A dual function antibiotic-transporter conjugate exhibits superior activity in sterilizing MRSA biofilms and killing persister cells

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Supporting Information

Contents

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a. Abbreviations ***p. 2***

b. Supplemental Tables, Figures, and Schemes ***p. 3 – 8***

c. General Synthetic Methods, Experimental Procedures, and Characterization Data ***p. 8 – 17***

d. References ***p. 18***

Abbreviations

Ahx	aminohexanoic
Cbz-Ahx-OH	*N*-Benzyloxycarbonyl-6-aminohexanoic acid
CFU	colony forming units
CPP	cell-penetrating peptide
DCM	dichloromethane
DIPEA	*N,N*-diisopropylethylamine
DMF	dimethylformamide
DMSO	dimethyl sulfoxide
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
Fl	fluorescein
GR-MoTr	guanidinium-rich molecular transporter
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
MBEC	minimum biofilm eradication concentration
MeCN	acetonitrile
MeOH	methanol
MHB	Mueller-Hinton Broth
MIC	minimum inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *Staphylococcus aureus*
ND	not determined
OD	optical density
PBS	phosphate buffered saline
PI	propidium iodide
Pd/C	palladium on carbon
RP	reverse phase
S. aureus	*Staphylococcus aureus*
SSTI	soft skin and tissue infection
SYTO 9	a green fluorescent dye that is permeant to cells
TB	Trypan Blue
TFA	trifluoroacetic acid
TSB	Tryptic Soy Broth
USA300/400	methicillin-resistant *S. aureus* strains
V	vancomycin
VISA	vancomycin-intermediate *S. aureus*
VSE	vancomycin-susceptible *Enterococci*
VRE	vancomycin-resistant *Enterococci*

Table SI-1. Median MBEC values (μM) for V-r8, V-r4 ,and comparative antibiotic MBEC data[a]

Strain	*V-r8 (TFA)*	*V-r8 (HCl)*	*V-r4 (TFA)*	*Antibiotic MBEC data in strain 29213*
MSSA (29213)	20 (16-25)	16	ND	Oritavancin: 22 (12-32)
MRSA (USA400MW2)	10 (6-26)	10 (4-16)	48[b]	Dalbavancin: 14 (8-20)
MRSA (USA300LAC)	9.5 (3-16)	ND[c]	ND	Vancomycin: $\geq$ 500

[a] MBEC values are medians from 2-4 independent experiments, where all treatments were performed in TSB. Parentheses indicate the range of values obtained in experiments. V-r8 (TFA) and vancomycin data is reproduced from Table 1 in the main text.
[b] In our first V-r4 MBEC experiment, we obtained an MBEC value of >16 μM (highest concentration tested). In a second experiment, we tested higher concentrations of V-r4 and obtained an MBEC of 48 μM (reported in table).
[c] We determined the MBEC of V-r8 (HCl) in PBS buffer in USA300LAC (the strain used for *in vivo* assays), as *in vivo* treatments were performed in PBS. We obtained a median MBEC of 2.5 μM (range 1-4 μM)

Table SI-2. Median MIC values (μM) for V, V-r8 (TFA), V-r8 (HCl), r8, and V + r8[a]

Strain	*V*	*V-r8 (TFA)*	*V-r8 (HCl)*	*r8*	*V + r8*
MSSA (29213)	0.50 (0.50-0.63)	1.8 (1.0-2.0)	1.3 (1.0-1.5)	60 (40-80)	0.50
MRSA (USA400 MW2)	0.50 (0.31-0.63)	0.94 (0.63-1.0)	0.75 (0.50-1.0)	20	0.50
MRSA (USA300 LAC)	0.50 (0.31-0.50)	2.0 (1.8-2.0)	1.5 (1.0-2.0)	40	0.50
VISA (700699)	4.0	12 (8.0-16)	ND	16	2.0
VSE (*E. faecium* 35567)	0.38 (0.25-0.5)	0.56 (0.13-1.0)	0.19 (0.13-0.25)	>64	0.5
VSE (*E. faecalis* OG1RF)	1.5 (1.0-2.0)	2.0	2.0	>64	1.3 (0.5-2.0)
VRE (*E. faecium* 51559)	512	4.0	ND	>64	32
VRE *(E. faecalis* 51575)	128	32	24 (16-32)	>64	48 (32-64)
VRE (*E. faecalis* V583)	16	ND	4.0	ND	3.0 (2.0-4.0)

[a] MIC values are medians from 2-7 independent experiments. Parentheses indicate range of values obtained in experiments. No range indicates values were the same across experiments. V, V-r8 (TFA), r8, and V + r8 MICs for MSSA and two MRSA strains are reproduced from Table 1 in the main text.

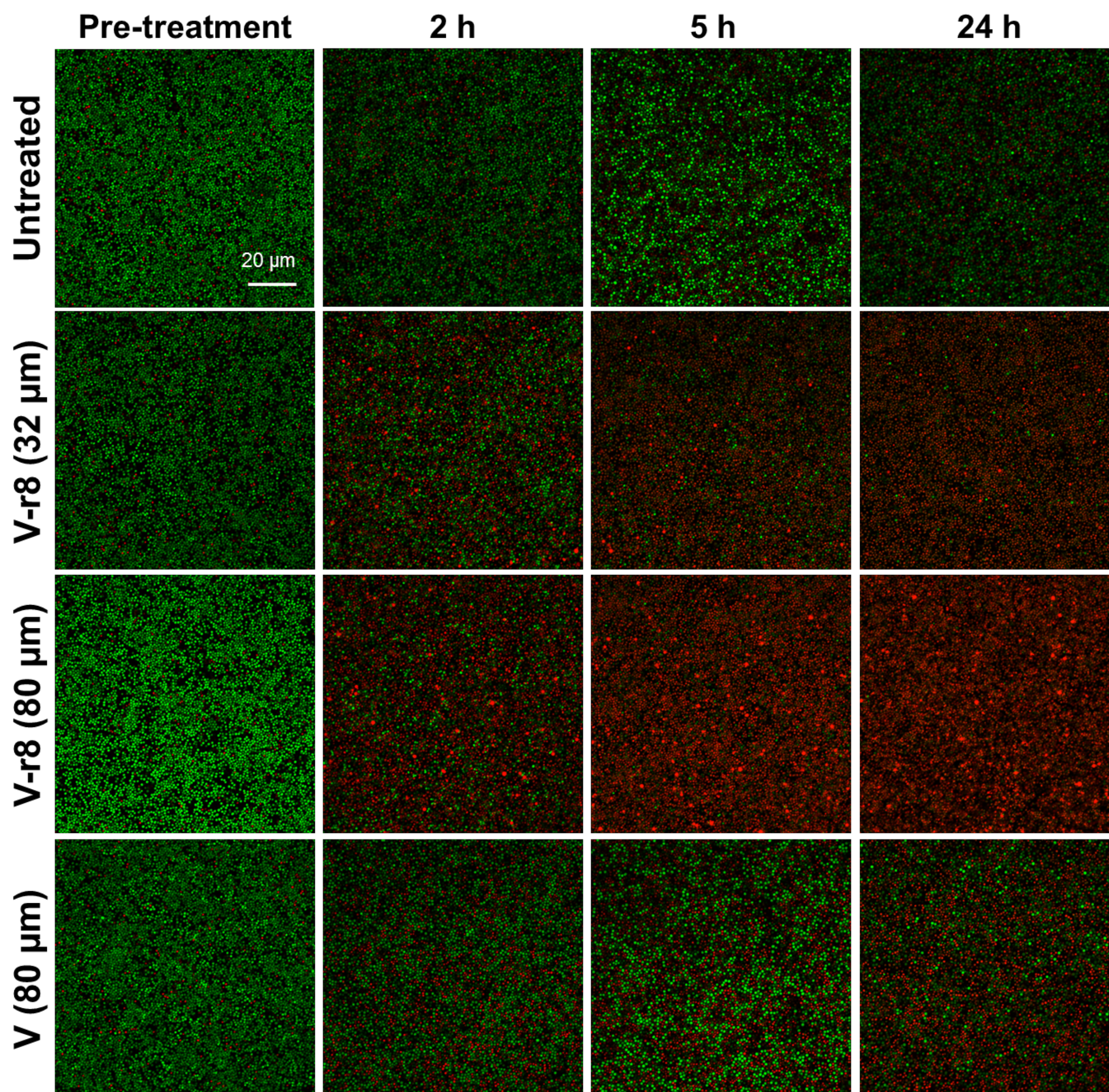


Figure SI-1. Evaluation of V-r8 biofilm killing over a period of 24 h. Confocal microscopy images of MRSA USA400 MW2 biofilms treated with concentrations of V-r8 at 32 or 80 µM demonstrate its ability to rapidly eliminate biofilm-associated bacteria, in contrast to vancomycin at 80 µM (SYTO9: green, stains all viable cells; PI: red, stains dead cells).

