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Tight Binding of a Dimeric Derivative of Vancomycin with Dimeric L-Lys-D-Ala-D-Ala

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Abstract: The ligand/receptor pair consisting of a synthetic dimeric derivative of vancomycin (**V**), linked at the C terminus by *p*-xylylenediamine (**V**-CONHCH$_2$C$_6$H$_4$CH$_2$NHCO-**V**), and a dimeric derivative of L-Lys-D-Ala-D-Ala, [CH$_2$CON$^{\epsilon}$H(N$^{\alpha}$-Ac)-L-Lys-D-Ala-D-Ala-CO$_2^-$]$_2$, provides a new system with which to study the influence of divalency on the strength of binding. A competitive assay using affinity capillary electrophoresis (ACE) has been developed and used to estimate the dissociation constant of the divalent complex ($K_d^d \approx 1.1$ nM) and the enhancement in binding ($\sim 10^3$) relative to the corresponding monomeric interaction between unmodified monomeric vancomycin and diacetyl-L-Lys-D-Ala-D-Ala.

Introduction

Divalent interactions in molecular recognition involving two linked ligands interacting with two linked receptors have been studied in many systems, including dimers of cyclodextrin with divalent ligands,[1−3] dimers of sialyl Lewis x with E-selectin,[4] dimers of immunophilin ligands such as FK506 and cyclosporin A with receptors active in controlling cell signal transduction,[5] and divalent antibodies with a surface antigen.[6,7] We have begun to develop a new model system—based on polyvalent derivatives of vancomycin (**V**) and of its ligand, D-Ala-D-Ala (**DADA**)—to study the physical organic chemistry and physical biochemistry of polyvalency. The binding of **V** with **DADA** has been extensively characterized thermodynamically and structurally,[8−14] especially by Williams[15−18] and Griffin.[19−21] There is inferential evidence that spontaneous noncovalent dimerization of vancomycin and structurally related glycopeptides is responsible for their tight binding to **DADA** groups in the bacterial cell wall,[22] and thus that simultaneous interaction of the dimer with the two ligands contributes to their antibiotic action.[23−26] We now describe a system comprising synthetic dimeric derivatives of vancomycin and of **DADA** that demonstrates an increase in the strength of binding of these components of approximately 10^3 relative to the analogous binding of monovalent vancomycin and **DADA**. These complexes do not have the geometry required to model the head-to-tail dimeric structure hypothesized for the action of the unmodified antibiotic, but they suggest the entropic advantage that can be obtained through divalency.

Results and Discussion

We chose *p*-xylylenediamine as the linker R$_d$ in the dimeric derivative of vancomycin **V**-R$_d$-**V** because it is structurally rigid and easily modified synthetically. Previous studies have showed that modification at the C-terminus of **V** has little effect on its

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(1) Many dimers of cyclodextrin have been reported as divalent receptors, and a wide range of enhancement of affinity of dimers has been observed. Recently, Zhang and Breslow described a careful study of cyclodextrin dimers (*J. Am. Chem. Soc*. **1996**, *118*, 8495−8496 and references therein) and concluded that the binding constant of β-cyclodextrin (β-CD) with cholesterol was 1.7×10^4 M^{-1}, while that of a dimer of β-CD with cholesterol was 5.5×10^6 M^{-1}; the enhancement was therefore a factor of 300.

(2) Beeson, J. C.; Czarnik, A. W. *Bioorg. Med. Chem.* **1994**, *2*, 297−303.

(3) Venema, F.; Rowan, A. E.; Nolte, R. J. M. *J. Am. Chem. Soc.* **1996**, *118*, 257−258.

(4) Defrees, S. A.; Kosch, W.; Way, W.; Paulson, J. C.; Sabesan, S.; Halcomb, R. L.; Huang, D.-H.; Ichikawa, Y.; Wong, C.-H. *J. Am. Chem. Soc*. **1995**, *117*, 66−79. The concentration of the 3,6-linked SLe^x dimer required to block adhesion of 50% of the cells was 0.2 mM, and the value for the tetrasaccharide monomer was 1.2 mM.

(5) Spencer, D. M.; Wandless, T. J.; Schreiber, S. L.; Crabtree, G. R. *Science* **1993**, *262*, 1019−1024.

(6) Hornick, C. L.; Karush, F. *Isr. J. Med. Sci.* **1969**, *5*, 163−170. The binding constant of rabbit γG anti-2,4-dinitrophenyl (DNP) antibody with the monovalent hapten N$^{\epsilon}$-DNP-lysine was 6×10^6 M^{-1}, while that of the antibody with conjugated bateriophage ϕX174 with DNP groups was 3.5×10^{11} M^{-1}; the enhancement was a factor of 6×10^4.

(7) Hornick, C. L.; Karush, F. *Immunochemistry* **1972**, *9*, 325−340.

(8) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 773−787.

(9) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 789−803.

(10) Sheldrich, G. M.; Jones, P. G.; Kennard, O.; Williams, D. H.; Smith, G. A. *Nature* **1978**, *271*, 223−224.

(11) Sheldrick, G. M.; Paulus, E.; Vertesy, L.; Hahn, F. *Acta Crystallogr.* **1995**, *B51*, 89−98.

