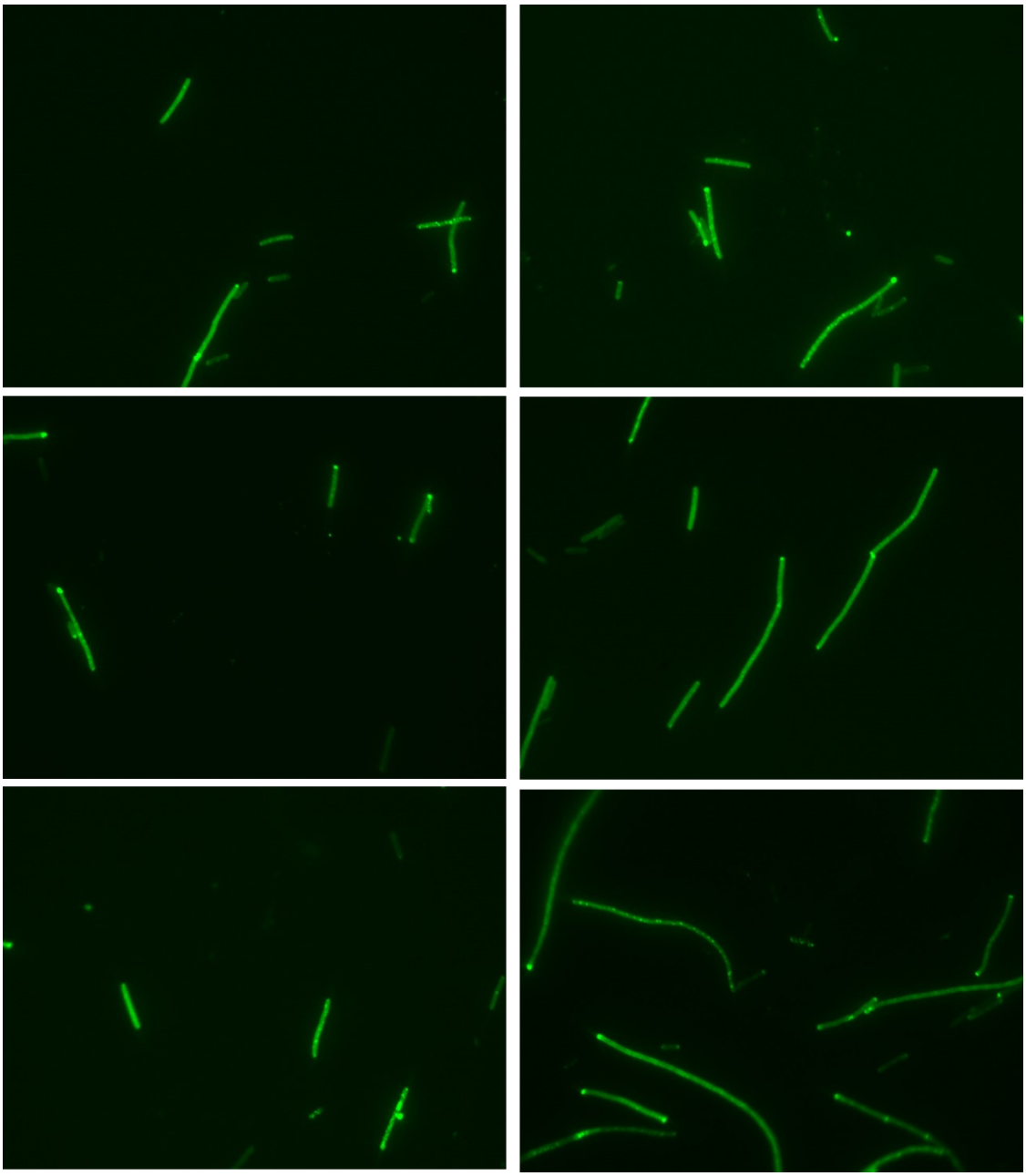