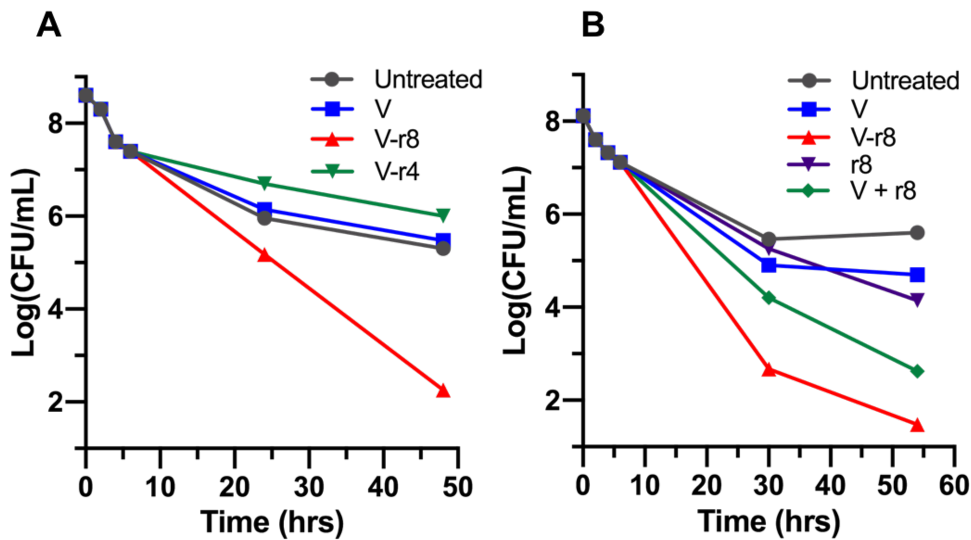


Figure SI-2. Evaluation of V-r4, V+r8, and r8 activity against MRSA USA300 LAC persister cells in comparison with V-r8. (A) Comparative activity of V, V-r4, and V-r8 against persister cells. (B) Comparative activity of V, V-r8, r8, and V+r8 against persister cells. Persister cells were generated by treatment of MRSA cells with ciprofloxacin at 40 µM for 6 h. All treatments were performed at 20 µM and each panel represents an independent experiment.

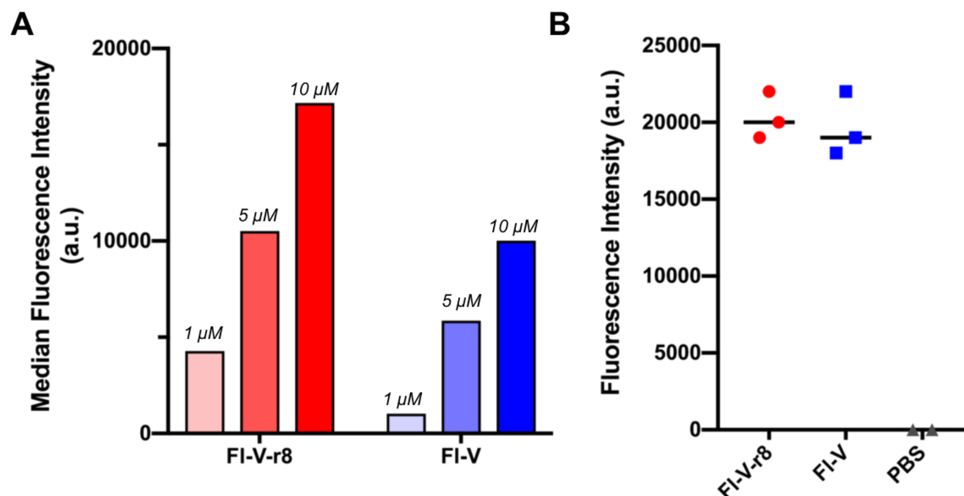


Figure SI-3. (A) Whole-cell fluorescence analysis of MRSA USA400 MW2 treated with Fl-V or Fl-V-r8. Cells treated with Fl-V or Fl-V-r8 exhibit concentration-dependent fluorescence after washing away extracellular excess compound as determined by FACS. Each bar represents the median fluorescence obtained from a population of ~2500 cells from a representative experiment. (B) Fluorescence measurements of Fl-V and Fl-V-r8 stock solutions at matched concentration. Each data point (n=3) represents a fluorescence measurement of a separately prepared 1.25 µM stock solution. PBS (n=2) was measured as a control, and black bars represent median fluorescence values.

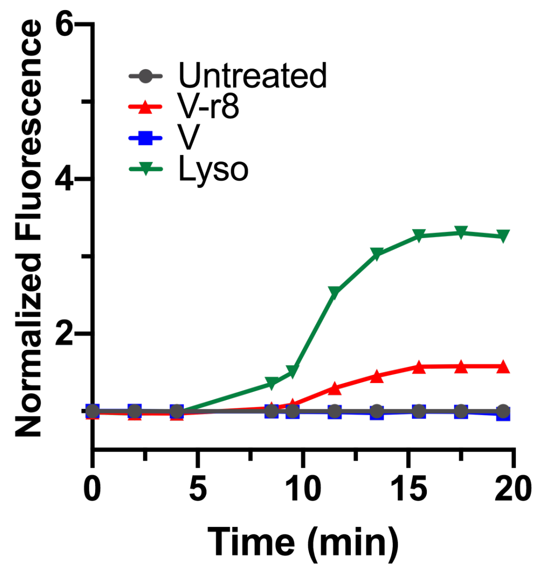


Figure SI-4. Evaluation of PI uptake and fluorescence as a reporter for the perturbation of membrane barrier function upon treatment with V-r8, V, or lysostaphin, where treatments were performed at 1 μM for V and V-r8, and 12.5 μg/mL lysostaphin (all ~1X MIC). Experiments were performed with exponential-phase bacteria resuspended in HEPES-Glucose (H-G) buffer.

