Design, Synthesis, and Characterization of a High-Affinity Trivalent System Derived from Vancomycin and L-Lys-D-Ala-D-Ala

Jianghong Rao, Joydeep Lahiri, Robert M. Weis,† and George M. Whitesides*

Contribution from the Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

Received July 26, 1999

Abstract: A trivalent derivative of vancomycin, tris(vancomycin carboxamide), [C_6H_3-1,3,5-($CONHC_6H_4$-4-$CH_2NHCOV)_3$ (R_tV_3; **V** = vancomycin)], binds an analogous trivalent derivative of D-Ala-D-Ala, $R'_tL'_3$, (C_6H_3-1,3,5-[$CON^{\epsilon}H(N^{\alpha}$-Ac)-L-Lys-D-Ala-D-Ala$]_3$) in water with a dissociation constant that is approximately 4×10^{-17} M, as estimated by HPLC using a competitive assay against $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala (**L**). This binding is one of the tightest known for low molecular weight organic species. The dissociation of $R_tV_3 \cdot R'_tL'_3$ in the presence of an excess of **L** could be followed by HPLC. The kinetics of dissociation are quite different from those of monovalent tight-binding systems such as avidin and biotin. In particular, the rate of dissociation of the aggregate $R_tV_3 \cdot R'_tL'_3$ is rapid in the presence of monovalent **L** at concentrations greater than the value of the dissociation constant for the complex of **L** with **V**; by contrast, the rate of dissociation of biotin·avidin is independent of the concentration of biotin. Two mechanisms by which the dissociation may occur are postulated and discussed. Calorimetric measurements for the trivalent system indicate that the enthalpy of association is ∼−40 kcal/mol, about three times that of **V** + **L**, and thus the entropy of association is ∼−18 kcal/mol, approximately 4.5 times that of **V** + **L**.

Introduction

We are developing vancomycin (**V**) and D-Ala-D-Ala (DADA) as a model system with which to study the physical-organic chemistry and physical biochemistry of polyvalency.[1,2] Vancomycin binds to peptides terminating in D-Ala-D-Ala with dissociation constants ranging from about 100 μM (for *N*-acetyl-D-Ala-D-Ala) to about 1 μM (for diacetyl-L-Lys-D-Ala-D-Ala and similar compounds).[3,4] The geometry of these complexes is well-defined (Scheme 1),[5−7] and their thermodynamics has been extensively described by Williams and co-workers.[8−12] Vancomycin is rigid, and the values of thermodynamic parameters for its association with *N*-acetyl-D-Ala-D-Ala (at 298 K, $\Delta G° = -5.2$ kcal/mol; $\Delta H° = -8.5$ kcal/mol; $\Delta S° = -11.1$ e.u.; $-T\Delta S° = 3.3$ kcal/mol) reflect this rigidity.[8] The simplicity, conformational rigidity, and ease of modification of vancomycin and DADA, combined with the wealth of relevant structural, thermodynamic, and kinetic information obtained by Perkins, Williams, Griffin, Pratts, and others, make this system an attractive one with which to study the physical−organic chemistry of polyvalent interactions.

Polyvalent binding—the simultaneous interactions of multiple, linked ligands with multiple, linked receptors—is a subject of growing interest in biochemistry.[13] A range of important biological interactions—from the association of IgGs with the surface of a bacterium to the rolling of a leukocyte along the capillary endothelium near a site of inflammation—depend on oligo- or polyvalency.[13] In an ideal case, the contribution of polyvalency to the free energy of binding that would come from linking receptors and ligands would appear primarily in the $T\Delta S°$ term.[13] Thus, for example, the free energy of association of a trivalent receptor−ligand system might be related to that of the monomers by eqs 1−3 (Scheme 2). That is, the enthalpy of the trivalent process would be three times that of the monomer, while the loss in entropy (a term we assume, in the simplest case, to be dominated by translational entropy) would be approximately that of the monomer.