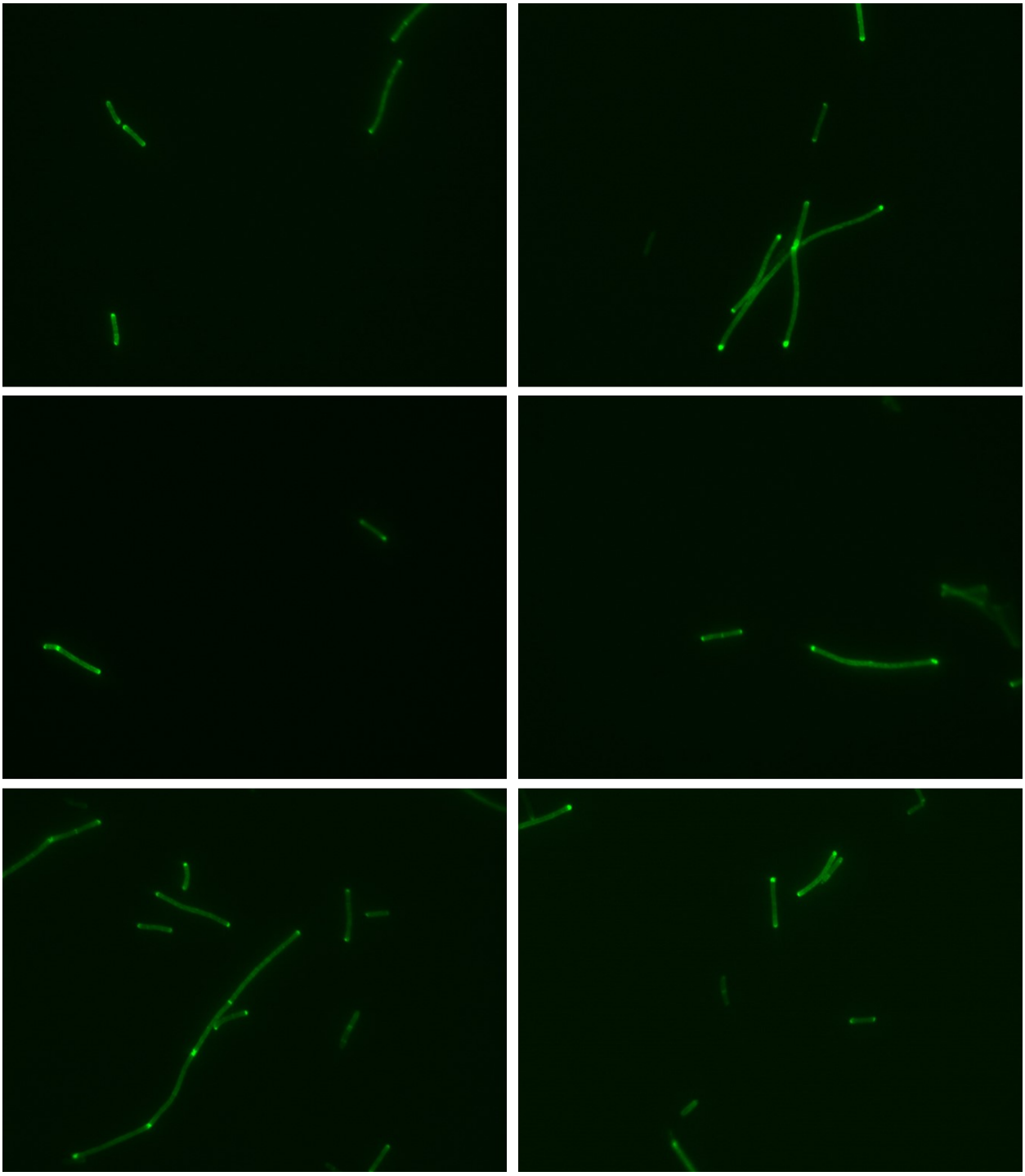
Supplementary Datasets