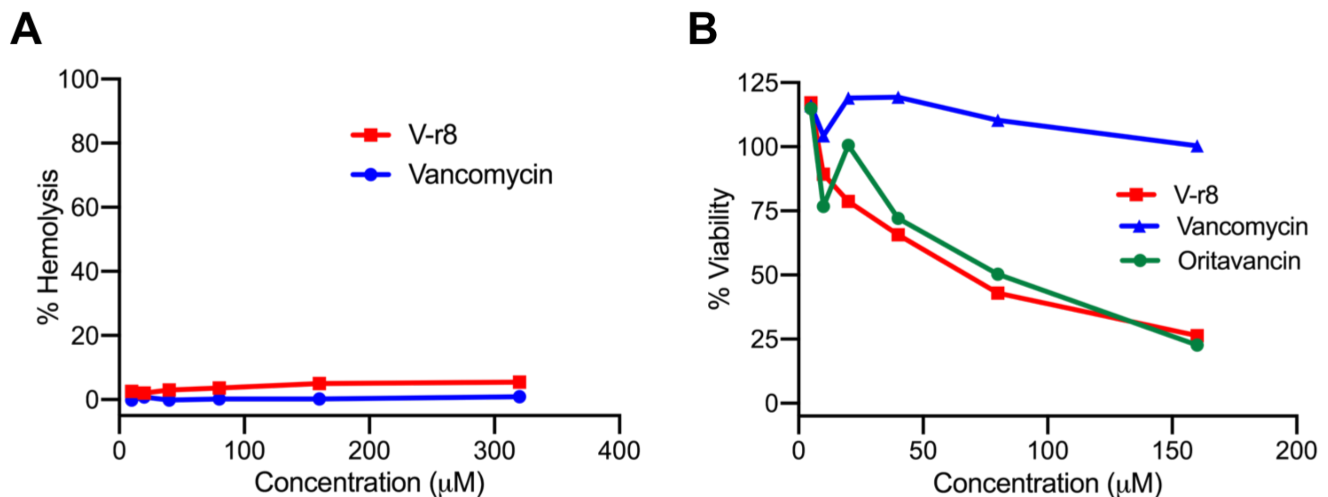
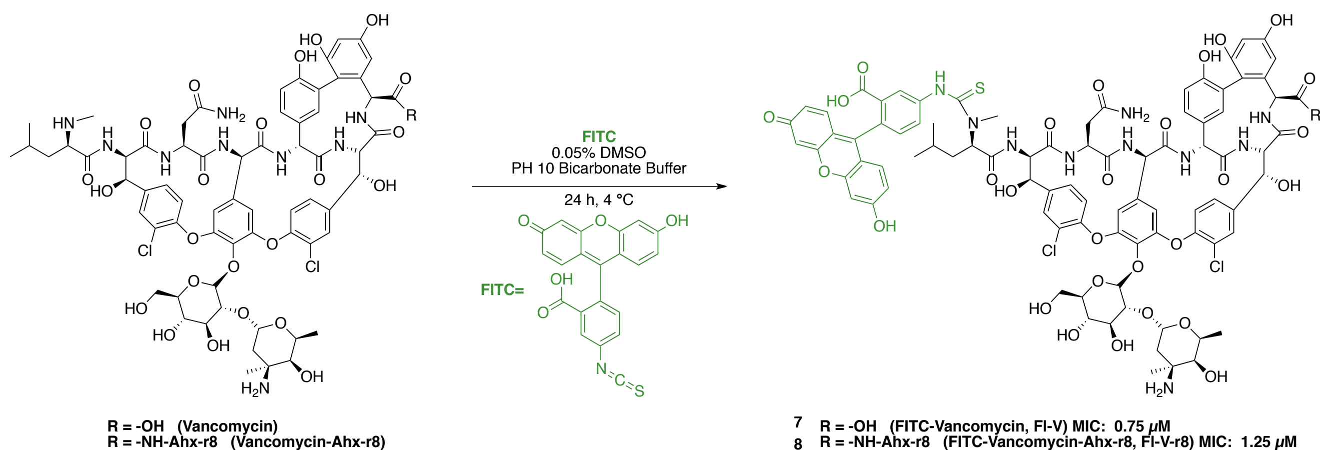


Figure SI-5. Evaluation of the hemolytic activity and cytotoxicity of V-r8 and comparator agents. A) Analysis of hemolytic activity, where 1% Triton X-100 was used as a positive control with 100% hemolysis. B) Cytotoxicity of V-r8 and comparator agents as measured using an MTT assay, where data from compound-treated cells was normalized to untreated cells.

Scheme SI-1: Synthesis of Fl-V and Fl-V-r8



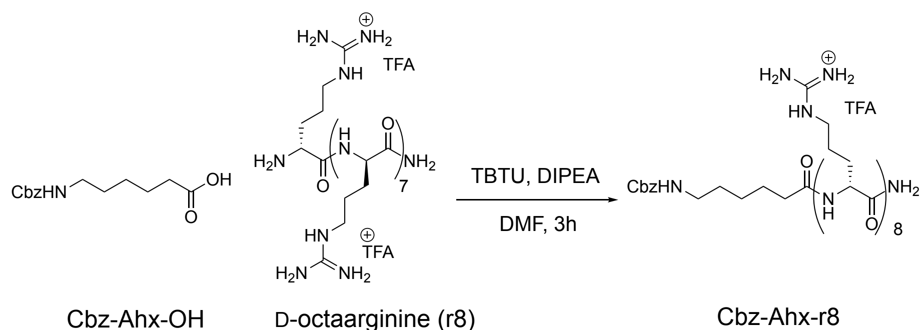
<u>*General Methods*</u>

Unless otherwise noted, all reactions were run under a nitrogen atmosphere in flame-dried glassware. Reactions were sealed with rubber septa or Teflon™-coated caps and stirred using Teflon™-coated magnetic stir bars. Solid reagents were measured on a Mettler Toledo AB104-S balance. Room temperature indicates an external temperature of 22-25 °C.

Anhydrous dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and methanol (MeOH) were obtained from Thermo Fisher. Amine base (DIPEA) were distilled over CaH_2 under nitrogen. Reagents were purchased from SERVA (Vancomycin hydrochloride), Bachem (Cbz-Ahx-OH), Thermo Fisher Scientific (fluorescein isothiocyanate, FITC), Novabiochem (peptide coupling reagents), Applied Biosystems (peptide resin) and UCB bioproducts (octa-D-arginine). RP-HPLC was carried out in an MeCN:H_2O gradient using a Shimadzu Prominence system equipped with a Restek-18 column (5 µm, 21x250 nm) or an Agilent Eclipse XDB-C18 5µm semi-preparative column (9.4x250 mm). NMR spectra were measured on a Varian INOVA 500 (^{1}H at 500 MHz, ^{13}C at 125 MHz), a Varian 400 (^{1}H at 400 MHz, ^{13}C at 100 MHz), or a Varian INOVA 600 MHz (^{1}H at 600 MHz, ^{13}C at 150 MHz) magnetic resonance spectrometer, as noted. Deuterated solvents were obtained from Cambridge Isotope Laboratories, Inc. ^{1}H chemical shifts are reported relative to the residual solvent peak (d4-methanol = 3.31 ppm or DMSO = 2.50 ppm)[1] as follows: chemical shift (δ), multiplicity (s=singlet, bs=broad singlet, d=doublet, t=triplet, q=quartet, m=multiplet), coupling constant(s) in Hz, integration. ^{13}C chemical shifts are reported relative to the residual deuterated solvent ^{13}C signals (d4-methanol = 49.00 ppm or DMSO = 39.52 ppm). High-resolution mass spectra (HRMS) were obtained at the Vincent Coates Mass Spectrometry Laboratory, Stanford, CA 94305. Matrix-assisted laser desorption/ionization (MALDI) were obtained at the Protein and Nucleic Acid Facility (PAN), Stanford, CA 94305.

