SUPPORTING INFORMATION

A Vancomycin-Arginine Conjugate Inhibits Growth of Carbapenem-resistant *E. coli* and Targets Cell-Wall Synthesis

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SUPPORTING METHODS

I. Synthesis and Characterization of Vancomycin Conjugates

General information

All reactions were conducted in oven- or flame-dried glassware under an argon atmosphere unless otherwise noted. Reactions were concentrated under reduced pressure with a rotary evaporator or a lyophilizer unless otherwise noted. Commercial reagents were used as received without any further purification unless otherwise noted. For all reactions, dry solvents from Acros Organics were used. NMR spectra were acquired on a Varian INOVA 600, Varian INOVA 500, or Varian 400 magnetic resonance spectrometer. ^{1}H chemical shifts are reported relative to the residual solvent peak (DMSO = 2.50 ppm, H_2O = 4.79 ppm) as follows: chemical shift (δ), multiplicity (br. = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, or combinations thereof), integration, coupling constant(s) in Hz. High-resolution mass spectra (HRMS) were acquired at the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford.

Representative procedure for syntheses of vancomycin-amino acid conjugates

The synthesis was adapted from a literature procedure.[31] Vancomycin hydrochloride (20 mg, 13.4 µmol) was dissolved in 0.5 mL dimethylsulfoxide (DMSO) and 0.5 mL dimethylformamide (DMF). The corresponding amide-protected amino acid (26.8 µmol, 2.0 equiv) was added and the mixture was cooled to 0°C. Then diisopropylethylamine (DIEA) (12 µL, 67 µmol, 5.0 equiv) was added, followed by PyBOP (10.5 mg, 20.1 µmol, 1.5 equiv). The reaction mixture was allowed to warm to room temperature and was stirred for another 5 h. The desired product was purified by preparative reversed-phase HPLC (water/acetonitrile, 0.1 % TFA) and the respective trifluoroacetate salt was obtained as a white solid (single peak by HPLC).

Synthesis and characterization of vancomycin-ornithine and lysine derivatives.

Cbz/Boc protected amino acid (Cbz-Lys(Boc)-OH, 100mg, 0.263 mmol, 1 equiv) was mixed with ammonia in water (NH_3 H_2O, 0.25 mL, 28% in water, density 0.9 g/mL), EDC (75.6 mg, 0.390 mmol, 1.5 equiv), HOBt (53.3 mg, 0.39 mmol, 1.5 equiv) in DMF (2.75 mL) at 0 °C for 7 h, then warmed to room temperature. The reaction mixture was washed twice with H_2O and extracted three times with EtOAc. The combined organics were washed with saturated NaCl and dried over Na_2SO_4. The mixture was then filtered through a vacuum filter and concentrated *in vacuo*. The desired product, Cbz/Boc protected lysine amine (Cbz-L-Lys(Boc)-NH_2), was isolated through column chromatography in 10% MeOH/Dcm. The Cbz-Lys(Boc)-NH_2 (33.5 mg, 0.0883 mmol, 1 equiv) was dissolved in THF (1 mL) and mixed with Pd/C (4.7 mg, 10 wt.%). The black suspension was stirred under a hydrogen-filled balloon (H_2) at room temperature for 12 h. The reaction mixture was exposed to air and filtered through a celite column to remove Pd/C, where no pressure was applied. The reaction vial and column were rinsed with THF thoroughly. After concentrating and drying *in vacuo*, the deprotected L-Lys(Boc)-NH_2 (15 mg, 0.0611 mmol, 2 equiv) was dissolved in DMF (0.8 mL)

under N_2 in a 3 dr vial containing a stir bar. Vancomycin (40 mg, 0.0306 mmol, 1 equiv) was dissolved in a second vial in DMSO (1 mL) under N_2 and added to the first vial dropwise using a syringe. The mixture was cooled to 0 °C while stirring. PyBOP (34 mg, 0.0611 mmol, 2 equiv) was dissolved in DMF (0.2 mL) and added dropwise into the stirring reaction, followed by the addition of DIEA (26.7 µL, 153 µmol, 5 equiv). The reaction was stirred for 5 min and then warmed to room temperature. After 16 h, the reaction mixture was diluted in H_2O and lyophilized. The crude product (yellow oil) was purified by RP-HPLC, 10-30% CH_3CN/H_2O with 0.1% TFA over 30 min. The appropriate fractions were isolated from a single peak by HPLC and lyophilized to afford a white powder. The Boc group was deprotected in 1M MeOH/HCl for 2-4 h at room temperature.

