Supporting Information

General.

Chemical reagents and solvents were used as received from commercial sources. $^1$H NMR spectra were obtained at 400 MHz in DMSO-d$_6$ or CD$_3$OD. Chemical shifts are expressed as parts per million using the solvent peak as the internal standard and coupling constants are reported in hertz. Preparative HPLC was carried out on an Agilent Series 1100 system equipped with a Varian Dynamax Microsorb C-18 (100Å, 250 x 21.4 mm) column at a flow rate of 8 mL/min. LC-MS was carried out on an Agilent Series 1100 system equipped with a Zorbax SB C-18 column and a Series 1100 MSD at a flow rate of 0.4 mL/min. Fluorescence spectra were recorded on a Perkin-Elmer LS-50B. Surface plasmon resonance spectroscopy was performed on a Biacore™ 3000 system using commercially available CM-5 (carboxymethylated dextran) chips.

General Procedure for Synthesis of Linked Vancomycin Dimers$^1$: Compound 6.$^3$

To a stirring solution of vancomycin hydrochloride (Acros) (100 mg, 67 µmol, 2.2 equiv) in DMSO (1 mL) was added a solution of p-xylylenediamine (Aldrich) (4.1 mg, 30 µmol, 1 equiv) in DMF (1 mL). The mixture was cooled to 0 °C and a solution of HBTU (Novabiochem) (27 mg, 71 µmol, 2.3 equiv) in 0.2 mL DMF was added, followed by 26 µL (150 µmol, 5 equiv) of iPr$_2$EtN. Stirring at 0 °C was continued for an additional 15 min and diethyl ether (10 mL) was added to form a white precipitate. The solid was collected by centrifugation (7100 rpm, 10 min) and triturated with diethyl ether (10 mL). The crude product was purified by HPLC (5→95% CH$_3$CN (0.1% TFA) in H$_2$O (0.1% TFA) over 60 min). The combined product fractions were lyophilized to afford 61 mg (60%) of 6, as the tetra-trifluoroacetate salt: The $^1$H NMR (400 MHz, DMSO-d$_6$) spectrum showed resonances attributable to vancomycin as well as the linker region, as was previously reported$^3$ [spectrum attached at the end of Supp. Inf.]: LC-MS (ESI) (10→90% CH$_3$CN (0.1% TFA) in H$_2$O (0.1% TFA) over 20 min) $t_R = 6.34$ min (purity = 98%), m/z calcld for C$_{140}$H$_{161}$Cl$_4$N$_2$O$_{46}$ [M+3H]$^3+$: 999.3, found 999.9; HRMS (ESI) m/z calcld for C$_{140}$H$_{161}$Cl$_4$N$_2$O$_{46}$ [M+3H]$^3+$: 999.3204, found 999.3232; [M+4H]$^4+$: 749.7421, found 749.7430.

Compound 5.$^2$

The title compound was synthesized according to the general procedure from vancomycin hydrochloride (100 mg, 67 µmol, 2.2 equiv) and 1,6-hexanediamine (Aldrich) (3.5 mg, 30 µmol, 1 equiv) to afford 55 mg (53%) of 5, as the tetra-trifluoroacetate salt: The $^1$H NMR (400 MHz, DMSO-d$_6$) spectrum showed resonances attributable to vancomycin as well as the linker region, as was previously reported$^2$ [spectrum attached at the end of Supp. Inf.]: LC-MS (ESI) (10→90% CH$_3$CN (0.1% TFA) in H$_2$O (0.1% TFA) over 20 min) $t_R = 6.23$ min (purity = 97%), m/z calcld for C$_{138}$H$_{165}$Cl$_4$N$_2$O$_{46}$ [M+3H]$^3+$: 992.7, found 993.8; HRMS (ESI) m/z calcld for C$_{138}$H$_{165}$Cl$_4$N$_2$O$_{46}$ [M+3H]$^3+$: 992.6641, found 992.6682; [M+4H]$^4+$: 744.7499, found 744.7510.