(12) Popieniek, P. H.; Pratt, R. F. *Anal. Biochem.* **1987**, *165*, 108−113.

(13) Loll, P. J.; Bevivino, A. E.; Korty, B. D.; Axelsen, P. H. *J. Am. Chem. Soc.* **1997**, *119*, 1516−1522.

(14) Chu, Y.-H.; Whitesides, G. M. *J. Org. Chem.* **1992**, *57*, 3524−3525.

(15) Pierce, C. M.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 2* **1995**, *2*, 153−157.

(16) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332−1339.

(17) Kannan, R.; Harris, C. M.; Harris, T. M.; Waltho, G. P.; Skelton, N. J.; Williams, D. H. *J. Am. Chem. Soc.* **1988**, *110*, 2946−2953.

(18) Bongini, A.; Feeney, J.; Williamson, M. P.; Williams, D. H. *J. Chem. Soc., Perkin Trans. 2* **1981**, 201−206.

(19) Shi, Z.; Griffin, J. H. *J. Am. Chem. Soc.* **1993**, *115*, 6482−6486.

(20) Sundram, U. N.; Griffin, J. H. *J. Org. Chem.* **1995**, *60*, 1102−1103.

(21) Sundram, U. N.; Griffin, J. H.; Nicas, T. I. *J. Am. Chem. Soc.* **1996**, *118*, 13107−13108.

(22) Beauregard, D. A.; Williams, D. H.; Gwynn, M. N.; Knowles, D. J. C. *Antimicrob. Agents Chemother.* **1995**, *39*, 781−785.

(23) Gerhard, U.; Mackay, J. P.; Maplestone, R. A.; Williams, D. H. *J. Am. Chem. Soc.* **1993**, *115*, 232−237.

(24) Groves, P.; Searle, M. S.; Waltho, J. P.; Williams, D. H. *J. Am. Chem. Soc.* **1995**, *117*, 7958−7964.

(25) Mackay, J. P.; Gerhard, U.; Beaurgard, D. A.; Westwell, M. S.; Searle, M. S.; Williams, D. H. *J. Am. Chem. Soc.* **1994**, *116*, 4581−4890.

(26) Westwell, M. S.; Bardsley, B.; Dancer, R. J.; Try, A. C.; Williams, D. H. *J. Chem. Soc., Chem. Commun.* **1996**, 589−590.

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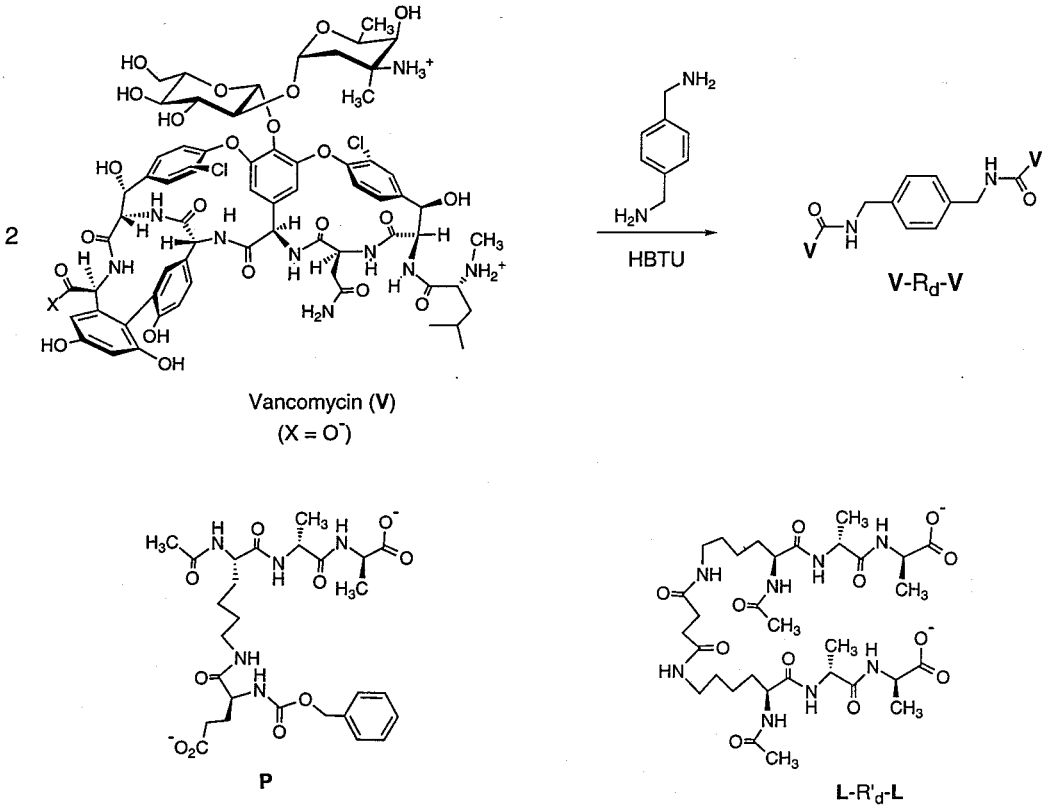
Scheme 1. The Dimeric Derivative of Vancomycin **V**-R_d-**V** and Ligands **L**-R'$_d$-**L** and **P**. HBTU: 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium Hexafluorophosphate