Experimental Procedures and Characterization Data

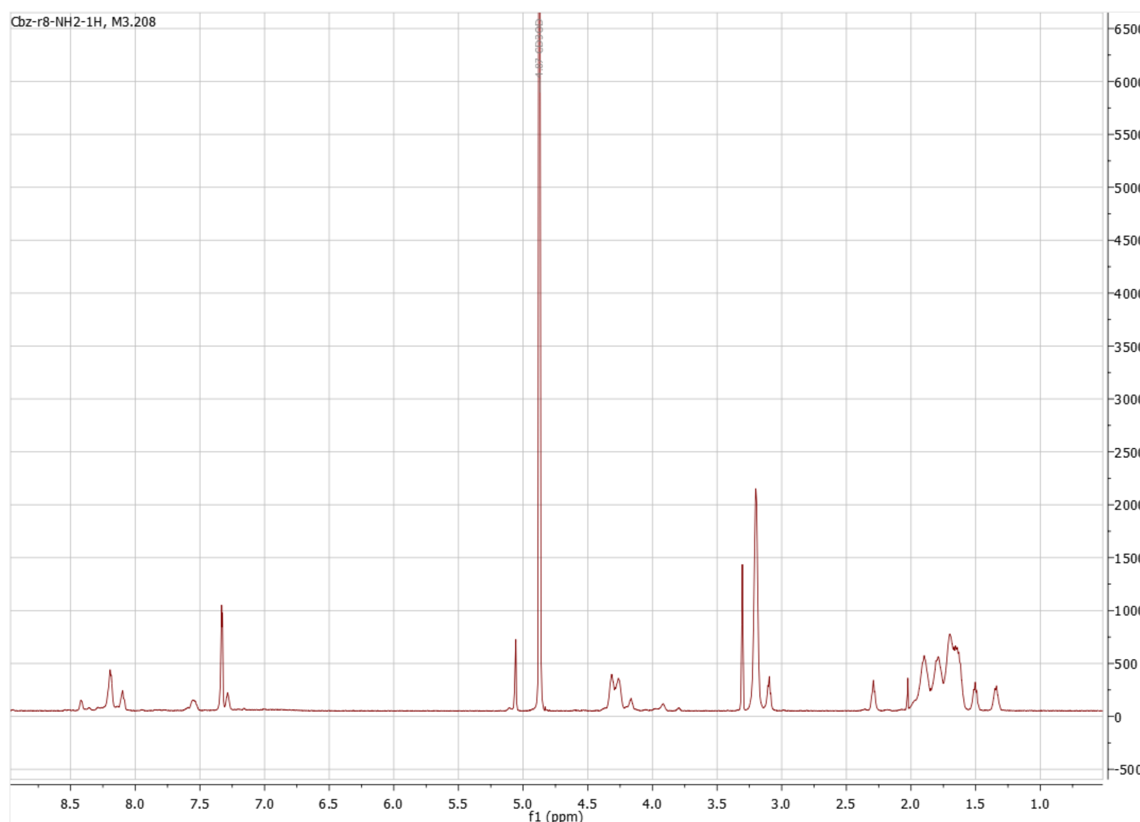
Synthesis and Characterization.



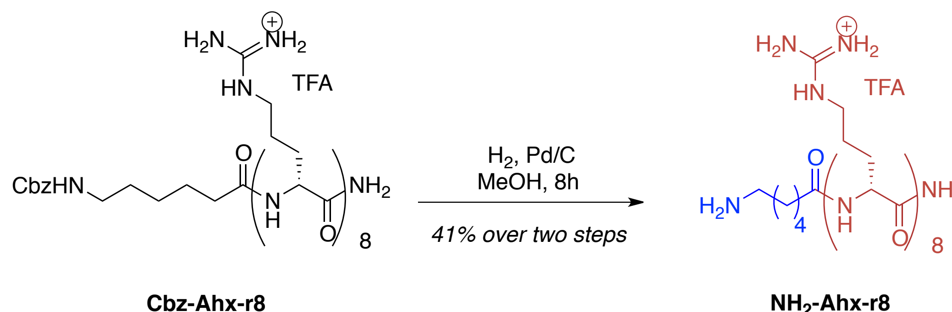
Cbz-ahx-r8 (TFA)

Cbz-Ahx-OH (17.3 mg, 0.065 mmol, 1 molar equiv) and TBTU (41.7 mg, 0.13 mmol, 2 molar equiv) were dissolved in anhydrous DMF (300 μL). To a separate vial was added octa-D-arginine (TFA) (142.7 mg, 0.065 mmol, 1 molar equiv) in dry DMF (0.5 mL). Both vials were flushed with nitrogen (N_2). The D-octaarginine solution was transferred from the original vial via a syringe to the flask containing Cbz-Ahx-OH, and the original vial was rinsed with two 400 μL portions of dry DMF. The reaction mixture had an orange tint. Upon addition of freshly distilled DIPEA (45 μL, 0.26 mmol, 4 molar equiv) to the reaction, the reaction mixture turned more orange and clear. The reaction mixture stirred at room temperature under N_2 and was monitored by LC-MS and stopped after three hours. The DMF was removed by lyophilization and the reaction mixture was purified by RP-HPLC on a preparative C18 column, 5-70% CH_3CN/H_2O with 0.1% TFA over 30 min. The appropriate fractions were lyophilized and the product was isolated as a white solid (51.6% yield, one peak by HPLC).

^{1}H-NMR (CD_3OD, 600 MHz): δ 8.43 – 8.09 (m, 8H), 7.34 – 7.28 (m, 5H), 5.06 (s, 2H), 4.33 – 4.25 (m, 8H), 3.21 (dd, *J* = 1.2, 0.3 Hz, 16H), 3.10 (t, *J* = 6.6 Hz, 2H), 2.30 (t, *J* = 6.5 Hz, 2H), 2.03 – 1.62 (m, 34H), 1.51 (t, *J* = 7.2 Hz, 2H), 1.38 – 1.32 (m, 2H) ppm.
^{13}C-NMR (CD_3OD, 126 MHz): δ 177.1, 176.9, 176.7, 176.5, 174.8, 174.6, 174.5, 174.1, 163.5, 163.2, 162.9, 162.7, 162.6, 158.6, 138.4, 129.4, 128.9, 128.6, 119.3, 116.9, 67.3, 55.7, 55.6, 54.9, 54.3, 42.0, 41.9, 41.6, 36.5, 30.6, 29.8, 29.7, 29.6, 29.5, 28.9, 27.4, 26.5, 26.4, 26.3 ppm.
HRMS (ES+ *m/z*): Calculated for $C_{62}H_{119}N_{34}O_{11}^{2+}$: 757.4782 (M+2H)/2. Found: 757.4867 (M+2H)/2.
T_R: 14 minutes.



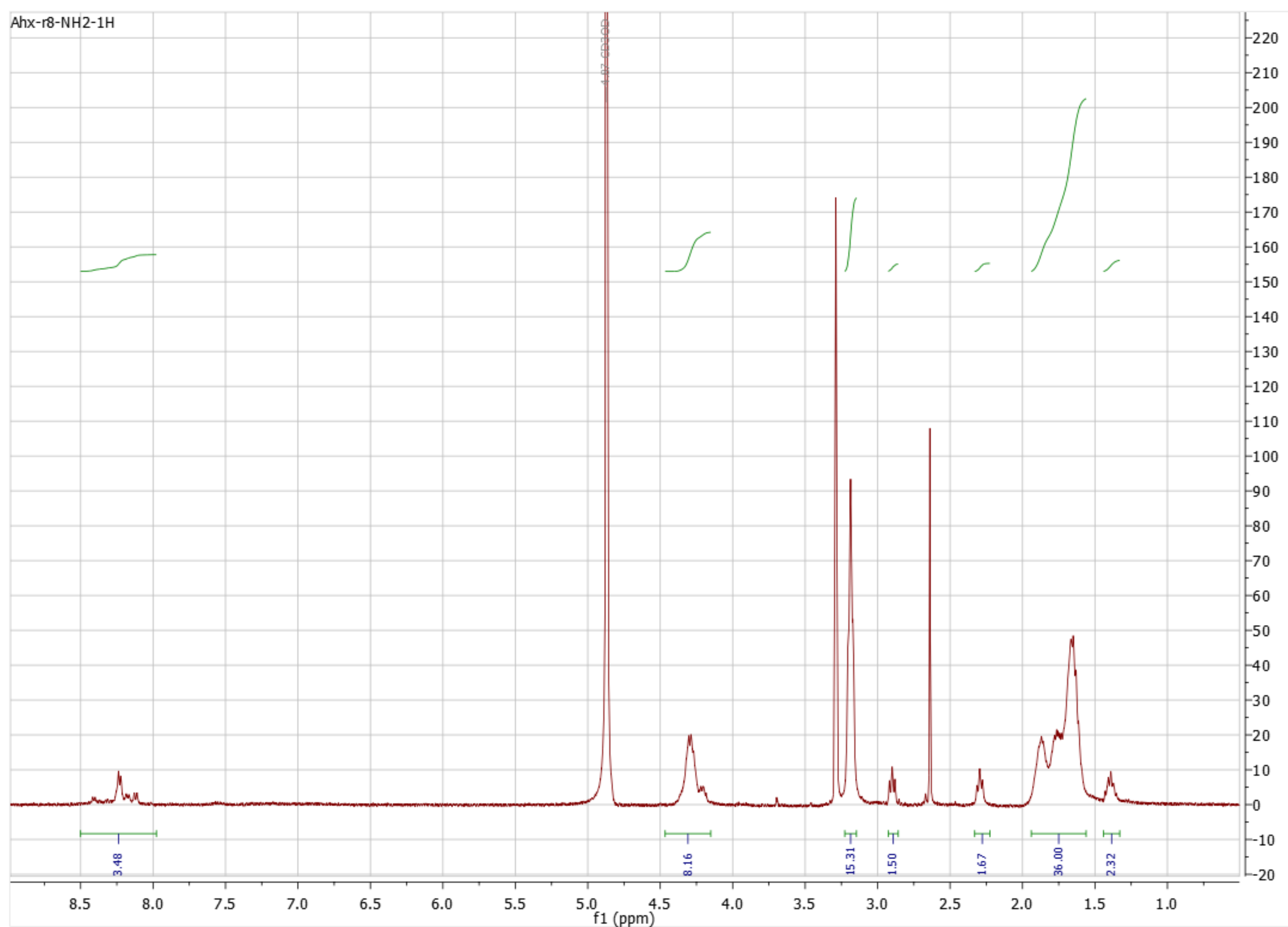
^{1}H NMR spectrum for Cbz-ahx-r8 (TFA)