To a solution of 5 (15.6 mg, 4.6 µmol, 1 equiv) in 300 µL of pyridine/H2O (1:1, v/v) was added phenylisothiocyanate (Aldrich) (1.63 µL 13.7 µmol, 3 equiv). After stirring for 6 h, diethyl ether (5 mL) was added to form a yellow precipitate. The precipitate was collected by centrifugation (7100 rpm, 10 min) and the resultant pellet was triturated with water (2 mL) and was dried overnight in vacuo. The crude bis-thiourea species was taken up in 700 µL of 5% TFA in CH2Cl2 and stirred for 2 h. Methanol (1 mL) was added and the resultant solution was evaporated in vacuo. The white residue was dissolved in methanol (0.5 mL) and precipitated with diethyl ether (5 mL). The crude product was collected by centrifugation (7100 rpm, 10 min) and was purified by HPLC (5 → 48% CH3CN (0.1% TFA) in H2O (0.1% TFA) over 70 min). The combined product fractions were lyophilized to afford 8 mg (55%) of 3, as the tetra-trifluoroacetate salt: The 1H NMR (400 MHz, DMSO-d6) spectrum showed resonances characteristic of desleucyl-vancomycin4 (δ 2.89 and 2.11, asparagine β-protons), loss of resonances arising from the N-Me-Leucine δ-protons of 5 (δ 0.97-0.79), presence of resonances arising from the two vancosamine methyl groups (δ 1.29 and 1.08) as well as the presence of resonances arising from the linker: δ 3.13 (-HNCH2CH2CH2-), 1.47 (-HNCH2CH2CH2-), 1.30 (-HNCH2CH2CH2-)] [spectrum attached at the end of Supp. Inf.]; LC-MS (ESI) (10 → 90% CH3CN (0.1% TFA) in H2O (0.1% TFA) over 20 min) tR = 5.16 min (purity = 98%), (2 → 90% MeOH (0.1% TFA) in H2O (0.1% TFA) over 20 min) tR = 11.92 min (purity = 96%), m/z calcd for C124H139Cl4N18O44 [M+3H]3+: 907.9, found 908.5.

Compound 4.

The title compound was synthesized from 6 (15.8 mg, 4.6 µmol, 1 equiv) according to the general procedure to afford 6.5 mg (44%) of 4, as the tetra-trifluoroacetate salt: The 1H NMR (400 MHz, DMSO-d6) spectrum showed resonances characteristic of desleucyl-vancomycin4 (δ 2.90 and 2.10, asparagine β-protons), loss of resonances arising from the N-Me-Leucine δ-protons of 6 (δ 0.97-0.79), presence of resonances arising from the two vancosamine methyl groups (δ 1.29 and 1.08) as well as the presence of resonances arising from the linker: δ 7.19 (phenyl CHs), 4.45 (-CH2PhCH2-) [spectrum attached at the end of Supp. Inf.]; LC-MS (ESI) (10 → 90% CH3CN (0.1% TFA) in H2O (0.1% TFA) over 20 min) tR = 5.22 min (purity = 97%), (2 → 90% MeOH (0.1% TFA) in H2O (0.1% TFA) over 20 min) tR = 11.80 min (purity = 97%), m/z calcd for C126H134Cl4N18O44 [M+2H]2+: 1371.3772, found 1371.3820; [M+3H]3+: 914.5872, found 914.5898.

g-N-succinyl-(dansyl-Lys-D-Ala-D-Ala)2.

Fmoc-D-Ala-Wang-PS resin (Novabiochem) (208 mg, 0.15 mmol, 0.72 mmol/gram) was suspended in 3 mL of CH2Cl2 for 30 min and filtered under N2. The Fmoc group was removed by treatment with 20% piperidine/DMF (v/v) (3 mL, 10 min, 3x) and the resin was washed (DMF, CH2Cl2, 3 x 3 mL ea.) and filtered under N2. A mixture of Fmoc-D-Ala-OH (93 mg, 0.30 mmol, 2 equiv), PyBOP (156 mg, 0.30 mmol, 2 equiv), iPr2EtN
(114 µL, 0.66 mmol, 4.4 equiv) in DMF (1.2 mL) was added to the resin and was shaken for 1h. The resin was washed, dried and subjected to the same coupling conditions to ensure near-quantitative acylation evidenced by a negative qualitative Kaiser test. Extension to the resin-bound Fmoc-tripeptide was achieved by subjecting to identical conditions for Fmoc deprotection and coupling (vide supra) except Fmoc-Lys(Dde)-OH (Novabiochem) was used in place of Fmoc-d-Ala-OH. The resin-bound Fmoc-tripeptide was treated with 20% piperidine/DMF (v/v) (3 mL, 5 min, 4x) to remove the Fmoc group, and was washed (DMF, CH₂Cl₂, MeOH, THF, 3 x 3 mL ea.). A solution of dansyl-chloride (Sigma) (120 mg, 0.45 mmol, 3 equiv), iPr₂EtN (157 µL, 0.9 mmol, 6 equiv) in dry THF (2 mL) was added and the resin was shaken overnight. The resin was washed (THF, CH₂Cl₂, MeOH, DMF, 2 x 3 mL ea.) and was suspended in 2% hydrazine/DMF (v/v) (3 mL, 5 min, 3 x) to remove the Dde group. After washing (DMF, CH₂Cl₂, 3x3 mL ea.), the filtered resin was treated with a mixture of succinic acid (9 mg, 0.075 mmol, 0.5 equiv), PyBOP (156 mg, 0.3 mmol, 2 equiv), iPr₂EtN (114 µL, 0.66 mol, 4.4 equiv) in DMF (2.0 mL) and was shaken for 24h. The resin was washed (DMF, CH₂Cl₂, MeOH, 3 x 4 mL ea.) and dried overnight in vacuo. The dry resin was suspended in CH₂Cl₂ for 2 h, filtered under N₂, and treated with TFA/triisopropylsilane/H₂O (95:2.5:2.5, v/v) for 4h. The solution was added into 10 mL of diethyl ether to form the crude product precipitate, which was purified by HPLC (5→95% CH₃CN (0.1% TFA) in H₂O (0.1% TFA) over 55 min). The combined product fractions were lyophilized to afford a white powder (35 mg, 42%): ¹H NMR (400 MHz, CD3OD) δ 8.53 (d, J = 8.5, 4H) 8.25 (d, J = 7.4, 2H) 7.71-7.60 (m, 4H) 7.49 (d, J = 7.6, 2H) 4.31 (q, J = 7.4, 2H) 4.18-4.09 (m, 2H) 3.64 (q, J = 5.1, 2H) 3.04 (s, 12H) 2.83-2.68 (m, 4H) 2.39 (s, b, 4H) 1.61-1.41 (m, 4H) 1.38 (d, J = 7.4, 6H) 1.22-0.86 (m, 14H); LC-MS (ESI) (10→90% CH₃CN (0.1% TFA) in H₂O (0.1% TFA) over 10 min) tR = 6.47 min (purity = 98%), calcd for C₅₂H₇₃N₁₀O₁₄S₂ [M+H]⁺: 1125.5, found 1125.8.