affinity for **DADA**.[19] We synthesized **V**-R_d-**V** by coupling two molecules of **V** with one of *p*-xylylenediamine (Scheme 1). The ^{1}H-NMR spectroscopy and ESI-MS of the product confirmed its structure. We formed the dimeric derivative of D-Ala-D-Ala, **L**-R'$_d$-**L**, by coupling the ϵ-amino groups of the lysyl moieties of the tripeptide, N$^\alpha$-Ac-L-Lys-D-Ala-D-Ala, with the two carboxylic acid groups of a succinic acid (Scheme 1). Examination of CPK molecular models of the complex suggested that **L**-R'$_d$-**L** fitted well into both pockets of **V**-R_d-**V**.

We first performed a fluorescence titration to compare the binding of **V**-R_d-**V** to a monomeric ligand and to **L**-R'$_d$-**L**. On complexation with vancomycin, there is an increase in the fluorescence intensity of the ligand N$^\alpha$-dansyl-N$^\epsilon$-Ac-L-Lys-D-Ala-D-Ala (f**L**).[12] A solution containing **V**-R_d-**V** ($\sim$1 μM) and f**L** ($\sim$2 μM) had stronger fluorescence than did a solution of f**L** ($\sim$2 μM) alone; this difference in the fluorescence intensity between the two solutions reflected the binding of f**L** to **V**-R_d-**V**. We titrated the solution containing **V**-R_d-**V** ($\sim$1 μM) and f**L** ($\sim$2 μM) with **L**-R'$_d$-**L** (Figure 1). Upon addition of **L**-R'$_d$-**L**, the fluorescence intensity of the solution decreased; this decrease reflected the dissociation of the complex of **V**-R_d-**V** with f**L**, as a result of competitive binding of **L**-R'$_d$-**L** to **V**-R_d-**V**. We defined ΔF as the difference in the fluorescence intensity between the solution being titrated and a reference solution containing only f**L** ($\sim$2 μM). Before the addition of **L**-R'$_d$-**L**, ΔF was at its initial value, ΔF_i. At the end of the titration, there was no or little complexation between **V**-R_d-**V** and f**L**, and ΔF was at its final value, ΔF_f. A similar titration was performed using the monomeric ligand diacetyl-L-Lys-D-Ala-D-Ala (**L**) for comparison. The observation that a plot of the data from the titration of **V**-R_d-**V** and f**L** with **L**-R'$_d$-**L** was nearly linear indicated that the binding of **L**-R'$_d$-**L** to **V**-R_d-**V** was tight and stoichiometric at that concentration (Figure 1).

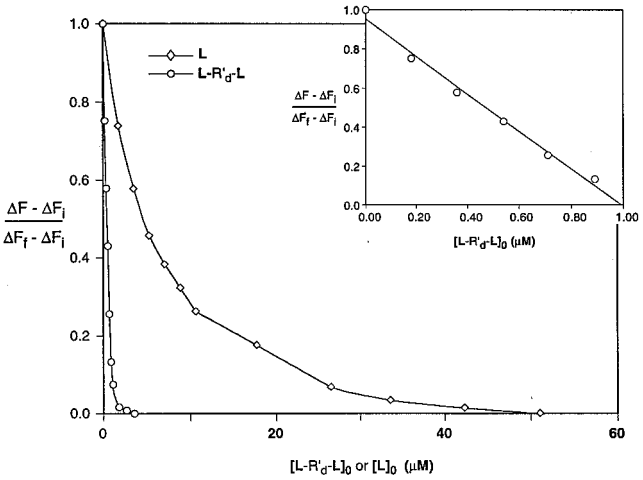
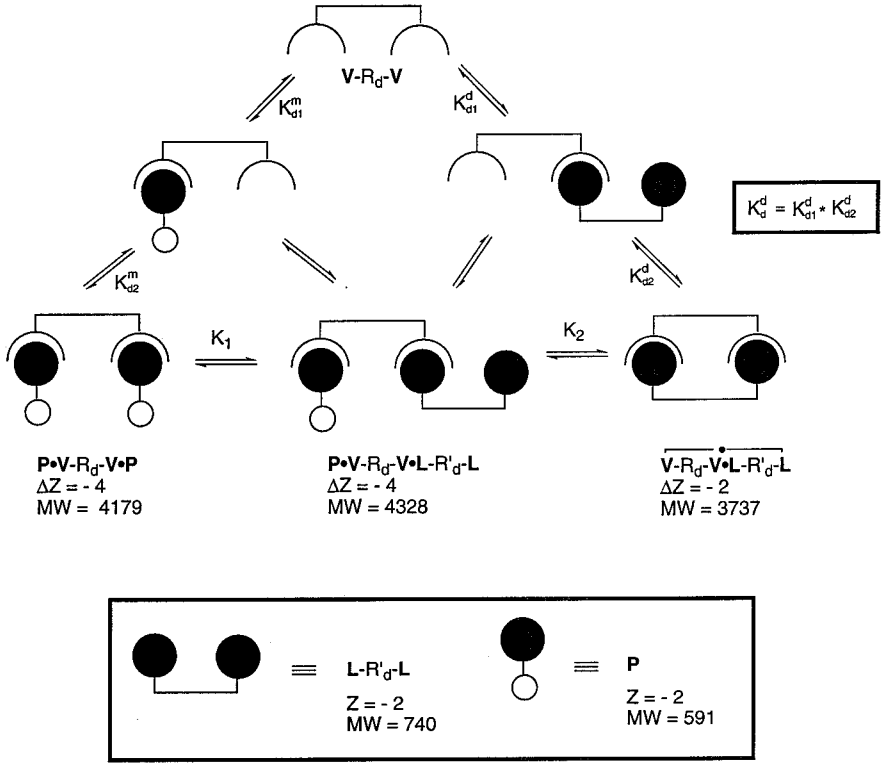