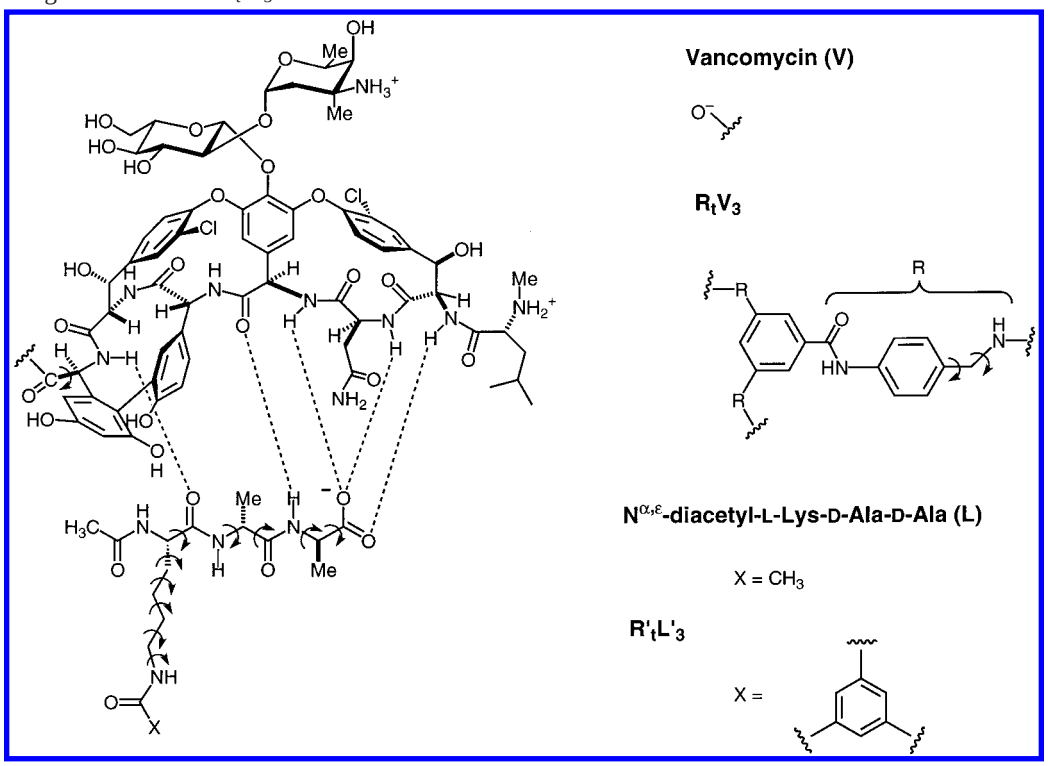
In practice, this formulation of a trivalent association neglects obvious complexities of real molecules. In particular, association might introduce favorable or unfavorable enthalpic terms due to interactions among the linking groups, receptors and ligands, and the modifications of the receptor and ligand required to link them may also modify their association. The entropic term will include contributions from conformational, rotational, and vibrational terms, and from solvent release, in addition to a term due to translational entropy. In trying to understand the interplay of these terms, it seemed to us sensible to study a system in

* To whom correspondence should be addressed.
† Department of Chemistry, University of Massachusetts, Amherst, Massachusetts 01003.

(1) Rao, J.; Whitesides, G. M. *J. Am. Chem. Soc.* **1997**, *119*, 10286.
(2) Rao, J.; Lahiri, J.; Lyle, I.; Weis, R. M.; Whitesides, G. M. *Science* **1998**, *280*, 708.
(3) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 773.
(4) Nieto, M.; Perkins, H. R. *Biochem. J.* **1971**, *123*, 789.
(5) Sheldrich, G. M.; J., P. G.; Kennard, O.; Williams, D. H.; Smith, G. A. *Nature* **1978**, *271*, 223.
(6) Schafer, M.; Schneider, T. R.; Scheldrick, G. M. *Structure* **1996**, *4*, 1509.
(7) Loll, P. J.; Bevivino, A. E.; Korty, B. D.; Axelsen, P. H.; *J. Am. Chem. Soc.* **1997**, *119*, 1516.
(8) Williams, D. H. *Acc. Chem. Res.* **1984**, *17*, 7, 364.
(9) Kannan, R.; Harris, C. M.; Harris, T. M.; Waltho, G. P.; Skelton, N. J.; Williams, D. H. *J. Am. Chem. Soc.* **1988**, *110*, 0, 2946.
(10) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332.
(11) Bongini, A.; Feeney, J.; Williamson, M. P.; Williams, D. H. *J. Chem. Soc., Perkin Trans.* **1981**, *2*, 201.
(12) Williams, D. H.; Cox, J. P. L.; Doig, A. J.; Gardner, M.; Gerhard, U.; Kaye, P. T.; Lal, A. R.; Nicholls, I. A.; Salter, C. J.; Mitchell, R. C. *J. Am. Chem. Soc.* **1991**, *113*, 3, 7020.
(13) Mammen, M.; Choi, S.-K.; Whitesides, G. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2754 and references therein.