Supplementary data S1: Microscopy images of *B. subtilis* expressing GFP::minD post-treatment with compounds and when left untreated.

i) Microscopy images of *B. subtilis* expressing GFP-MinD post-treatment with VanNHdipi at 0.4 μM. A total of 38 cells were counted and observed, of which 6 cells were found to be depolarized.

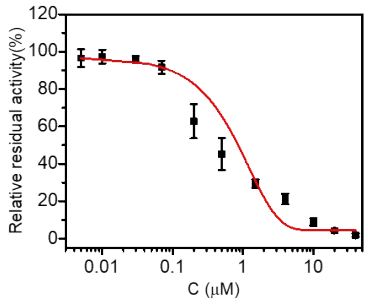
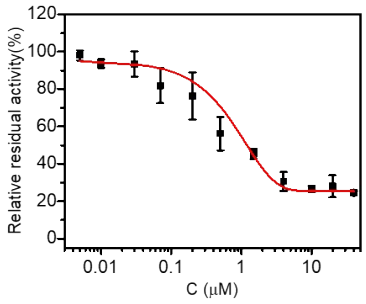


ii) Microscopy images of *B. subtilis* expressing GFP-MinD that were treated with water (Control). MinD can be observed at the poles of the bacterial cells.

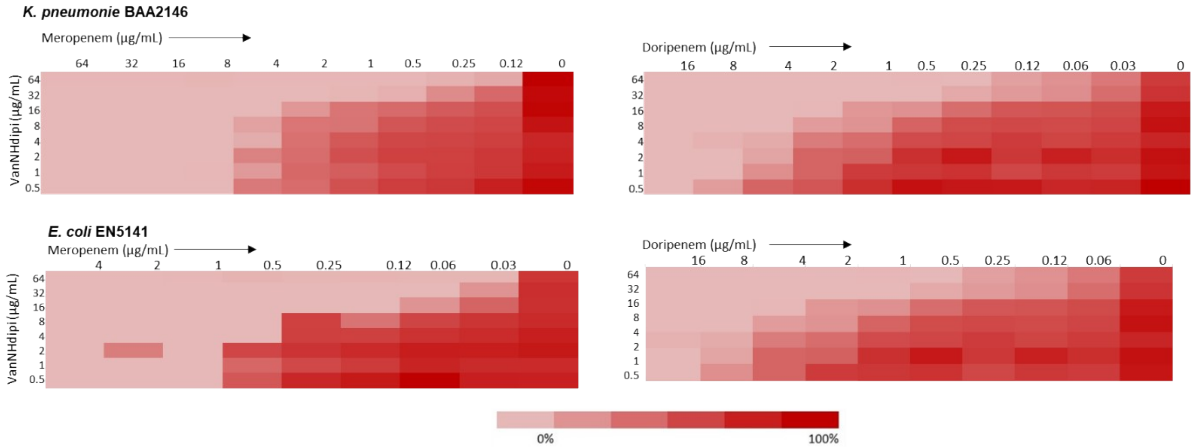


Supplementary data S2. Checkerboard assay to assess the effect of the combination of

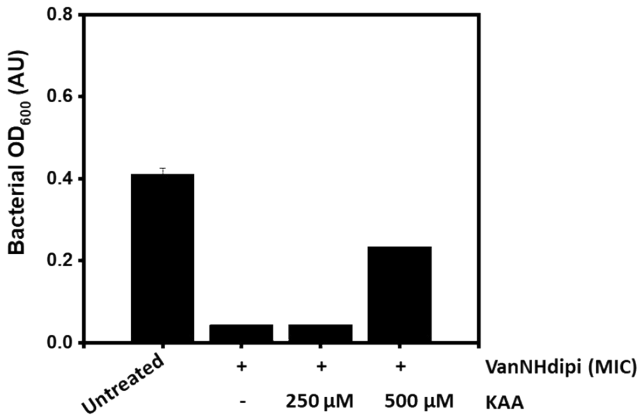
Supplementary data S4. Curves for the inhibition of MBL enzyme activity determined using the absorption method.