Fluorescence titration experiments.

Solutions of either dansyl-Lys(Ac)-d-Ala-d-Ala⁵ or ε-N-succinyl-(dansyl-Lys-d-Ala-d-Ala)₂ (1 µM in 10 mM HEPES, 6 mM NaCl, pH = 7.4) were incubated in a 4 mL quartz cuvette (Starna) at 298K, for 10 min immediately prior to performing titrations. The emission spectrum of the peptide was measured through irradiation at 330 nm. Three emission scans were average and recorded. The titrant solution containing the compound of interest (1-6) was added and the solution was agitation and allowed to equilibrate for 5 min prior to recording the emission spectrum. To avoid dilution, 1 µM peptide was added into the titrant. Three emission scans were averaged and recorded for each titration data point.

Titrations of dansyl-Lys(Ac)-d-Ala-d-Ala or ε-N-succinyl-(dansyl-Lys-d-Ala-d-Ala)₂ by 1, 5, and 6 were performed until no further changes were detected on their emission (2~6 µM). Data derived from the titration of dansyl-Lys(Ac)-d-Ala-d-Ala by 1, 5 and 6 were analyzed by non-linear regression using the program SigmaPlot 2001 (SPSS Inc.) and the following expression derived from a 1:1 equilibrium association model:

\[
F-F₀ = ΔQ/2*((Pep₀+Cpd₀+Kₐ)(((Pep₀+Cpd₀+Kₐ)²)⁴*Pep₀*Cpd₀))/2); \]

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where $F$ is the fluorescence observed after each addition of the compound (1, 5 or 6), $F_0$ is the fluorescence of the dansyl-Lys(Ac)-D-Ala-D-Ala alone, $\Delta Q$ is the constant proportional to the difference between the quantum yields of the complexed dansyl-Lys(Ac)-D-Ala-D-Ala and free dansyl-Lys(Ac)-D-Ala-D-Ala. $\text{Pep}_0$ is the initial concentration of the peptide and $\text{Cpd}_0$ is the initial concentration of the compound (1, 5 or 6) after each addition. $\Delta Q$ and $K_d$ were the two unknown parameters obtained by least-squares curve-fitting.

Estimation of the lower limit of the $K_d$ of compounds 2-4 for either dansyl-Lys(Ac)-D-Ala-D-Ala or $\varepsilon$-N-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$ were carried out by addition of higher concentrations (20–60 $\mu$M) of titrant. The compounds at these high concentrations were themselves substantially fluorescent, requiring the subtraction of such contributions to the emission intensity from the titration profile. The addition of ~60 $\mu$M of 2-4 resulted in no significant change on the fluorescence emission spectrum of either dansyl-Lys(Ac)-D-Ala-D-Ala or $\varepsilon$-N-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$ (after correction for background), indicating no binding and that the $K_d$’s are greater than 60 $\mu$M (example shown in Figure 1b).