Synthesis of vancomycin-aspartic acid derivative (V-D)

Fmoc-Asp(O*t*Bu)-OH (205.8 mg, 0.5 mmol) and NH_4OH (~30%) (60 µL, 1.0 mmol) were coupled as previously described in the representative procedure. The desired product was then purified via column chromatography (ethyl acetate/pentane 2:1) and treated with 20% piperidine in DMF for ~1 h to remove the Fmoc group. The reaction mixture was lyophilized and the remaining residue was further used without any further purification and characterization. The sidechain-protected aspartic acid amide was then coupled to vancomycin and subsequently isolated as previously described in the representative procedure. Then, the coupling product was dissolved in 85% aqueous H_3PO_4/H_2O to cleave off the *tert*-butyl protecting group. At 48 h, LC-MS analysis still showed the presence of protected coupling product. Nevertheless, the desired, deprotected product was isolated via by preparative reversed-phase HPLC (water/acetonitrile, 0.1 % TFA) and the respective trifluoroacetate salt was obtained as a white solid.

Synthesis of vancomycin-diarginine derivative (V-RR).

Boc-Arg-OH hydrochloride (34.2 mg, 0.11 mmol) and H-Arg-NH_2 hydrochloride (27.2 mg, 0.11 mmol) were coupled as previously described in the representative procedure. The reaction mixture was lyophilized and to the remaining residue 1.5 mL 1.25M HCl in methanol was added. The reaction mixture was stirred for 9h at room temperature and lyophilized afterwards. The remaining residue was dissolved in 200 µL DMF and used for the vancomycin coupling reaction as previously described in the representative procedure without any further purification and characterization.

II. Characterization Data and Spectra

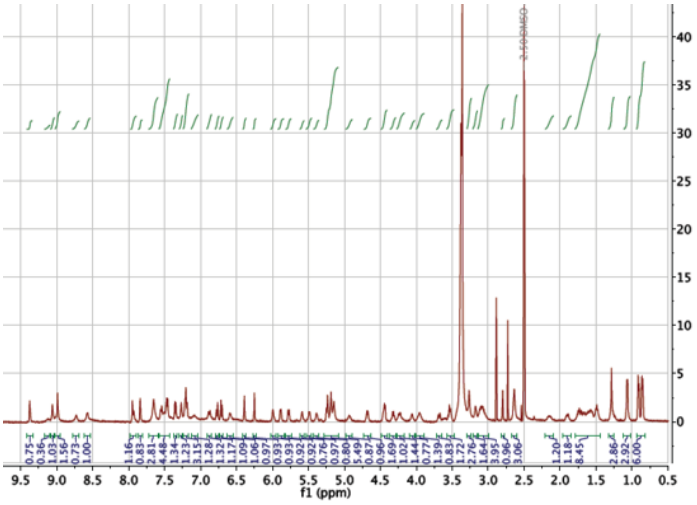
V-R

^{1}H NMR (d^6-DMSO, 500 MHz): δ = 9.38 (s, 1H), 9.06 (s, 1H), 8.99 (s, 2H), 8.73 (s, 1H), 8.57 (s, 1H), 7.96-7.90 (m, 1H), 7.84 (s, 1H), 7.65 (s, 3H), 7.57-7.43 (m, 5H), 7.35 (d, 1H, *J* = 8.2 Hz), 7.27 (s, 1H), 7.23-7.17 (m, 3H), 7.09 (s, 1H), 6.88 (d, 1H, *J* = 11.1 Hz), 6.79-6.75 (m, 1H) 6.73-6.69 (m, 1H), 6.59 (s, 1H), 6.39 (s, 1H), 6.25 (s, 1H), 5.99 (s, 1H), 5.89 (d, 1H, *J* = 5.6 Hz), 5.78 (d, 1H, *J* = 7.9 Hz), 5.59 (s, 1H), 5.49 (s, 1H), 5.39 (s, 1H), 5.27 – 5.10 (m, 6H), 4.93 (s, 1H), 4.68 (d, 1H, *J* = 6.8 Hz), 4.48-4.41 (m, 2H), 4.32 (q, 1H, *J* = 7.4 Hz), 4.23 (d, 2H, *J* = 12.5 Hz), 4.06 (s, 1H), 3.95 (s, 2H), 3.68 (d, 1H, *J* = 11.0 Hz), 3.56-3.50 (m, 2H), 3.27 (s, 3H), 3.18 (s, 2H), 3.14-3.01 (m, 4H), 2.80 (s, 1H), 2.64 (s, 3H), 2.15 (s, 1H), 1.90 (d, 1H, *J* = 11.7 Hz), 1.78-1.44 (m, 9H), 1.28 (s, 3H), 1.07 (d, 3H, *J* = 6.3 Hz), 0.88 (dd, 6H, *J* = 26.3, 6.0 Hz) ppm.