10.1021/ja992648l CCC: $19.00
Published on Web 03/08/2000

Scheme 1. Model of Complexes of Vancomcyin or Trimeric Vancomycin Derivative R_tV_3 with $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala (**L**) or Trimeric Ligand of DADA $R'_tL'_3$[a]



[a] The dotted lines represent intermolecular hydrogen bonds.[9] Examinations of the model of the complex $R_tV_3 \cdot R'_tL'_3$ reveal frozen rotors in R_tV_3 and $R'_tL'_3$ upon complexation, indicated by curved arrows: Three rotors are frozen in each subunit of R_tV_3; six more rotors are frozen in each L-lysine subunit of $R'_tL'_3$ than in **L** in monovalent binding of **V** and **L**.

which the interacting components were relatively rigid, and in which $\Delta S°$ was relatively small for the monomers. The system comprising vancomycin and L-Lys-D-Ala-D-Ala met these criteria.

Both vancomycin and DADA can be modified by synthesis,[14,15] and relatively straightforward procedures can be used to generate polyvalent derivatives of both.[1] Both the ϵ-amino group of the lysine group in L-Lys-D-Ala-D-Ala and the carboxylate group of vancomycin can be modified relatively easily with little influence on the binding constant.[4,14] Divalency has been inferred to be important in the biological activity of some of vancomycin-type antibiotics.[16−18] Williams et al. have suggested that spontaneous dimerization (with head-to-tail geometry) of these antibiotics contributes to their tight binding to DADA groups in the bacterial cell wall.[19] We have examined the interaction of a synthetic dimer of vancomycin (**V**-CONHCH$_2$C$_6$H$_4$CH$_2$NHCO-**V**; **V**= vancomycin) with a corresponding dimeric derivative of DADA [CH$_2$CON$^\epsilon$H(N^α-Ac)-L-Lys-D-Ala-D-Ala]$_2$, and demonstrated an enhancement in binding of approximately 10^3, relative to unmodified monomeric vancomycin and $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala.[1] Here we describe a trivalent system that comprises trimeric derivatives of vancomycin and DADA and that shows remarkably tight binding in water. A preliminary report of this work has appeared.[2] Understanding the origins of this tight binding—and of the contribution of conformational mobility to it—helps to clarify the nature of oligovalent interactions.