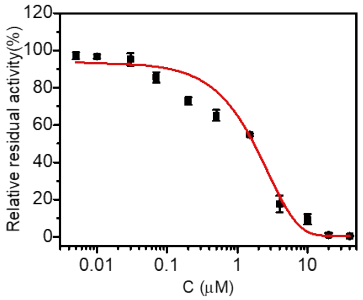
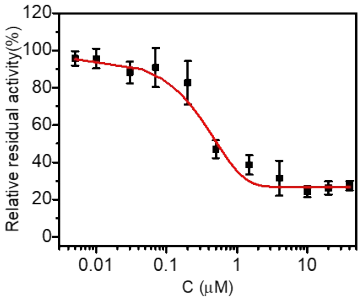
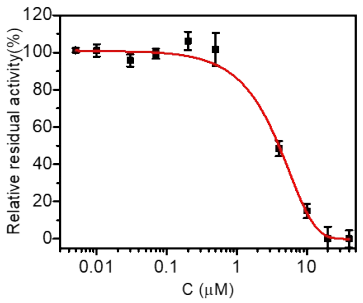
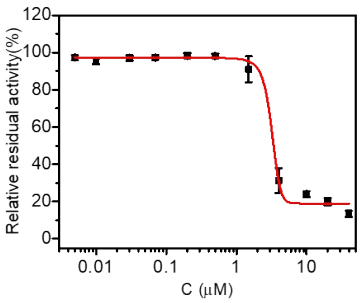
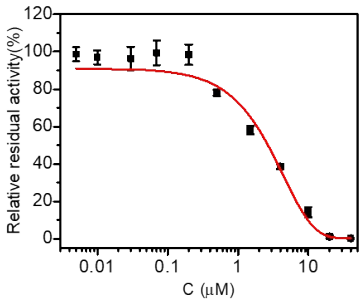
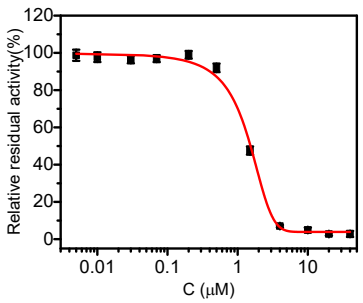
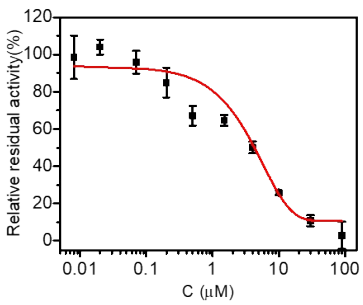
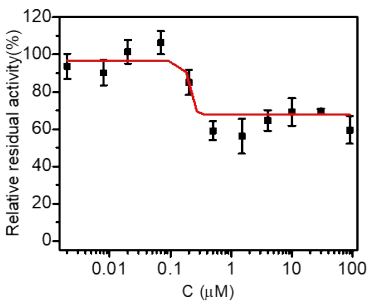
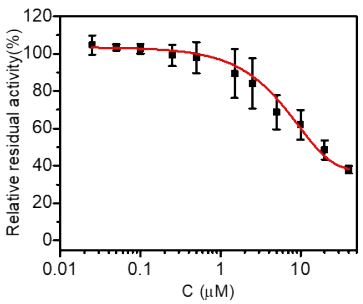
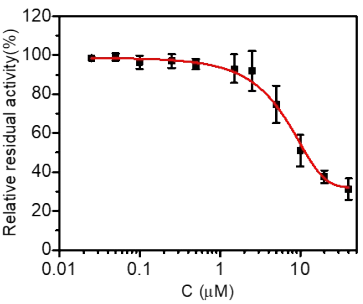
MBL	VanNHdipi	Dipi-Van
NDM-1 (Class B1)		

VanNHDipi (**2**) with meropenem and doripenem against NDM-1 positive (C and D) *K. pneumoniae* BAA2146 and *E. coli* EN5141.

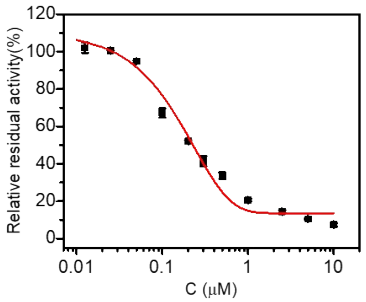
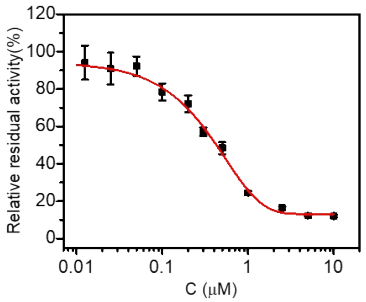
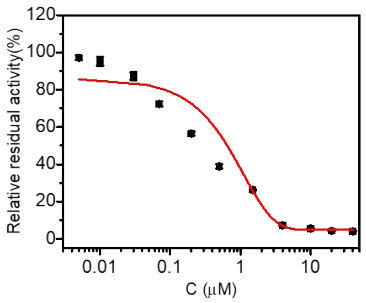
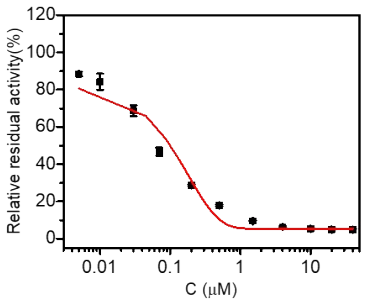
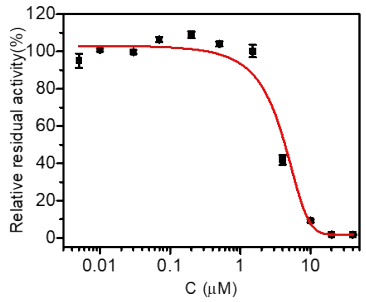
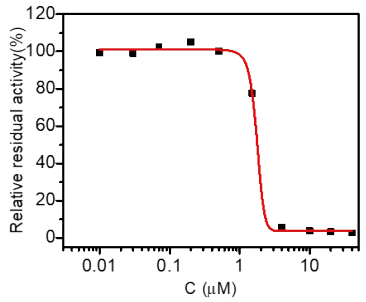
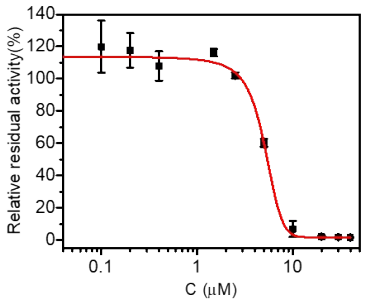
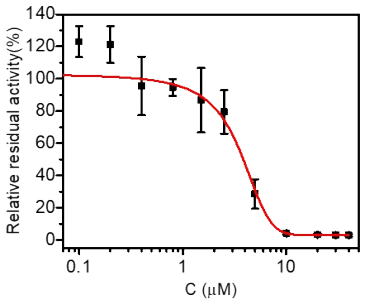