Figure 1. The competitive fluorescence titration of a solution containing **V**-R_d-**V** ($\sim$1.0 μM) and f**L** ($\sim$2.0 μM) with **L**-R'$_d$-**L** and with **L**; the expanded inset shows the region from [**L**-R'$_d$-**L**] = 0 to 1.0 μM. The excitation wavelength was 330 nm and the fluorescence intensity was monitored at 550 nm at 24 °C. ΔF, ΔF_i, and ΔF_f are defined as in the text.

In order to establish the influence of divalency accurately, we have developed a new assay using affinity capillary electrophoresis (ACE).[27] In general, ACE measures the change of mobility of a receptor as a function of the concentration of a ligand in the CE buffer; Scatchard analysis yields the affinity constant. This assay is based on competition of species for the active sites (Scheme 2); the reagents were chosen so that the influence of their charges on the electrophoretic mobility of the aggregate indicated the composition of the aggregate. Ligand

(27) For review of general principles of ACE, see: Chu, Y.-H.; Avila, L.; Gao, J.; Whitesides, G. M. *Acc. Chem. Res.* **1995**, *28*, 461−468 and references therein.

Scheme 2. Schematic Representation of Equilibrium among All Possible Species in the Competitive Assay[a]



[a] The K_{d1}^{m} and K_{d2}^{m} are the two dissociation constants for the complex **P•V**-R_d-**V•P**; K_{d1}^{d} and K_{d2}^{d} are the two dissociation constants for the complex $\overline{\text{V-R}_d\text{-V•L-R'}_d\text{-L}}$ (the divalent species). The equilibrium constant K_1 defines the exchange reaction between **P•V**-R_d-**V•P** and **P•V**-R_d-**V•L**-R'_d-**L**; K_2 is the equilibrium constant for the exchange between **P•V**-R_d-**V•L**-R'_d-**L** and $\overline{\text{V-R}_d\text{-V• L-R'}_d\text{-L}}$.

P was designed for this purpose; it was a monovalent ligand but had the same number of carboxylate groups as ligand **L**-R'_d-**L**.

There are four amino groups on the **V**-R_d-**V**, and at pH 7.0 this compound is expected to have a net charge between +3 and +4; **P** has a net charge of −2 at pH 7.0. The complex of **V**-R_d-**V** with **P** has a net negative charge at pH 7.0 (the complex emerged after the neutral marker when [**L**-R'_d-**L**] = 0; Figure 2). This observation indicated that **V**-R_d-**V** bound 2 equiv of **P** and formed a 1:2 complex.

We began the development of the assay by establishing the average dissociation constant, K_d^m ("m" defines monovalent binding), of the aggregate **P•V**-R_d-**V•P**. We assumed the two active sites of **V**-R_d-**V** to be independent and identical, and we estimated a value of $K_d^m \approx 4.8\ \mu$M;[28] this value is comparable to those observed by others for similar compounds.[21] On the basis of this value, we selected a concentration of **P** for the assay system that was sufficiently high to saturate both active sites of **V**-R_d-**V** ([**P**] = 1.0 mM $\approx 200K_d^m$). At this concentration, the only vancomycin-derived species in solution at significant concentration is **P•V**-R_d-**V•P** (Scheme 2). Since **P** has a net charge of −2 at pH 7.0, the difference in charge, ΔZ, between this complex and **V**-R_d-**V** is −4 (eq 1).

$$\Delta Z = Z(\textbf{P•V}\text{-R}_d\text{-}\textbf{V•P}) - Z(\textbf{V}\text{-R}_d\text{-}\textbf{V}) = -4 \qquad (1)$$