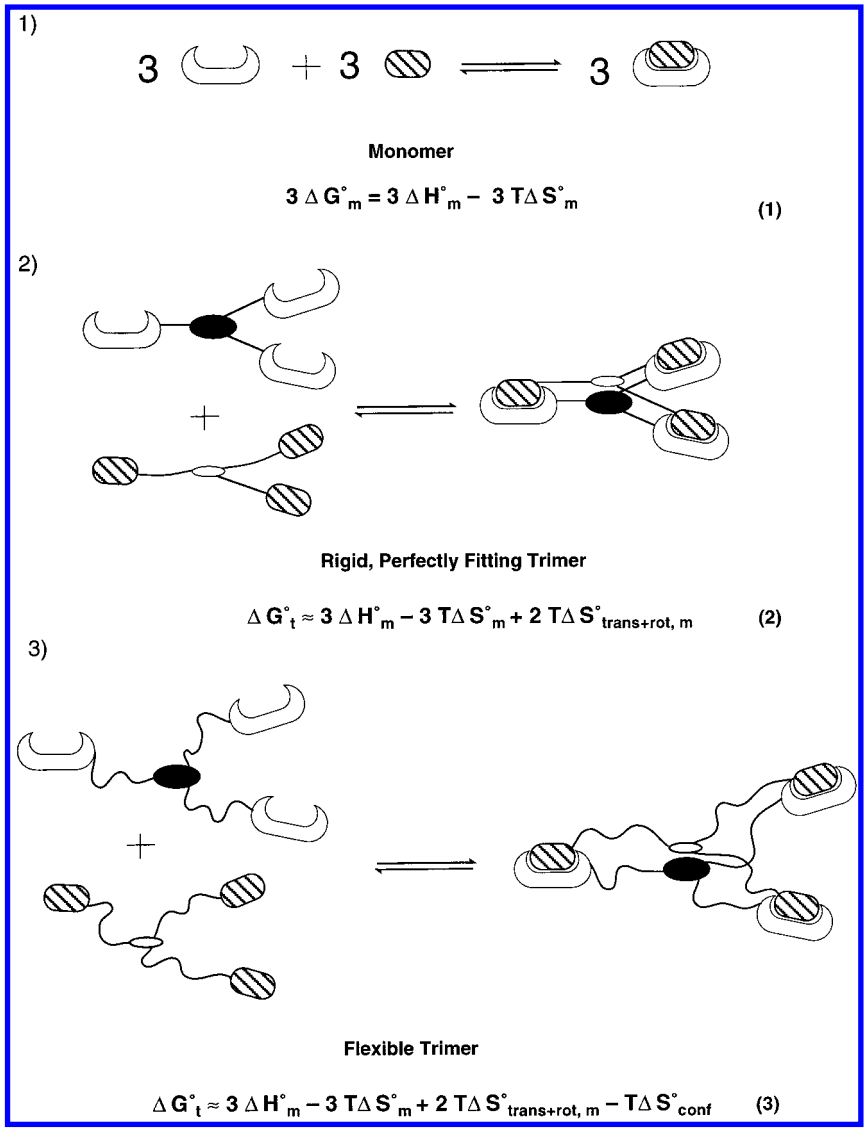
Results

Design and Synthesis of Trivalent Derivatives of Vancomycin, R_tV_3, and of DADA, $R'_tL'_3$. The overall design for these two derivatives was based on the positions available for modification, and on the geometry of the complex (Scheme 1; we use the subscript "t" to indicate trivalent derivatives). The basic strategy was to use the ϵ-amino group of the lysine group of the ligand, and the single carboxylic acid of vancomycin, to form links to central hubs. The design of the central hub for R_tV_3 was critical to the success of our study. It had to be: (i) rigid, to minimize the loss of conformational entropy on binding, and (ii) appropriately reactive, to allow preparation of the desired trivalent derivative of vancomycin. A rigid triamine was designed and synthesized for this purpose (Scheme 3). Coupling of the triamine with vancomycin mediated by 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) was carried out successfully in a mixture of dimethylformamide and dimethyl sulfoxide. Purification of the reaction residue by reverse-phase HPLC afforded the desired product R_tV_3.

The R_tV_3 hexatrifluoroacetate was isolated as a white foam after lyophilization. The ^{1}H NMR spectrum showed the resonances expected for the hub and vancomycin units. The ESI-MS exhibited an ion at *m/z* 4818.8; this value is consistent with the average calculated molecular weight of 4817.4 for the parent ion (M + H$^+$). Obtaining a pure sample of R_tV_3 was very difficult. We made a substantial effort to purify R_tV_3 and were able to obtain a small amount of R_tV_3 sample that was almost pure (as judged by HPLC analysis; Figure 1A). We used this

(14) Sundram, U. N.; Griffin, J. H. *J. Org. Chem.* **1995**, *60*, 0, 1102.
(15) Shi, Z.; Griffin, J. H. *J. Am. Chem. Soc.* **1993**, *115*, 5, 6482.
(16) Gerhard, U.; Mackay, J. P.; Maplestone, R. A.; Williams, D. H. *J. Am. Chem. Soc.* **1993**, *115*, 232.
(17) Groves, P.; Searle, M. S.; Waltho, J. P.; Williams, D. H. *J. Am. Chem. Soc.* **1995**, *117*, 7958.
(18) Mackay, J. P.; Gerhard, U.; Beaurgard, D. A.; Westwell, M. S.; Searle, M. S.; Williams, D. H. *J. Am. Chem. Soc.* **1994**, *116*, 4581.
(19) Beauregard, D. A.; Williams, D. H.; Gwynn, M. N.; Knowles, D. J. C. *Antimicrob. Agents Chemother.* **1995**, *39*, 781.