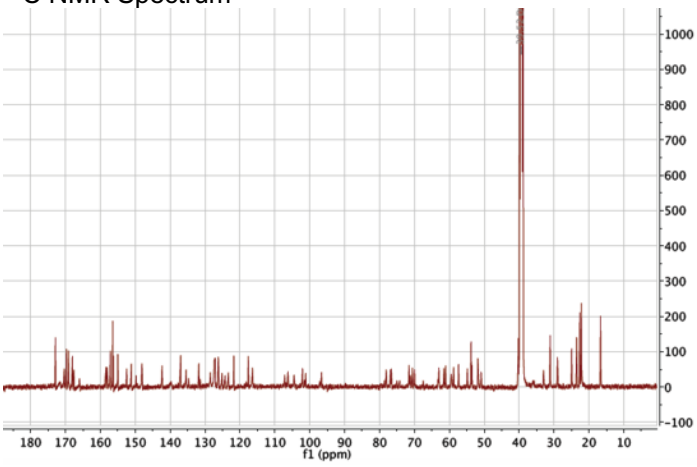
^{13}C NMR (d^6-DMSO, 500 MHz): δ = 173.03, 170.51, 169.90, 169.28, 168.16, 167.74, 166.11, 158.52, 158.27, 157.28, 156.61, 156.45, 155.17, 152.64, 151.31, 149.91, 148.28, 142.49, 137.15, 135.61, 134.90, 131.94, 131.53, 128.66, 127.53, 127.37, 127.26, 126.39, 126.32, 125.34, 124.42, 123.51, 121.92, 117.76, 116.60, 107.36, 106.40, 104.68, 102.25, 101.73, 101.34, 96.83, 78.76, 78.27, 77.04, 76.79, 75.18, 74.37, 71.72, 71.36, 70.76, 70.11, 67.65, 63.16, 61.76, 61.26, 59.59, 58.94, 57.54, 55.01, 53.94, 53.86, 53.68, 52.01, 51.06, 40.39, 40.02, 39.85, 39.69, 39.52, 39.35, 39.19, 39.02, 33.20, 31.26, 29.21, 25.13, 23.72, 22.79, 22.32, 16.86 ppm.

HRMS (ES+, *m/z)* calculated for $C_{72}H_{91}O_{24}N_{14}Cl_2^{3+}$: 535.1897, Found: 535.1905.

^{1}H NMR spectrum for V-R:



^{13}C NMR Spectrum

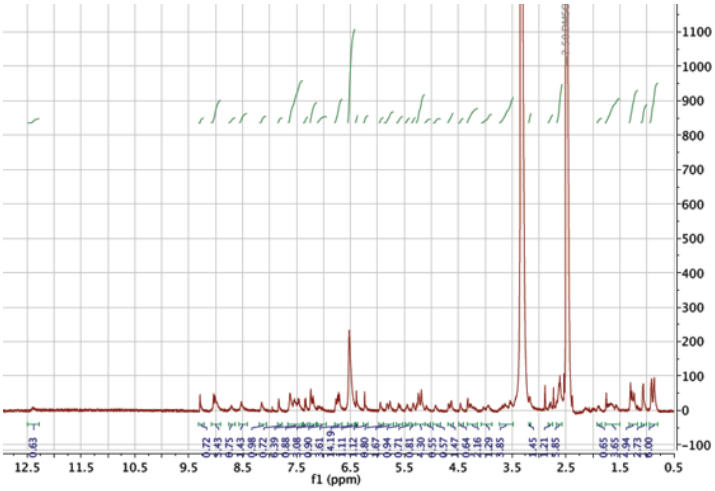


V-D

^{1}H NMR (d^{6}-DMSO, 600 MHz): δ = 12.39 (s, 1H) 9.29 (s, 1H), 9.08-8.91 (m, 3H),
8.71 (s, 1H), 8.52 (s, 1H), 8.14 (s, 1H), 7.83 (s, 1H), 7.66-7.38 (m, 6H), 7.34 (d, 1H, *J* = 8.2 Hz), 7.25-7.15 (m, 3H), 7.06 (br. s, 1H), 6.80-6.65 (m, 4H), 6.52 (s, 14H), 6.39 (s, 1H), 6.24 (s, 1H), 5.94 (s, 1H), 5.85-5.72 (m, 2H), 5.65-5.53 (m, 1H), 5.44 (s, 1H), 5.34 (s, 1H), 5.27 – 5.12
(m, 4H), 5.09 (s, 1H), 4.92 (s, 1H), 4.69-4.59 (m, 1H), 4.46 (s, 1H), 4.34-4.13 (m, 2H), 4.07-3.87 (m, 1H), 3.74-3.48 (m, 4H), 3.18 (s, 1H), 2.82-2.76 (m, 1H), 2.67-2.57 (m, 6H), 1.90
(s, 1H), 1.78-1.50 (m, 3H), 1.33 – 1.19 (m, 4H), 1.07 (d, 2H, *J* = 6.4 Hz), 0.88 (dd, 5H, *J* = 22.8, 6.1 Hz) ppm.