We then examined the mobility of the **V**-R_d-**V** group in a buffer containing [**P**] = 1.0 mM as a function of the concentration of **L**-R'_d-**L** added to this buffer. These species will be present at equilibrium: **P•V**-R_d-**V•P**, **P•V**-R_d-**V•L**-R'_d-**L**, and $\overline{\text{V-R}_d\text{-V•L-R'}_d\text{-L}}$ (the divalent species).[29] The values of ΔZ (defined by analogy with eq 1) for the first two of these species is −4; that for the divalent species is −2. The mass of these three species is similar: in particular, that of the first and second differs by only 3.5%. We therefore expect the influence of differences in mass (or, more properly, hydrodynamic drag) on electrophoretic mobilities, μ, to be small among these three aggregates. Thus only the fraction of **V**-R_d-**V** present in divalent species $\overline{\text{V-R}_d\text{-V•L-R'}_d\text{-L}}$ will change the electrophoretic mobility relative to that of **P•V**-R_d-**V•P**. Analysis of μ for species containing the **V**-R_d-**V** group as a function of [**L**-R'_d-**L**] thus indicates the relative concentration of the divalent complex $\overline{\text{V-R}_d\text{-V•L-R'}_d\text{-L}}$ and allows the value of K_d of that species to be determined relative to the known value for the species **P•V**-R_d-**V•P** (eqs 2, 3). We define the dissociation constant of the divalent complex $\overline{\text{V-R}_d\text{-V•L-R'}_d\text{-L}}$ as K_d^d (the superscript "d" signifies divalent binding), R as the ratio of the change in the electrophoretic mobility of **V**-R_d-**V** on adding **L**-R'_d-**L** $\Delta\mu$, to its maximal value $\Delta\mu_{max}$; Scatchard analysis uses eq 2 to estimate K, the dissociation constant of $\overline{\text{V-R}_d\text{-V•L-R'}_d\text{-L}}$ relative to the two dissociation constants of **P•V**-R_d-**V•P**, K_{d1}^m and

(28) At 280 nm, **P** has little UV absorbance. This value was an average of three independent runs, determined by UV difference spectroscopy according to the literature reported method (ref 8).

(29) Our analysis does not take into account another possible 1:2 complex of **V**-R_d-**V**, **L**-R_d-**L•V**-R_d-**V•L**-R'_d-**L**. The value of ΔZ for this complex is −4; that is the same as that for **P•V**-R_d-**V•P**. The presence of a small amount of **L**-R'_d-**L•V**-R_d-**V•L**-R'_d-**L** should, therefore, not change the electrophoretic mobility μ.

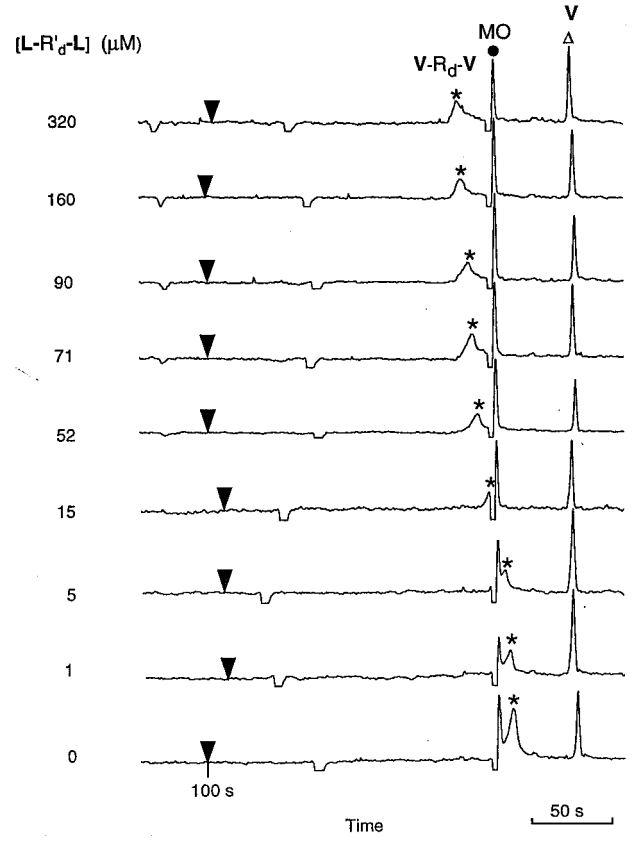


Figure 2. Representative electropherograms of a mixture of **V**-R_d-**V** and **V** in the running buffer (20 mM phosphate; pH 7.0) containing **L**-R'$_d$-**L** and 1.0 mM **P**; the concentrations of **L**-R'$_d$-**L** were as indicated in the plot. Mesityl oxide (MO) was used as a neutral marker (●); the **V**-R_d-**V** group is represented by * and **V** by △. The inverted peaks are due to the loss of ligands in the buffer as a result of initial binding. The time marker (▼) corresponds to 100 s after injection for all samples.

K_{d2}^m (eq 3).[30]

$$\frac{R}{[\mathbf{L}\text{-R'}_d\text{-}\mathbf{L}]} = -\left(\frac{K_1}{[\mathbf{P}]} + \frac{K}{[\mathbf{P}]^2}\right)R + \frac{K}{[\mathbf{P}]^2} \qquad (2)$$

$$K = K_1K_2 = \frac{K_{d1}^m K_{d2}^m}{K_{d1}^d K_{d2}^d} = \frac{(K_d^m)^2}{K_d^d} \qquad (3)$$

The electrophoretic mobility of **V**-R_d-**V** changed systematically with the concentration of **L**-R'$_d$-**L**. Figure 2 shows a representative series of electropherograms of **V**-R_d-**V** in 20 mM phosphate buffer at pH 7.0 containing 1.0 mM **P** and various concentrations of **L**-R'$_d$-**L**. We used unmodified vancomycin as the control to estimate the effect of the hydrodynamic drag on the complex. Figure 3 indicates that the change in the electrophoretic mobility of vancomycin was insignificant on addition of **L**-R'$_d$-**L**; thus the effect of the small differences in the hydrodynamic drag of the aggregates of **V**-R_d-**V** with **P** and **L**-R'$_d$-**L** on their electrophoretic mobility was negligible. Hence we can interpret the data in terms of the effect of divalency on binding. Scatchard analysis yielded the value of $K \approx 20$ mM (Figure 3). We therefore estimated K_d^d for the divalent species

V-R_d-**V**•**L**-R'$_d$-**L** to be 1.1 nM (eq 3).