**Figure 1.** a) Titration of dansyl-Lys(Ac)-D-Ala-D-Ala (1 $\mu$M) with 1 (black triangles), 5 (gray triangles), 6 (open triangles), 3 (gray circles), 2 (black circles). These were uncorrected for changes in background due to receptor fluorescence as their contribution was minimal (<10%). Curves correspond to best-fit lines obtained by non-linear regression of the data to equation 1. b) No fluorescence enhancement is observed upon addition of 4 (60 $\mu$M) to $\varepsilon$-N-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$ (1 $\mu$M) after correction for background.
**Coupling of peptides to SPR chips.**

A Biacore™ CM-5 chip was docked in a Biacore™ 3000 biosensor and deionized water was run at 10 µL/min over the flow cells (20 min). Equal volumes of N-hydroxysuccinimide (50 µL, 100 mM in deionized water) and EDCI (50 µL, 400 mM in deionized water) were combined, and 50 µL of this solution was injected over flow cells 1, 2 and 3 (50 µL, 10 µL/min). Solutions of Ac-Lys-D-Ala-D-Lac (Bachem) or Ac-Lys-D-Ala-d-Ala (Sigma) (20 mg/mL) in NaOAc buffer (10 mM, pH = 8.5) were subsequently injected (70 µL, 10 µL/min) across flow cells 2 and 3, respectively. Finally, cells 1, 2 and 3 were inactivated by injection (50 µL, 10 µL/min) of aqueous ethanolamine (1 M, pH = 8).

**SPR Binding Expts.**

Solutions of compound 4 or 6 (2 µM-3 nM) in HBS buffer (Biacore™) (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) were centrifuged at 14000 rpm for 10 min to remove all particulate materials. Each compound solution was eluted (150 µL, 30 µL/min) serially over cells 1, 2, 3, followed by HBS buffer (100 µL, 30 µL/min). After each association / dissociation cycle, the surfaces were regenerated by injection of 10 mM HCl (10 µL x 2, 30 µL/min) followed by HBS buffer (30 µL/min), until a stable baseline was achieved.

**SPR Data Analysis.**

All obtained sensorgram data were adjusted such that the time of each injection was set to 0 seconds (t = 0). Data obtained from the control flow cell (cell 1) was subtracted from all data obtained from peptide-derivatized flow cells (cell 2 or 3) inorder to correct for bulk refractive index changes and non-specific binding.

The $K_d$ of compound 6 binding to Ac-Lys-D-Ala-d-Ala was measured by Scatchard analysis of the sensorgram data using the following equation:

$$\frac{R_{eq}}{C} = -\frac{R_{eq}}{K_d} + \frac{R_{max}}{K_d}$$

Where $R_{eq}$ is the response reached at equilibrium (Fig. 2a), $C$ is the initial concentration of compound 6, and $R_{max}$ is the maximal possible response. A plot of $R_{eq}/C$ vs. $R_{eq}$ derived from the sensorgram data resulted in a biphasic Scatchard plot (Fig 2b). The biphasic nature of this Scatchard plot arises from the presence two binding modes (in this case monovalent and divalent binding) with different affinities. Linear regression of these two linear regions gave lines with slopes of $-1/K_d$, allowing for calculation of the $K_d$ of 245 and 5 nM for the monovalent and divalent binding, respectively. 6
Figure 2. a) SPR sensorgrams obtained by eluting 6 (250 nM, 200 nM, 167 nM, 125 nM, 83.3 nM, 63 nM, 31.3 nM, 15.6 nM, 7.8 nM) over a Ac-Lys-D-Ala-D-Ala derivatized cell. \( R_{eq} \) corresponds to the response reached at equilibrium. b) Scatchard analysis of the data. The two linear phases were separately analyzed by linear regression to obtain the slopes that corresponding to \(-1/K_d\).

Elution of 4 and 6 at high concentrations led to large levels of non-specific adsorption to all studied surfaces. Non-specific adsorption was minimized by addition of carboxymethyl-dextran\(^7\) (Carbomer Inc., ~500,000 Da, 0.1 mmol -COOH per gram dextran, 0.5 mg/mL) to the buffer (HBS) and compound stock solutions. These new conditions allowed the measurement of 4 binding to the peptide derivatized-surfaces at higher concentrations (25 \( \mu \)M). At these higher concentrations, modest binding of 4 to Ac-Lys-D-Ala-D-Ala derivatized surfaces was observed, but no binding to Ac-Lys-D-Ala-D-Lac derivatized surfaces was detected (Figure 3).

Figure 3. a) SPR sensorgrams obtained by eluting 4 (25 \( \mu \)M) over Ac-Lys-D-Ala-D-Ala and Ac-Lys-D-Ala-D-Lac derivatized cells.

References

1) The procedure was followed according to ref. 2, with minor modifications. A shorter reaction time and fewer equivalents of HBTU was employed inorder to suppress guanidylated side-products that were difficult to separate via HPLC.
1
(vancomycin)

5

2
(desleucyl-vancomycin)

3
1
(vancomycin)

2
(desleucyl-vancomycin)

4