Scheme 2. Thermodynamics of Binding for a Monovalent System and a Trivalent System with Rigid and Flexible Linking Groups (eqs 1−3)[a]



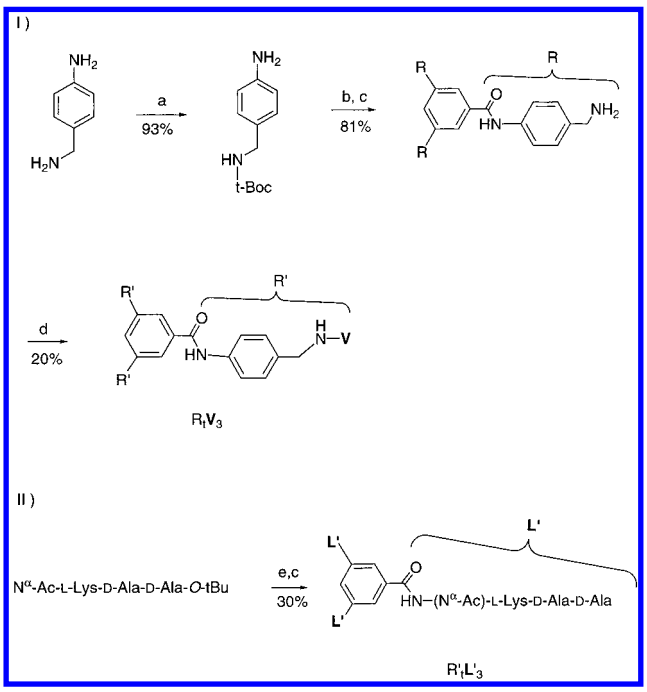
[a] (1) The total entropic cost of association of three monovalent receptors and three monovalent ligands is 3 $T\Delta S°_m$. (2) If the three receptors and three ligands are connected by a perfect rigid linking group (that is, one in which there is neither conformational entropy loss nor enthalpic strain of binding), the entropy loss of binding is approximately 3 $T\Delta S°_m$ − 2 $T\Delta S°_{trans+rot,m}$ (eq 2; $T\Delta S°_{conf} = 0$). (3) Flexible trimers may associate without enthalpic strain but with loss in conformational entropy (eq 3; $T\Delta S°_{conf} < 0$). If $-T\Delta S°_{conf}$ is too large, monovalent binding will dominate. $\Delta G°_m$, $\Delta H°_m$ and $T\Delta S°_m$ are the free energy, enthalpy, and entropy of monovalent binding, respectively; $T\Delta S°_{trans+rot,m}$ is the translational and rotational entropy of monovalent binding. $\Delta G°_t$ is the free energy of trivalent binding, and $T\Delta S°_{conf}$ is the loss in conformational entropy in the two *intra*molecular association events.

pure sample for calorimetric measurements and for some HPLC experiments. Most of the samples we used in the HPLC studies contained approximately 10% impurities (estimated from the HPLC integration; Figure 1B); these impurities are indicated in the HPLC chromatograms.

A similar strategy was applied to the design of $R'_tL'_3$. We used a 1,3,5-benzene tris(carboxamide) as the core so that the structure was compatible in symmetry with R_tV_3. We synthesized a protected tripeptide precursor, N^{α}-Ac-L-Lys-D-Ala-D-Ala-*O*-*tert*-butyl ester. Coupling of this compound with 1,3,5-benzene tris(carbonyl chloride) afforded protected $R'_tL'_3$ (Scheme 3). After deprotection, $R'_tL'_3$ was purified by reverse phase HPLC and characterized by ^{1}H NMR spectroscopy and ESI-MS.

Association of R_tV_3 with a Monovalent Ligand. We began the analysis of binding of derivatives of DADA to R_tV_3 by measuring the binding constant of the vancomycin groups in this species with $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala (**L**). In the analysis, we assumed the binding of **L** to each site of R_tV_3 to be an independent event with the same affinity (defined by K_d^m; "m" indicates a monovalent association). In the absence of any information to the contrary, the assumption that the binding events are independent is physically reasonable: the three active sites of R_tV_3 are indistinguishable, they are far apart and separated by a rigid spacer, and there is no obvious mechanism for them to interact with one another (the head-to-tail dimerization observed in monomeric vancomycin [16−18] seems to be unlikely here, since the three vancomycin units are linked together in a head-to-head fashion). We defined the three stepwise dissociation constants as K_{d1}^m, K_{d2}^m, and K_{d3}^m, respectively (eqs 4−6). After making statistical corrections for the number of binding sites available at each step, the relationships between K_d^m and the three stepwise dissociation constants are

Scheme 3[a]