In terms of the binding free energy, $\Delta G°$, the value of $K_d^d \approx$ 1.1 nM corresponds to 11.9 kcal/mol; the corresponding value for vancomycin with diacetyl-L-Lys-D-Ala-D-Ala (**L**) is about

(30) See Supporting Information for detailed derivations.

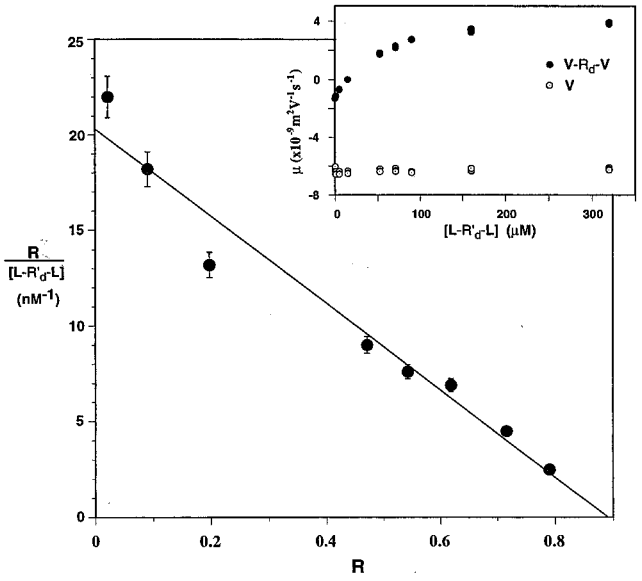


Figure 3. Scatchard plot of data from the ACE assays; $R = \Delta\mu/\Delta\mu_{max}$. The intercept along *y*-axis of the straight line indicates that $K/[\mathbf{P}]^2$ has a value of 20 nM^{-1} (eq 2): this value establishes $K = 20$ nM$^{-1} \times [\mathbf{P}]^2 = 20$ mM. The inset plots the electrophoretic mobilities of **V**-R_d-**V** and of **V** as a function of concentrations of **L**-R'$_d$-**L** in the running buffer.

8 kcal/mol.[8, 9] The second binding site in the divalent ligand therefore contributes an additional 3.9 kcal/mol to the binding energy. If we make the plausible assumption that K_{d1}^m and K_{d1}^d are indistinguishable, the ratio of K_{d2}^m to K_{d2}^d which, in fact, equals *K*, gives the effective molarity characterizing the second binding event in the divalent system.[31] We estimated this effective molarity to be 20 mM. For concentrations of **P** lower than 20 mM, intramolecular binding will, therefore, be more favorable than intermolecular binding in a system containing **P** and **L**-R'$_d$-**L**.

In a study of the binding of Ac**DADA** and *N*-decanoyl-D-Ala-D-Ala (N-dec-**DADA**) with ristocetin A in the presence of sodium dodecylsulfate, Williams *et al.* reported that there is a favorable intramolecular binding between a noncovalent ristocetin A dimer and N-dec-**DADA** anchored in micelles, and that intramolecularity gives an overall contribution of free energy to the binding of 3.5 kcal/mol.[25]

Conclusions

By tethering two molecules of vancomycin and two of **DADA** we have assembled and demonstrated a new model system with which to study divalency. This model system has several advantages. (i) It is biologically relevant. The study helps to understand the antibacterial action of vancomycin-type glycopeptides. A tight binder might be a potential antibiotic candidate.[21] (ii) The structures are relatively rigid, and the effect of entropy on binding is small. (iii) The well-defined specificity of the interaction between vancomycin and **DADA** minimizes nonspecific interaction that might complicate the analysis of the origin of increased binding energy.

The ACE competition assay we developed here in principle can be applied to other systems. It has all the advantages of the ACE method,[27] including the ability to measure the influence of divalency on binding. It is easy to apply; for analysis using ACE, this method requires only a competing monomeric ligand

(31) Page, M. I.; Jencks, W. P. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 1678−1683.

having one binding site with the same charge as that of the dimeric ligand of interest.