HRMS (ES+, *m/z)* calculated for $C_{70}H_{83}O_{26}N_{11}Cl_2^{2+}$: 781.7438, Found: 781.7430.

^{1}H NMR spectrum for V-D:

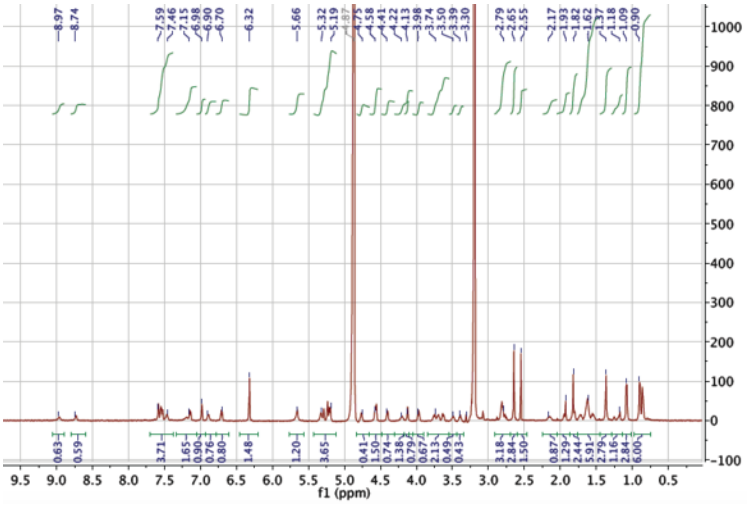


V-O

^{1}H NMR (d4-MeOH, 500 MHz): δ = 8.97 (s, 1H), 8.74(s, 1H), 7.58 (S, 1H), 7.58-7.46 (m, 4H), 7.20 (s, 1H), 7.14 (m, 2H), 6.98 (s, 1H), 6.89 (s, 1H), 6.71 (d, 1H), 6.32 (s, 2H), 5.66 (s, 1H), 5.37-5.13 (m, 5H), 4.76 (d, 1H), 4.56 (d, 2H), 4.40 (s, 1H), 4.20 (s, 2H), 4.12 (s, 1H), 3.97 (t, 1H), 3.80-3.66 (m, 2H), 3.49 (s, 1H), 3.39 (s, 1H), 2.87-2.73 (m, 3H), 2.65 (s, 3H), 2.55 (s, 2H), 2.15 (s, 1H), 1.94 (d, 1H), 1.81 (d, 3H), 1.62 (s, 6H), 1.37 (s, 3H), 1.18 (d, 1H), 1.08 (d, 3H), 0.88 (dd, 6H) ppm.

HRMS (ES+, m/z) $C_{71}H_{89}O_{24}N_{12}Cl_2^{3+}$: 781.2773, Found: 781.2776.

^{1}H NMR spectrum for V-O:

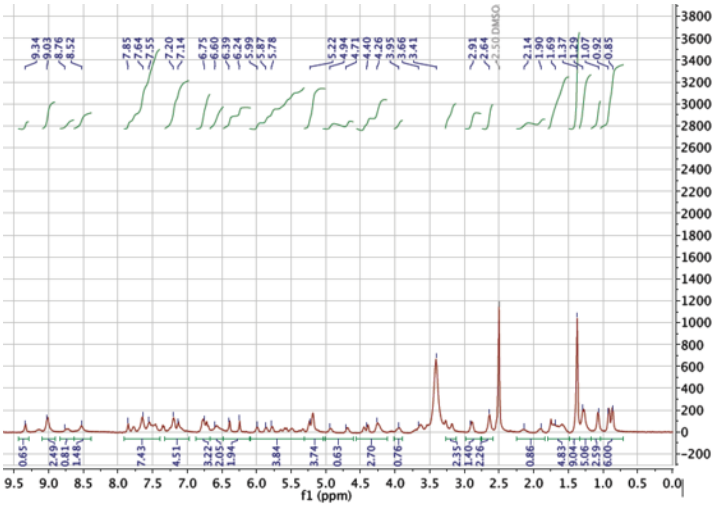


V-K

^{1}H NMR (d6-DMSO, 600 MHz): δ = 9.34 (s, 1H), 9.03 (m, 2H), 8.76 (s, 1H), 8.52 (m, 1H), 7.85-7.55 (m, 7H), 7.06 (s, 4H), 6.80-6.65 (m, 3H), 6.42 (s, 2H), 6.26 (s, 2H), 5.72 (m, 4H), 5.48-5.20 (m, 4H), 4.74-4.47 (m, 3H), 4.20 (m, 5H), 4.02 (m, 3H), 3.91 (m, 1H), 3.18 (s, 2H), 2.96 (m, 1H), 2.84 (s, 2H), 2.74 (s, 2H), 2.63 (s, 2H), 1.82-1.58 (m, 5H), 1.49-1.37 (m, 9H), 1.33-1.19 (m, 5H), 1.07-0.92 (m, 3H), 0.88 (m, 6H) ppm.