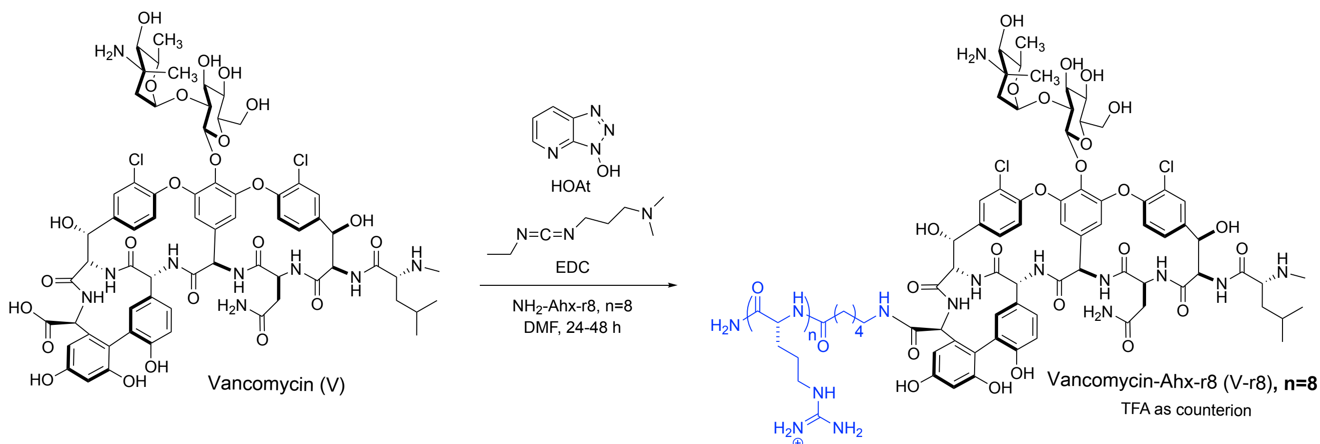
<u>NH_2-ahx-r8 (TFA)</u>

Cbx-Ahx-r8 (TFA) (45.2 mg, 0.186 mmol, 1 molar equiv) and Pd/C (2 mg, 10 w/w%) were dissolved in anhydrous methanol (200 µL). The reaction vial was flushed with N_2, then hydrogen gas (H_2). The reaction mixture stirred at room temperature under an H_2-filled balloon at 1 atm for 5 h, then was filtered through Celite and concentrated *in vacuo* to yield the deprotected peptide. The peptide was further purified by RP-HPLC on a preparative C18 column, 5-70% CH_3CN/H_2O with 0.1% TFA over 30 min. The appropriate fractions were lyophilized and the product was isolated as a white solid (64% yield, one peak by HPLC).

^{1}H-NMR (CD_3OD, 600 MHz): δ 8.42 – 8.10 (m, 3H), 4.32 – 4.29 (m, 8H), 3.21 (m, 15H), 2.92 (t, J = 7.1 Hz, 2H), 2.32 (t, J = 7.2 Hz, 2H), 2.04 – 1.66 (m, 36H), 1.43 – 1.40 (m, 2H) ppm.
^{13}C-NMR (CD_3OD, 101 MHz): δ 176.6, 176.4, 175.2, 174.7, 174.6, 174.5, 174.4, 174.0, 163.1, 162.8, 162.7, 55.7, 55.4, 55.3, 55.1, 54.9, 54.8, 54.2, 42.0, 41.9, 40.5, 36.3, 30.3, 29.7, 29.6, 29.5, 28.2, 27.1, 26.3, 26.2 ppm.
HRMS (ES+ m/z): Calculated for $C_{54}H_{114}N_{34}O_9^{2+}$: 690.4835 (M+2H)/2. Found: 690.4670 (M+2H)/2.
T_R: 5.4 minutes.



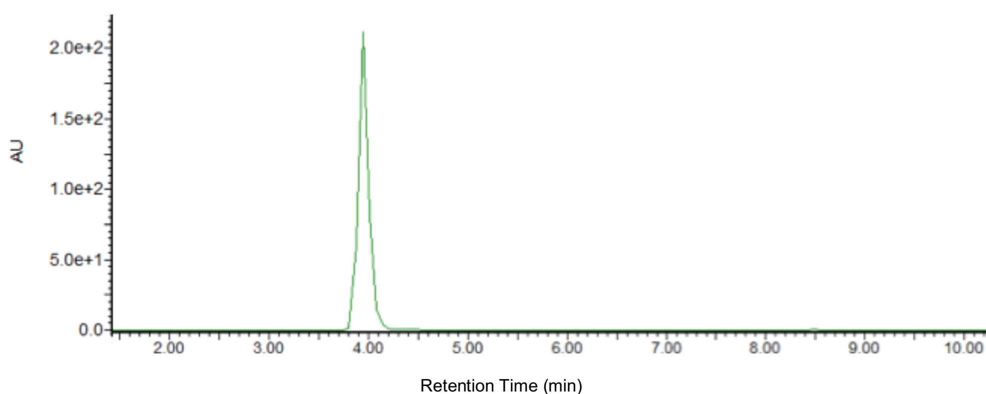
^{1}H NMR spectrum for NH_2-ahx-r8 (TFA)