Experimental Section

General Procedure. Chemical solvents were obtained from Aldrich. The ^{1}H-NMR spectra were recorded at 400 MHz on a Bruker spectrometer. Chemical shifts are reported in parts per million downfield of tetramethylsilane. Reverse-phase HPLC was carried out with a Waters Model 600E chromatography system and Vydac C18 columns. A 4.6 mm i.d. column was used for analytical purpose and a 21.4 mm i.d. column was used for preparative separations. Linear gradients of 0.2% trifluoroacetic acid (TFA) in acetonitrile and 0.2% TFA in water were used in HPLC elution. ACE binding study was performed on an ISCO Model 3140 or a Beckman P/ACE 5010 system. The fluorescence binding study was performed using a Perkin-Elmer fluorescence spectrophotometer, Model MPF-4. Vancomycin hydrochloride was purchased from Sigma and used without further purification. Amino acids were purchased from Sigma except D-Ala-O-tBu from BACHEM Bioscience, and peptide coupling reagent 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Applied Biosystem.

Synthesis of Vancomycin Derivative V-R$_d$-V. The dimeric vancomycin **V**-R$_d$-**V** was synthesized according to a reported coupling protocol.[20] To the solution of 100 mg of vancomycin hydrochloride (67 μmol) in 0.5 mL of dry dimethyl sulfoxide (DMSO) were added 0.5 mL of dry dimethylformamide (DMF) and 4.5 mg (0.5 equiv) of *p*-xylylenediamine. The mixture was cooled to 0 °C, and 38 mg (1.5 equiv) of HBTU was added, followed by 20 mg (2.3 equiv) of diisopropylethylamine (DIEA). The solution was allowed to warm to room temperature and stirred overnight. Analytical reverse-phase HPLC showed nearly complete loss of vancomycin and appearance of a much less polar major product. Removal of the solvent afforded 100 mg of crude product, and 20 mg of this crude product was purified by preparative reverse-phase HPLC and lyophilized to afford 8.6 mg (2.5 μmol, 38%) of the dimeric derivative of vancomycin, **V**-R$_d$-**V**, as its trifluoroacetate salt. The ^{1}H-NMR spectroscopy showed resonances attributable to vancomycin as well as the linker: a new triplet at δ 7.18 (amide N*H*), a single at δ 7.14 (phenyl C*H*s), and a singlet at δ 4.45 (C*H*$_2$PhC*H*$_2$); ESI-MS exhibited an ion at *m*/*z* 2998.6, consistent with the molecular weight calculated for the parent ion (M + H$^+$), $C_{140}H_{159}N_{20}O_{46}Cl_4$, 2998.

Syntheses of Peptide Ligands. Peptide ligands **L**, **P**, f**L**, and **L**-R'$_d$-**L** were synthesized by solution method[32] and final products were purified by reverse phase HPLC. HBTU was used to activate all the peptide coupling in our syntheses. Benzyloxycarbonyl protection was used to protect amines and cleaved by palladium-catalyzed hydrogenation under a hydrogen balloon. The *tert*-butyl ester was used to protect the carboxylic acid and cleaved by 1:1 TFA/methylene chloride. Ligands **L**, **P**, and **L**-R'$_d$-**L** were synthesized from the same tripeptide precursor, N$^\alpha$-Ac-L-Lys-D-Ala-D-Ala-O-tBu.

***N*$^\alpha$-Carbobenzoxy-L-glutamyl-(*N*$^\alpha$-acetyl)-L-lysyl-D-alanyl-D-alanine (P).** Coupling of N$^\alpha$-Ac-L-Lys-D-Ala-D-Ala-O-tBu with N$^\alpha$-Z-L-Glu-γ-*tert*-butyl ester afforded the tetrapeptide ester. After cleavage of the *tert*-butyl ester, the free peptide was purified by HPLC. ^{1}H-NMR (DMSO-d_6) δ 8.13 (d, 1H), 8.07 (d, 1H), 7.99 (d, 1H), 7.85 (t, 1H), 7.37−7.29 (m, 5H), 5.0 (q, 2H), 4.30−4.20 (m, 1H), 4.17−4.13 (m, 2H), 3.93−3.88 (m, 1H), 3.03−2.96 (m, 2H), 2.20 (t, 2H), 1.90−1.80 (m, 1H), 1.81 (s, 3H), 1.75−1.65 (m, 1H), 1.65−1.40 (m, 2H), 1.40−1.36 (m, 2H), 1.36−1.16 (m, 2H), 1.26 (d, 3H), 1.17 (d, 3H); FAB-MS, for $C_{27}H_{39}N_5O_{10}Na$ (M + Na$^+$), calcd *m*/*z* 616.2595, found *m*/*z* 616.2595.

***N*$^\epsilon$,*N*'$^\epsilon$-[1,4-(1,4-Dioxobutanediyl)]bis(*N*$^\alpha$-acetyl-L-lysyl-D-alanyl-D-alanine) (L-R'$_d$-L).** To 98 mg (0.254 mmol) of N$^\alpha$-Ac-L-Lys-D-Ala-D-Ala-O-tBu and 15 mg (0.127 mmol) of succinic acid in 10 mL of acetonitrile solution were added 120 mg (0.317 mmol) of HBTU and 33 mg of (0.256 mmol) DIEA. The reaction was stirred at room temperature for 3 h; a white cloudy precipitate formed. The precipiate was collected after filtration and washed with saturated NaCl and ethyl acetate. The hexapeptide ester (56 mg) was obtained as white solid (0.066 mmol, 52%). The crude ester was deprotected with TFA and then fractionated by reverse phase HPLC. ^{1}H-NMR (DMSO-d_6) δ 8.14 (d, 2H), 8.08 (d, 2H), 8.00 (d, 2H), 7.77 (t, 2H), 4.32−4.24 (m, 2H), 4.20-4.12 (m, 4H), 2.97 (q, 4H), 2.25 (s, 4H), 1.82 (s, 6H), 1.60−1.40 (m, 4H), 1.36−1.27 (m, 4H), 1.27−1.17 (m, 4H), 1.27 (d, 6H), 1.17 (d, 6H); FAB-MS, for $C_{32}H_{55}N_8O_{12}$ (M + H$^+$), calcd *m*/*z* 743.3939, found *m*/*z* 743.3913.