HRMS (ES+, m/z) $C_{71}H_{91}O_{24}N_{12}Cl_2^+$: 1562.4808, Found: 1562.4811.

^{1}H NMR spectrum for V-K:

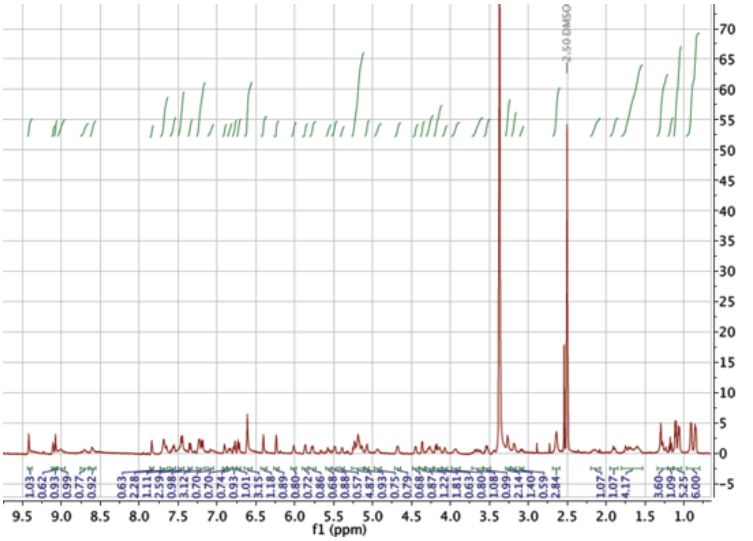


V-T

^{1}H NMR (d^6-DMSO, 500 MHz): δ = 9.42 (s, 1H), 9.11 (s, 1H), 9.08 (s, 1H), 9.01 (*br.* s, 1H) 8.71 (s, 1H), 8.60 (s, 1H), 7.84 (s, 1H), 7.73-7.63 (m, 2H), 7.60-7.53 (m, 1H), 7.50-7.43 (m, 3H), 7.35 (d, 1H, *J* = 8.3 Hz), 7.26-7.16 (m, 3H), 7.08 (s, 1H), 6.90 (s, 1H), 6.84 (d, 1H, *J* = 11.2 Hz) 6.79-6.75 (m, 1H) 6.73-6.69 (m, 1H), 6.61 (s, 3H), 6.40 (s, 1H), 6.24 (s, 1H), 6.01 (s, 1H), 5.87 (d, 1H, *J* = 6.2 Hz), 5.78 (d, 1H, *J* = 8.0 Hz), 5.60-5.53 (m, 1H), 5.52-5.45 (m, 1H), 5.39 (s, 1H), 5.27 – 5.12 (m, 5H), 5.08 (d, 1H, *J* = 5.2 Hz), 4.94 (s, 1H), 4.68 (d, 1H, *J* = 6.6 Hz), 4.45(s, 1H), 4.36 (d, 1H, *J* = 4.8 Hz), 4.31-4.22 (m, 1H), 4.21-4.11 (m, 2H), 4.07 (s, 1H), 3.94 (s, 1H), 3.72-3.58 (m, 1H), 3.57-3.49 (m, 1H), 3.27 (s, 2H), 3.18 (s, 1H), 3.11-3.05 (m, 1H), 2.64 (s, 3H), 2.20-2.07 (m, 1H), 1.95-1.85 (m, 1H), 1.80-1.53 (m, 4H), 1.34–1.21 (m, 4H), 1.17 (t, 1H, *J* = 7.3 Hz) 1.08 (dd, 5H, *J* = 21.9, 6.3 Hz), 0.88 (dd, 6H, *J* = 27.8, 5.7 Hz) ppm.

HRMS (ES+, *m/z)* calculated for $C_{70}H_{85}O_{25}N_{11}Cl_2^{2+}$: 774.7542, Found: 774.7547.

^{1}H NMR spectrum for V-T:

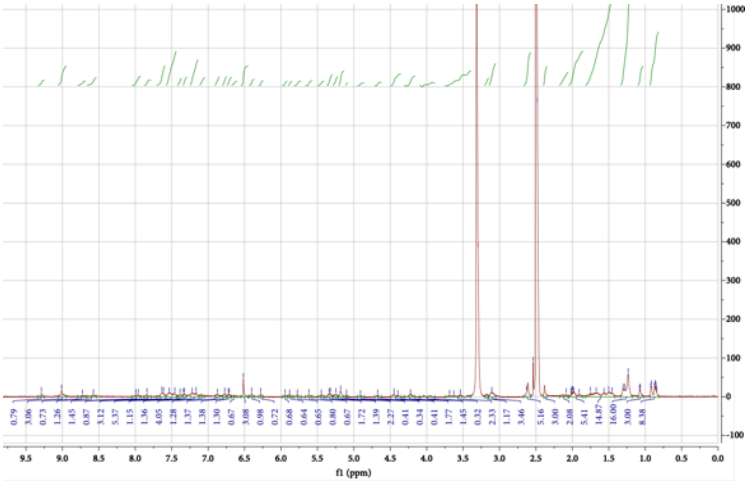


V-RR

^{1}H NMR (d^6-DMSO, 600 MHz): δ = 9.29 (s, 1H), 9.01 (s, 3H), 8.72 (s, 1H), 8.57 (s, 1H), 7.98 (d, 1H, *J* = 21.0 Hz), 7.84 (s, 1H), 7.70-7.58 (m, 3H), 7.57-7.43 (m, 5H), 7.38 (s, 1H), 7.33 (d, 1H, *J* = 8.3 Hz), 7.25-7.14 (m, 4H), 7.09 (s, 1H), 6.87 (s, 1H), 6.80-6.75 (m, 1H) 6.73-6.69 (m, 1H), 6.64 (s, 1H), 6.52 (s, 3H), 6.40 (s, 1H), 6.28 (s, 1H), 5.94 (s, 1H), 5.88 (s, 1H), 5.77 (s, 1H), 5.62 (s, 1H), 5.45 (s, 1H), 5.36-5.30 (m, 2H), 5.28 – 5.22 (m, 1H), 5.18 (s, 2H), 5.10 (s, 1H), 4.91 (s, 1H), 4.67 (s, 1H), 4.49-4.36 (m, 2H), 4.22 (s, 1H), 4.04 (s, 1H), 3.95 (s, 1H), 3.74-3.30 (m, 2H), 3.18 (s, 1H), 3.11 (s, 3H), 2.61 (s, 5H), 2.38 (s, 3H), 2.18-1.85 (m, 7H), 1.81-1.41 (m, 15H), 1.33 – 1.18 (m, 16H), 1.10-1.03 (m, 3H), 0.94-0.81 (m, 8H) ppm.

HRMS (ES+, *m/z)* calculated for $C_{78}H_{103}O_{25}N_{18}Cl_2^{3+}$: 578.2234, Found: 578.2233.

^{1}H NMR spectrum for V-RR:



III. Biological procedures

MIC Assays

MICs were determined using broth microdilution in accordance with CLSI methods.[32] One day prior to each MIC experiment, bacterial strains were streaked for single colonies on LB agar plates from frozen glycerol stocks stored at -80 °C. 3-5 colonies from each plate were harvested with a disposable inoculating loop and resuspended in 500 µL PBS. This suspension was diluted in PBS to an OD_{600} of 0.1 (~1x 10^8 CFU/ml), and the OD 0.1 suspension was diluted 1:100 in Mueller-Hinton Broth (MHB, Difco 257530) just prior to inoculating the treatment plate. 50 µL of inoculum was added to a treatment plate containing 2-fold serial dilutions of compound in MHB (50 µL of treatment/ well) to lend a final total volume of 100 µL/well and a final inoculum density of ~5×10^5 CFU/ml. The completed assay plate was sealed with Parafilm, placed in a lidded plastic tray lined with moistened paper towels, and incubated at 37 °C for 18-20 h. The MIC was read as the lowest treatment concentration where no bacterial growth occurred, as determined by OD_{600} measurements on a microplate reader. For experiments with 5 mM magnesium supplementation, a 50 mM $MgCl_2$ solution was prepared in MHB, filter-sterilized through a 0.22 µm filter, and added to treatment plate to achieve a desired final concentration of 5 mM.

Cytotoxicity Experiments

To assess the hemolytic activity of V-R, a 1 mL aliquot of single-donor human red blood cells (IPLA-WB3–22136, Innovative Research, Inc.) was centrifuged at 1000 g for 10 min. The supernatant was aspirated, and the resulting pellet of erythrocytes was resuspended in PBS and centrifuged again at 1000 g for 10 min. Upon two additional PBS washes and centrifugation cycles, the final erythrocyte pellet was resuspended in PBS to yield a 1% volume/volume suspension. A 100 µL portion of 1% erythrocyte solution was added to the wells of a V-bottomed 96 well microtiter plate. Treatments (100 µL) treatments were added to yield the desired final compound concentrations. Blank PBS was used as a negative control and 1% TX-100 as a positive control. The plate was incubated statically for 1 h at 37 °C and subsequently centrifuged for 5 min at 1500 g at room temperature. A 100 µL portion of the resulting supernatant was transferred to a flat-bottomed microtiter plate and analyzed on a microplate reader via absorbance measurements at 450 nm. Percent hemolysis was determined by dividing background-corrected absorbance measurements by background-corrected measurements for 1% TX-100.