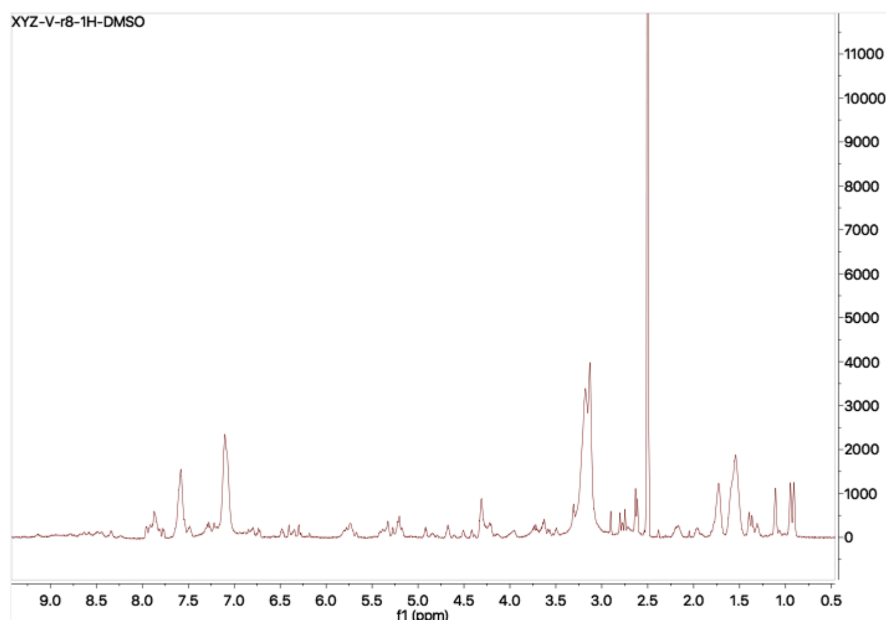
V-r8 (TFA)[2]

Vancomycin-HCl (37.1 mg, 0.025 mmol, 1.5 molar equiv) was added to an oven-dried vial containing a stir bar. HOAt (12.1 mg, 0.089 mmol, 5.4 molar equiv) and EDC-HCl (16.2 mg, 0.085 mmol, 5.1 molar equiv) were added to the vial. To a separate vial was added NH_2-ahx-r8 (TFA) (37.7 mg, 0.017 mmol, 1 molar equiv). Both vials were filled with argon and 0.5 mL dry DMF was added to each vial. The peptide was quantitatively transferred (rinsed twice with 0.2 mL DMF after the first transfer) to the vancomycin vial for a final concentration of 12 mM of peptide. N-methylmorpholine (10% total volume) was added dropwise and the reaction mixture turned from cloudy white to cloudy yellow. The reaction was stirred at room temperature for 24-36 h, at which point the reaction mixture turned clear. 1 mL HPLC grade H_2O was added slowly to dilute the reaction. The crude mixture was lyophilized overnight. The crude foam was redissolved in 1-1.5 mL HPLC grade H_2O and purified by RP-HPLC on a semi-preparative C18 column with 5-55% CH_3CN/H_2O with 0.1% TFA over 30 min. The appropriate fractions were lyophilized and the product was isolated as a TFA salt (41% yield, one peak by HPLC). The compound was stored as frozen aliquots in MQ water at -20 °C. Defrosted aliquots were used within 24 h for experiments. Aliquot concentrations were determined using Nanodrop UV-Vis spectrometer based on Beer's Law (λ=280 nm for vancomycin, ε=4200, path length=1 mm).

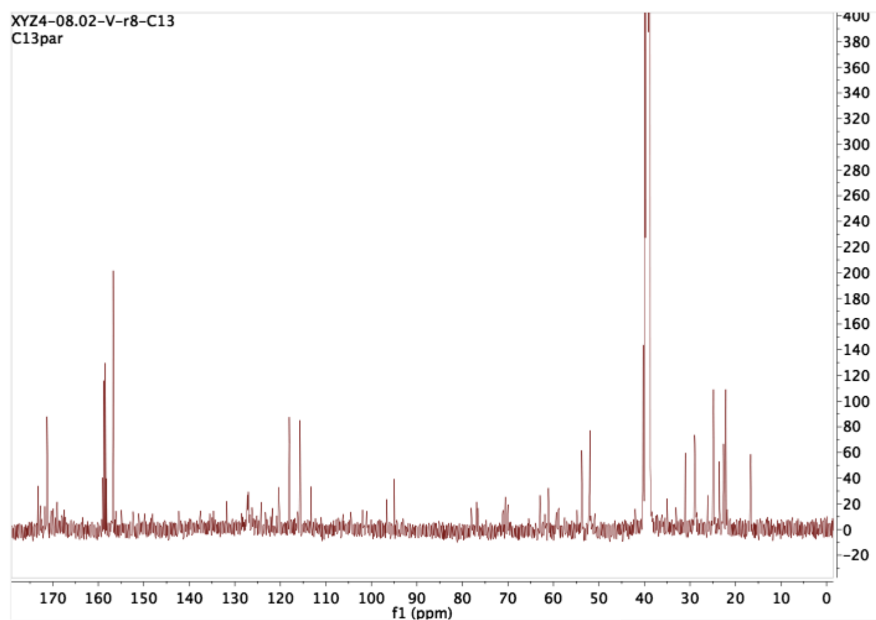
LC trace:



^{1}H-NMR (CD_3OD, 500 MHz): δ 9.16 – 8.74 (m, 1H), 8.25 (dt, *J* = 48.4, 4.9 Hz, 1H), 7.79 – 7.45 (m, 3H), 7.39 – 7.09 (m, 2H), 7.09 – 6.91 (m, 1H), 6.85 (d, *J* = 7.6 Hz, 1H), 6.54 – 6.28 (m, 1H), 5.81 (d, *J* = 15.6 Hz, 1H), 5.58 – 5.23 (m, 4H), 4.75 – 4.63 (m, 1H), 4.63 – 4.56 (m, 0.25 H), 4.50 – 4.18 (m, 8H), 4.18 – 4.01 (m, 1H), 4.00 – 3.70 (m, 2H), 3.70 – 3.58 (m, 1H), 3.21 (d, *J* = 11.0 Hz, 12H), 3.02 – 2.64 (m, 4H), 2.31 (s, 2H), 2.13 – 2.02 (m, 1H), 2.02 – 1.55 (m, 30H), 1.54 – 1.44 (m, 1H), 1.43 – 1.33 (m, 1H), 1.27 – 1.16 (m, 2H), 1.10 – 0.86 (m, 6H) ppm.
^{13}C-NMR: (DMSO, 126 MHz) 174.1, 173.1, 172.6, 172.1, 171.9, 170.7, 169.9, 159.8, 159.6, 159.3, 159.1, 157.8, 157.6, 157.0, 155.8, 150.6, 143.2, 138.4, 135.5, 132.6, 127.9, 126.9, 125.1, 122.6, 121.3, 118.9, 116.5, 114.1, 107.1, 105.4, 102.8, 101.9, 97.5, 95.8, 78.9, 77.7, 77.5, 71.4, 70.9, 63.8, 61.9, 60.2, 59.7, 55.7, 54.6, 52.8, 41.1, 35.9, 33.9, 31.9, 29.7, 26.9, 25.7, 25.8, 24.4, 23.5, 23.0, 17.5 ppm.
^{19}F-NMR: (CD_3OD, 376 MHz): δ –76 .9 ppm.
HRMS (ES+ *m/z*): Calculated for $C_{120}H_{185}Cl_2N_{43}O_{32}^{2+}$: 1406.1752 (M+2H)/2, $C_{120}H_{186}Cl_2N_{43}O_{32}^{3+}$: 937.0783 (M+3H)/3. Found: 1406.1726 (M+2H)/2, 938.0782 (M+3H)/3.
T_R: 3.91 minutes.