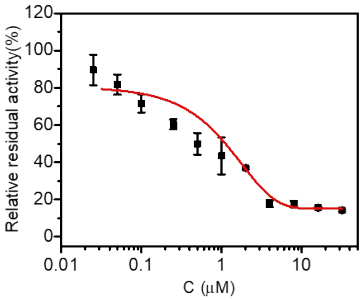
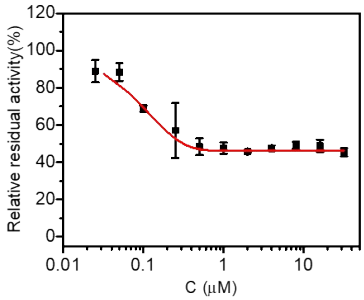
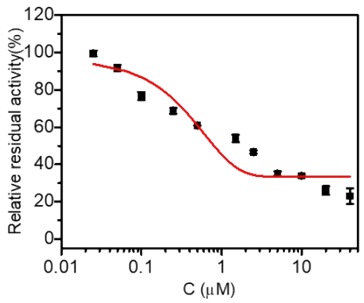
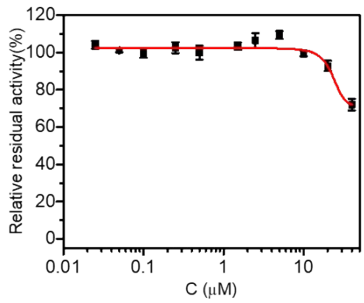
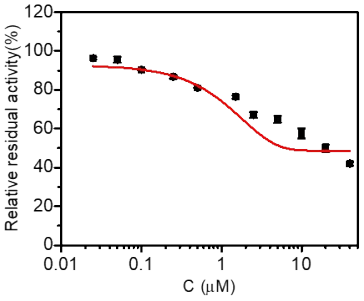
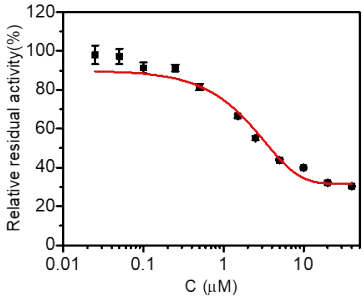
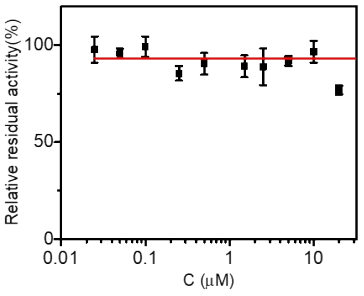
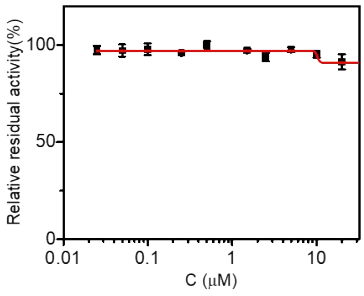
Supplementary data S3. OD_{600} of MRSA cultures post-treatment with VanNHDipi (at MIC) in the presence and absence of competing ligand KAA



NDM-12 (Class B1)		
VIM-1 (Class B1)		
VIM-27 (Class B1)		
IMP-1 (Class B1)		
L1 (Class B3)		

Supplementary data S5. Curves for the inhibition of MBL enzyme activity determined using fluorescence method

MBL	VanNHdipi	Dipi-Van
NDM-1 (Class B1)		
NDM-12 (Class B1)		
VIM-1 (Class B1)		
VIM-27 (Class B1)		

IMP-1 (Class B1)		
CphA (Class B2)		
L1 (Class B3)		
KPC-2 (Class A)		
AmpC (Class C)