Cellular cytotoxicity of V-R was assessed using an MTT percent viability assay with ATCC 5637 (HTB-9) cells. The experiments were performed in 96-well tissue culture plates seeded with 20,000 HTB-9 cells per well in RPMI media supplemented with 10% FBS and penicillin-streptomycin for 24 hours prior to treatments. A 96-well treatment plate containing desired concentrations of compounds in 100 µL media per well was prepared. The cells were subsequently washed once with media, and then the compound containing media from treatment plate was transferred into cell-containing plate using a multichannel pipet. The cells were incubated with compounds for 18 hours at 37 °C before performing the MTT assay. After the incubation period, cells were treated with MTT (10 µL, 5 mg/mL in RPMI medium per well) and further incubated for 2 hours at 37 °C. 100 µL of solubilizing solution (10% Triton X-100, 90% 0.1 N HCl in isopropanol) was added to each well with thorough mixing to lyse cells open. Colorimetry data was obtained on a plate reader (570 nm). Normalized viability was obtained by comparing the absorbance at 570 nm produced by the treated cells with that of control cells (no compound added).

Time-Kill Kinetics Experiments

For time-kill kinetics experiments, a stationary-phase culture of *E. coli* UTI89 was diluted to 1×10^6 CFU/mL in MHB, and 50 µL of this suspension was mixed with 50 µL of MHB containing twice the desired final concentrations of compounds. The plate was incubated at 37 °C with 200 rpm shaking, and CFU/mL were enumerated on LB agar at determined time points (detection limit: 3 log CFU/mL).

Phase Contrast Microscopy

An OD_{600} 0.5 suspension of *E. coli* 25922 was prepared in MHB from a stationary-phase culture. 25 µL of the suspension was added to a treatment plate and mixed with 25 µL of a 2X stock solution of compound, prepared in MHB. After 2 h incubation with 200 rpm shaking at 37 °C, 2.5 µL aliquots were removed from treatment wells and applied to glass microscope slides (SuperFrost plus) and wet-mounted with coverslips. Samples were imaged on an AxioObserver microscope using a 100x/1.4 oil immersion objective.

HPLC-MS

Bacterial samples were prepared for HPLC-MS analysis by diluting a stationary-phase culture of *E. coli* UTI89 1:500 and growing in MHB to an OD_{600} of 0.6 at 37 °C with 200 rpm shaking. The OD_{600} 0.6 culture was treated with 130 µg/mL of chloramphenicol for 15 min 37 °C with 200 rpm shaking and then subsequently aliquoted (25 mL per aliquot) for treatment with compounds, where an untreated sample was included as a control. Upon 1 hr treatment, the aliquots were centrifuged at 4 °C for 20 min at 3000 rpm. CFU/mL were enumerated on LB agar to determine cell density of samples. The cell pellets were washed with 1 mL HEPES/Glucose buffer (5 mM HEPES, 5 mM Glucose) and centrifuged at 8000 rpm for 5 min at room temperature. The supernatant was removed, and the bacteria were resuspended in 500 µL HEPES Buffer and heated at 100 °C for 15 min. The samples were centrifuged at 13,000 rpm for 5 min, and the supernatant was removed and lyophilized. The lyophilized lysate was mixed with 200 µL bio-grade water and filtered through a 0.2-micron filter. The filtered samples were analyzed by RP-HPLC with a 0-30% MeCN/H_2O gradient and a C18 column (Agilent Eclipse XDB-C18 5µm semi-preparative column, 9.4x250 mm). The injection volume was 500 µL and the flow rate was 5 mL/min, with a column temperature of 25 °C. Fractions corresponding to peaks of interest were collected and identified using a ZQ single quadrupole mass spectrometer with an electrospray ionization source. HPLC spectra are presented as raw spectra, noting that normalizing the spectra by cell density of sample would yield same result, where the untreated sample featured 1.4 and 2.1X CFU/mL as compared to V and V-R samples, respectively.