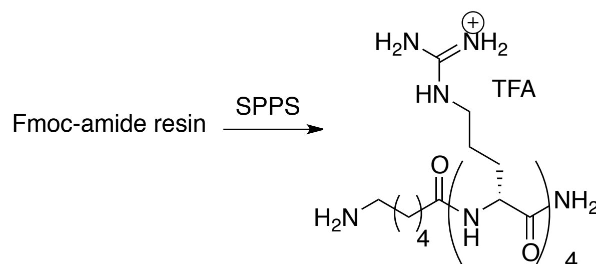
^{1}H NMR spectrum for V-r8 (TFA)



^{13}C NMR spectrum for V-r8 (TFA)

V-r8 (HCl) [3]

To the V-r8 (TFA) (11.9 mg) was added biological-grade water (12 mL). To this solution was added 100 mM HCl (aq) for a final concentration of ~10 mM. The solution sat at room temperature for one minute, then was lyophilized overnight. This procedure was repeated twice more. ^{1}H-NMR was the same as the spectrum before conversion. The conversion from TFA salt to Cl salt was confirmed by the absence of a peak by ^{19}F-NMR. The compound was stored as frozen aliquots in MilliQ water at -20 °C. Defrosted aliquots were used within 24 h for experiments. Concentrations of aliquots were determined using a Nanodrop UV-Vis spectrometer based on Beer's Law (λ=280 nm for vancomycin, ε=4200, path length=1 mm)



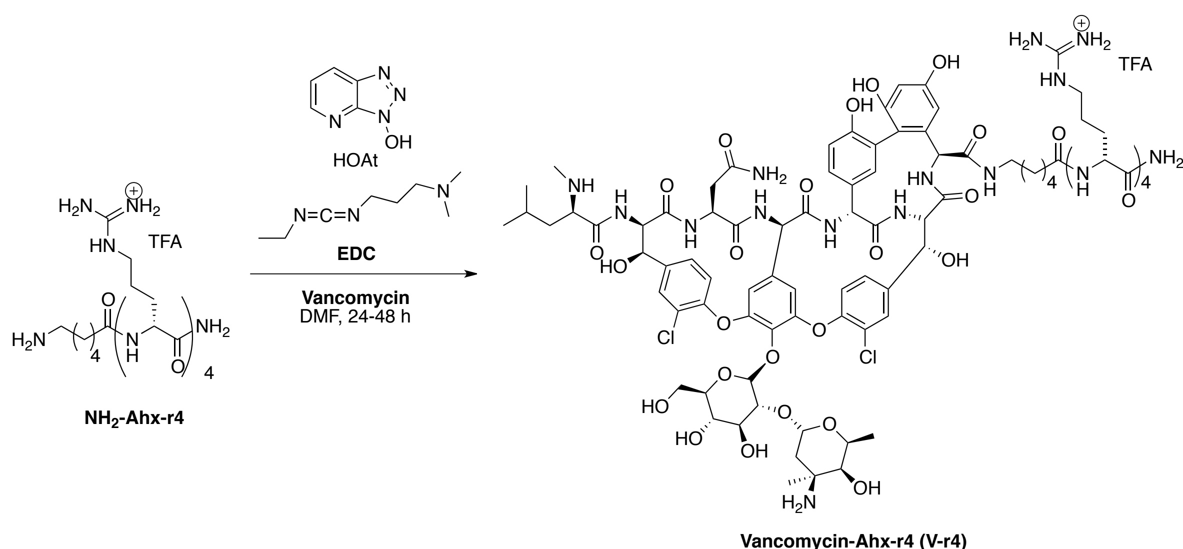
NH_2-ahx-r4[4]

Fmoc-Rink amide resin (169 mg, 0.11 mmol, 1 molar equiv) was mixed in DMF under nitrogen in a fritted peptide vessel for 20 min. The peptide vessel was drained via vacuum and the Fmoc-protecting group was removed by mixing resin in 8 mL of a 20% piperidine/DMF solution for 30 min. The vessel was drained via vacuum and washed with DMF (2x) and DCM (2x). A Kaiser resin test was performed on the resin by adding one drop each of (i) 5 g of ninhydrin in 100 mL ethanol, (ii) 80 g of liquefied phenol in 20 mL of ethanol, (iii) 0.001 M aqueous potassium cyanide in pyridine to a resin sample and the mixture was mixed by shaking and heated with a heat gun for 30 seconds. A positive test (indicating a free amine) resulted in blue-colored beads while a negative test (indicating a protected amine) resulted in dark red-colored beads.

After deprotection was complete, Fmoc-D-Arg (pbf)-OH (3.5 molar equiv) or Fmoc-Ahx-OH (3.5 molar equiv), HOBT (3.5 molar equiv), and HBTU (3.5 molar equiv) were dissolved in 10 mL DMF followed by the addition of DIPEA (10 molar equiv). The mixture was added to the resin and agitated for 2 h with a stream of nitrogen. The vessel was drained via vacuum and washed with DMF (2x) and DCM (2x) and a Kaiser Resin test was conducted to determine if the coupling was complete. The Fmoc deprotection and coupling sequence was repeated until the desired peptide was assembled.

After the final Fmoc deprotection, the resin was transferred to a 15 mL falcon tube and put under vacuum to dry for several hours. The peptide was deprotected and cleaved from the solid support by exposing the resin to a solution of 95% TFA and 5% triisopropylsilane. The mixture was mixed on a Labquake™ rotator for 24 h at room temperature. The solution was then filtered to remove the resin and concentrated under reduced pressure for 30 min to produce an oil. To the oil was added ~0.5 mL cold (0 °C) diethyl ether. The material was pelleted via centrifugation, and the ether layer was removed. The pellet was washed two additional times with dry diethyl ether before drying under vacuum. The crude peptide was dissolved in TFA/water then purified by RP-HPLC (5-70% CH_3CN/H2O with 0.1% TFA over 30 min) to afford a white solid (one peak by HPLC) after lyophilization.

^{1}H-NMR (CD_3OD, 600 MHz): δ 8.32 – 8.16 (m, 4H), 4.37 – 4.35 (m, 3H), 4.30 – 4.28 (m, 1H), 3.21 (s, 8H), 2.92 (t, *J* = 7.5 Hz, 2H), 2.30 (t, *J* = 7.4 Hz, 2H), 1.92 – 1.66 (m, 20H), 1.44 – 1.39 (m, 2H) ppm.
HRMS (ES+ *m/z*): Calculated for $C_{30}H_{63}N_{18}O_5^+$: 755.5278 (M+H). Found: 755.5213 (M+H).
T_R: 6 minutes.



V-r4 (TFA)

Vancomycin-HCl (20.2 mg, 0.014 mmol, 1.5 molar equiv) was added to an oven-dried vial containing a stir bar. HOAt (5.8 mg, 0.04 mmol, 5.0 molar equiv) and EDC-HCl (8.7 mg, 0.05 mmol, 5.3 molar equiv) were added to the vial. To a separate vial was added peptide NH2-ahx-r4 (10.3 mg, 0.01 mmol, 1.0 molar equiv). Both vials were filled with argon and 0.2 mL dry DMF was added to each vial. The peptide was quantitatively transferred (rinsed twice with 0.1 mL DMF after the first transfer) to the vancomycin vial for a final concentration of 12 mM of peptide. N-methylmorpholine (0.06 mL, 10% total volume) was added dropwise and the reaction mixture turned from cloudy white to cloudy yellow. The reaction was stirred at room temperature for 18 h, at which point