Diacetyl-L-lysyl-D-alanyl-D-alanine (L) was prepared using acetic anhydride in acetone to acetylate the ϵ-amine of N$^\alpha$-Ac-L-Lys-D-Ala-D-Ala-O-tBu. The *tert*-butyl ester was cleaved by TFA, and the peptide was purified by reverse phase HPLC. ^{1}H-NMR (DMSO-d_6) δ 8.14 (d, 1H), 8.08 (d, 1H), 8.00 (d, 1H), 7.77 (t, 1H), 4.32−4.24 (m, 1H), 4.20-4.12 (m, 2H), 2.96 (q, 2H), 1.82 (s, 3H), 1.76 (s, 3H), 1.58−1.40 (m, 2H), 1.36-1.17 (m, 2H), 1.27 (d, 3H), 1.17 (d, 3H); FAB-MS, for $C_{16}H_{29}N_4O_6$ (M + H$^+$), calcd *m*/*z* 373.2087, found *m*/*z* 373.2096.

***N*$^\alpha$-Dansyl-*N*$^\epsilon$-acetyl-L-lysyl-D-alanyl-D-alanine (fL)** was synthesized by coupling of N$^\alpha$-dansyl-N$^\epsilon$-Ac-L-Lys with D-Ala-D-Ala-O-tBu. TFA cleavage and reverse phase HPLC purification afforded the pale yellow fluorescent dansyl ligand with excitation maxima 330 nm and emission maxima 550 nm in water.[12] ^{1}H-NMR (D_2O) δ 8.65 (d, 1H), 8.34 (d, 1H), 8.27 (d, 1H), 7.94 (d, 1H), 7.77 (m, 2H), 4.14 (q, 1H), 3.91 (q, 1H), 3.58 (t, 1H), 3.34 (s, 6H), 2.56 (t, 2H), 1.78 (s, 3H), 1.44-1.40 (m, 2H), 1.27 (d, 3H), 1.02 (d,3H), 1.05−0.96 (m, 1H), 0.90−0.83 (m, 2H), 0.70−0.65 (m, 1H); FAB-MS, for $C_{26}H_{37}N_5O_7SNa$ (M + Na$^+$), calcd *m*/*z* 586.2311, found *m*/*z* 586.2322.

ACE Binding Study. The capillary tubing (Polymicro Technologies, Phoenix) was of uncoated fused silica with an internal diameter of 50 mm, a total length of 74 cm, and a length from the inlet to the detector of 44 cm. A representative sample for injection into the electrophoresis capillary consisted of **V**-R$_d$-**V** (0.7 mg/mL), 20 mM mesityl oxide (MO), and vancomycin (0.3 mg/mL). The sample solution (~8 nL) was introduced into the capillary by vacuum injection. The general conditions used during each CE experiment were as follows: voltage, 30 kV; current uncontrolled, but generally 30 mA; buffer, 20 mM phosphate (pH 7.0), 1.0 mM ligand **P**, 0−320 μM ligand **L**-R'$_d$-**L**; detection, 214 nm; temperature, 30 ± 2 °C.

Fluorescence Titration. The fluorescent-labeled ligand (f**L**, 2.0 μM) in phosphate buffer (20 mM and at pH 7.0) was used in the titration. The change in fluorescence intensity at 550 nm (excitation at 330 nm) was monitored with a Perkin-Elmer MPF-4 spectrofluorometer at 24 °C. The fluorescence intensity of a cell containing 3.0 mL of 2.0 μM f**L** and **V**-R$_d$-**V** (~1.0 μM) and a reference cell containing 3.0 mL of 2.0 μM f**L** was measured, respectively. Small aliquots of a 5.4 mM of **L**-R'$_d$-**L** solution in the same buffer was added to a cell containing 3.0 mL of 2.0 μM f**L** and **V**-R$_d$-**V** (~1.0 μM), and the fluorescence intensity decreased (reflecting the binding of **L**-R'$_d$-**L** to **V**-R$_d$-**V**). The titration was stopped until no more decrease of fluorescence intensity was observed. During the titration experiment, the same aliquots of buffer as that of **L**-R'$_d$-**L** solution were added to the reference cell as calibration. The same procedure was applied to the titration using ligand **L**.

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Supporting Information Available: A detailed derivation of eq 2 (5 pages). See any current masthead page for ordering and Internet access instructions.

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(32) Dourtolou, V.; Gross, B.; Lambropoulou, V.; Zioudrou, C. *J. Chem. Soc., Chem. Commun.* **1984**, 572−274.