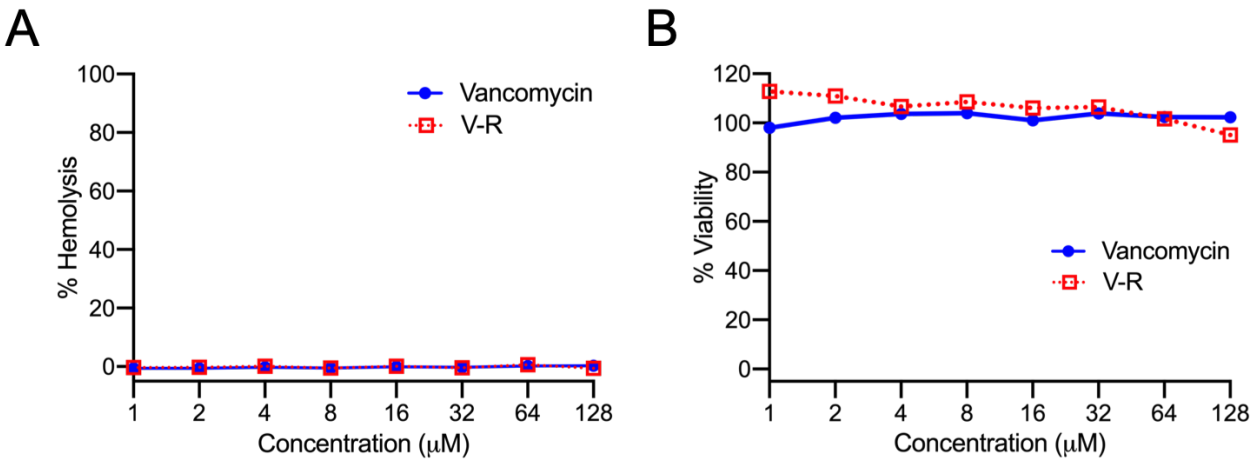
NPN Assay

Outer membrane permeabilization assays with the probe NPN were performed according to literature procedures.[15] Stationary-phase cultures of *E. coli* UTI89 were diluted 1:500 in MHB and grown to OD_{600} of 0.6 a at 37 °C with 200 rpm shaking. The bacteria were subsequently pelleted at 10,000 rpm for 5 min at room temperature and washed in HEPES/Glucose buffer (5 mM HEPES, 5 mM Glucose, pH 7.2). The bacteria were spun again and resuspended in buffer to yield a solution of bacteria with an n OD_{600} of 1.0. A 5 mM solution of NPN (Sigma-Aldrich) was prepared in acetone and then subsequently diluted in 1:10 in HEPES/Glucose buffer to prepare a 0.5 mM working solution for assays. A black-walled, clear bottom treatment plate was prepared a final bacterial concentration of OD_{600}=0.5 and 5 µM NPN, where bacterial inocula were prepared in HEPES-Glucose buffer. The plate was analyzed for 5 minutes on a fluorescence microplate reader, with excitation wavelength of 350 nm and emission wavelength of 420 nm at 37 °C. Antibiotic dilutions (prepared in HEPES/Glucose Buffer) were then added to the plate to yield desired final concentrations. The plate was reincubated at 37 °C on a microplate reader and scanned to assess impacts of antibiotic addition.

PI Assay

Inner membrane permeabilization studies were performed with the probe propidium iodide according to literature protocols.[15] Stationary-phase cultures of *E. coli* UTI89 were diluted 1:500 in MHB and grown to OD_{600} of 0.6 at 37 °C with 200 rpm shaking. The bacteria were subsequently pelleted at 10,000 rpm for 5 min at room temperature and washed in HEPES/Glucose buffer (5 mM HEPES, 5 mM Glucose, pH 7.2). The bacteria were spun again and resuspended in buffer to yield a solution of bacteria with an OD_{600} of 1.0. A 5 mM solution of PI was prepared in DMSO and was added to a final concentration of 10 µM to the bacterial culture. The bacterial culture was added to a black-walled, clear bottom 96 well plate and was incubated at 37 °C in a microplate with excitation wavelength of 535 nm and emission wavelength of 617 nm. The compounds were then added at desired final concentrations (compound solutions prepared in HEPES/Glucose buffer) and the fluorescence intensity was monitored post-addition.

SUPPORTING FIGURES and TABLES



Supporting Figure 1. *In vitro* toxicity assessment of V-R. A) Analysis of hemolytic activity in human erythrocytes, where percent hemolysis is expressed relative to 1% Triton X-100. B) Cytotoxicity assessment in HTB-9 bladder cells as measured by MTT assay, where % viability is expressed relative to untreated cells.

Supporting Table 1. Minimum Inhibitory Concentration (MICs, μM)[a] of Vancomycin-Arginine Peptide Conjugates

Compound	MIC
V-r8	32
r8	24 (16-32)
V+r8	24 (16-32)
V-r4	>32

[a] Median MICs from 2 independent experiments, with ranges provided in parentheses. MICs were determined in strain *E. coli* UTI89.

Supporting Table 2. Minimum Inhibitory Concentration (MICs, μM)[a] of V-K and V-R

Strain	V-R	V-K
E. coli 25922	8 (8-16)	12 (8-20)
E. coli UTI89	12 (8-16)	16
E. coli BAA-2469 (CR)	16 (8-16)	18 (12-24)

[a] Median MICs from 2-3 independent experiments, with ranges provided in parentheses.

Supporting Table 3. Minimum Inhibitory Concentration (MICs, μM)[a] of V-R upon 5 mM magnesium supplementation

Antibiotic	MIC	MIC + 5 mM Mg^{2+}
V-R	8	64 (32-64)
V	128	512 (256-512)
Chloramphenicol	32	32

[a] Median MICs from 2-3 independent experiments, with ranges provided in parentheses. Experiments were performed in strain UTI89.