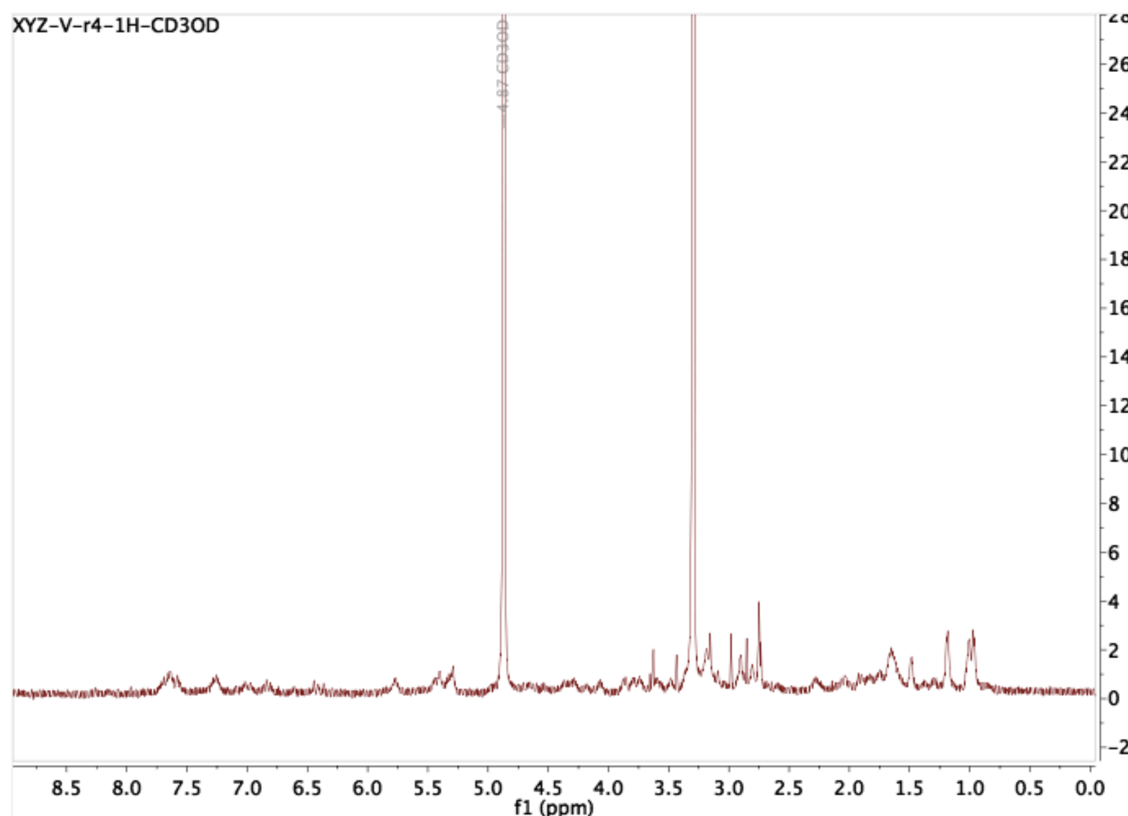
the reaction mixture had turned clear. 1 mL HPLC grade H_2O was added slowly to dilute the reaction. The crude mixture was lyophilized overnight and redissolved in 1 mL HPLC grade H_2O and purified by RP-HPLC on a semi-preparative C18 column with 5-70-100% CH_3CN/H_2O with 0.1% TFA over 40 min. The appropriate fractions were lyophilized and the product was isolated as a TFA salt (26% yield, one peak by HPLC). The compound was stored as frozen aliquots in MilliQ water at -20 °C. Defrosted aliquots would be used within 24 h for experiments. Concentration of aliquots were determined using Nanodrop UV-Vis spectrometer based on Beer's Law ((λ=280 nm for vancomycin, ε=4200, path length=1 mm).

^{1}H-NMR (CD_3OD, 500 MHz): δ 7.67 (t, J = 31.0 Hz, 3H), 7.29 (s, 2H), 7.16 – 6.95 (m, 1H), 6.87 (s, 1H), 6.61 – 6.26 (m, 1H), 5.81 (s, 1H), 5.38 (d, J = 55.8 Hz, 4H), 4.83 – 4.61 (m, 1H), 4.59 – 4.16 (m, 2H), 4.10 (s, 1H), 3.98 – 3.73 (m, 2H), 3.71 – 3.57 (m, 1H), 3.57 – 3.49 (m, 1H), 3.47 (q, J = 1.7 Hz, 1H), 3.26 – 3.10 (m, 4H), 3.02 (s, 1H), 3.00 – 2.90 (m, 1H), 2.90 – 2.87 (m, 1H), 2.86 – 2.81 (m, 1H), 2.78 (d, J = 7.2 Hz, 2H), 2.41 – 2.22 (m, 1H), 2.19 – 1.99 (m, 1H), 1.68 (s, 5H), 1.52 (s, 1H), 1.46 – 1.38 (m, 1H), 1.38 – 1.28 (m, 1H), 1.22 (s, 3H), 1.09 – 0.94 (m, 6H) ppm.

^{19}F-NMR: (CD_3OD, 376 MHz): δ –76 .9 ppm.

HRMS (ES+ *m/z*): Calculated for $C_{96}H_{137}Cl_2N_{27}O_{28}^{2+}$: 1093.4837 (M+2H)/2, $C_{96}H_{138}Cl_2N_{27}O_2^{3+}$: 729.3210(M+3H)/3. Found: 1094.4117 (M+2H)/2, 729.2594 (M+3H)/3.

T_R: 15 minutes.



^{1}H NMR spectrum for V-r4 (TFA)

Fl-V[5] (TFA)

Synthesis was adapted from literature procedure.[5] Vancomycin (HCl) (16.1 mg, 0.011 mmol, 1.0 molar equiv) was dissolved in 2.5 mL bicarbonate buffer ($Na_2CO_3/NaHCO_3$, pH=10) in an oven-dried vial charged with a stir bar. FITC (25 mg, 0.064 mmol, 6.0 molar equiv) was dissolved in 25 µL DMSO and added to the vancomycin vial. An orange precipitate formed immediately. The reaction was stirred at 4 °C overnight. The reaction mixture was filtered and purified by RP-HPLC, 10-30-90% CH_3CN/H_2O with 0.1% TFA over 30 min. The appropriate fractions were isolated and lyophilized to afford an orange powder (55% yield, one peak by HPLC).

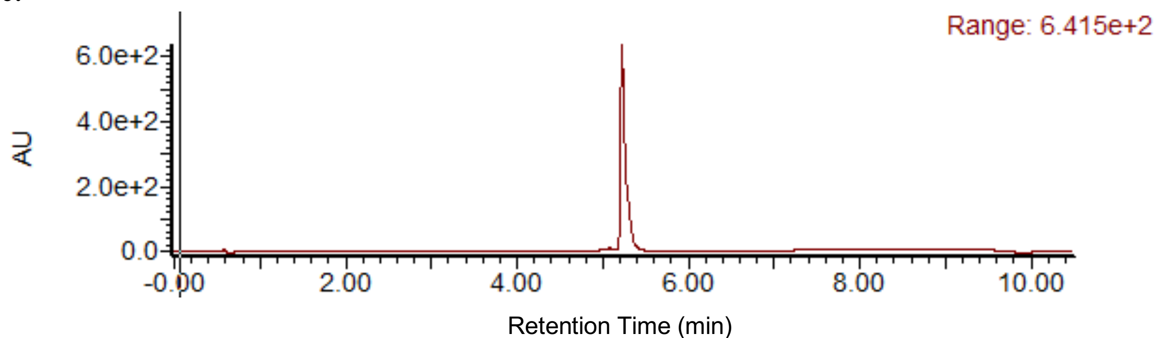
HRMS (ES+ *m/z*): Calculated for $C_{87}H_{86}Cl_2N_{10}O_{29}S^+$: 1836.470 (M+H). Found: 1839.464 (M+H) and 1696.379 (M-glucose+H, *m/z* found matched with literature where fluorescein was conjugated with secondary amine on the peptide core of vancomycin)[5]

T_R: 11.7 minutes. (10~80% CH_3CN/H_2O with 0.1% TFA over 15 min on analytical RP-HPLC)

Fl-V-r8 (TFA)

V-r8 (TFA) a(12.0 mg, 0.0037 mmol, 1.0 molar equiv) was dissolved in 1.8 mL bicarbonate buffer ($Na_2CO_3/NaHCO_3$, pH=10) in an oven-dried vial charged with a stir bar. FITC (8.9 mg, 0.023 mmol, 6.0 molar equiv) was dissolved in a separate vial in 10 µL DMSO and added. Reaction was stirred at 4 °C for 36 h and monitored by LC/MS. The reaction mixture was filtered and purified by RP-HPLC 10-90% CH_3CN/H_2O with 0.1% TFA over 30 min. The appropriate fractions were isolated and lyophilized to afford an orange powder (23% yield, one peak by HPLC).

LC trace:



MALDI-MS (*m/z*): Calculated 1020.28 (M-glucose+3H)/3. Found: 1020.29.

T_R: 5.34 minutes.

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