[a] (I) Synthesis of R_tV_3: (a) Di-*tert*-butyl dicarbonate/TEA, rt; (b) 1,3,5-benzene tris(carbonyl chloride), CH_2Cl_2, 2 h; (c) TFA/ CH_2Cl_2, 1 h; (d) vancomycin hydrochloride, HBTU, DIEA, rt, overnight. (II) Synthesis of $R'_tL'_3$: (e) 1,3,5-benzene tris(carbonyl chloride), DMF, rt

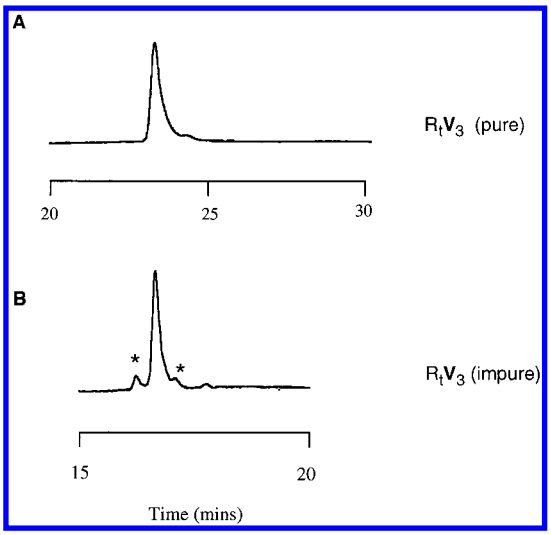


Figure 1. HPLC chromatograms of R_tV_3. (A) Pure R_tV_3 and (B) R_tV_3 containing about 10% impurities marked by asterisk signs (*).

defined in these equations. The overall dissociation constant of complex R_tV_3·3**L** can then be expressed as $(K_d^m)^3$ (eq 7).

$$R_tV_3{\cdot}\mathbf{L} \overset{K_{d1}^m}{\rightleftharpoons} R_tV_3{\cdot}\mathbf{L} \quad K_{d1}^m = K_{d1}^m/3 \tag{4}$$

$$R_tV_3{\cdot}2\mathbf{L} \overset{K_{d2}^m}{\rightleftharpoons} R_tV_3{\cdot}\mathbf{L} + \mathbf{L} \quad K_{d2}^m = K_d^m \tag{5}$$

$$R_tV_3{\cdot}3\mathbf{L} \overset{K_{d3}^m}{\rightleftharpoons} R_tV_3{\cdot}2\mathbf{L} + \mathbf{L} \quad K_{d3}^m = 3K_d^m \tag{6}$$

$$R_tV_3{\cdot}3\mathbf{L} \rightleftharpoons R_tV_3 + 3\mathbf{L} \quad K_{d1}^m K_{d2}^m K_{d3}^m = (K_d^m)^3 \tag{7}$$

Using difference UV spectroscopy,[3,4] we titrated R_tV_3 with **L** to estimate K_d^m. Scatchard analysis yielded the apparent average dissociation constant per binding site, which is, by our definition, K_d^m. The value of K_d^m obtained from the plot is approximately 5.0 μM (Figure 2). For comparison, Table 1 gives values of dissociation constants for other relevant compounds; the value of K_d^m is similar to those measured previously for monomers and dimers. This similarity makes it unlikely that there are large differences between the values for K_{d1}^m, K_{d2}^m, and K_{d3}^m.

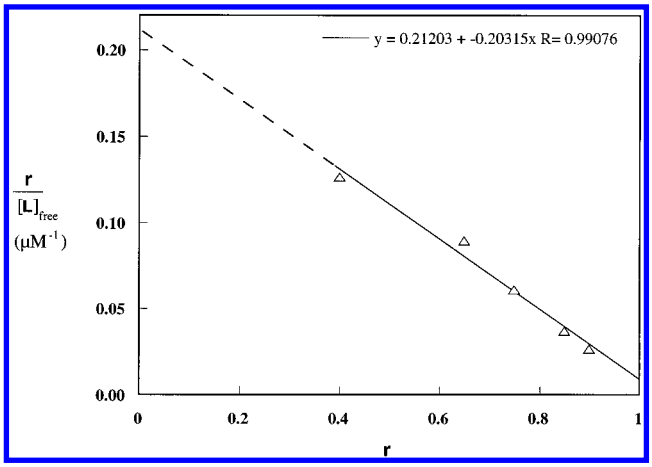


Figure 2. UV difference spectroscopy at 280 nm to determine K_d of **L** to R_tV_3 at pH 7.0 in the 20 mM phosphate buffer. R_tV_3 (2.1 μM, 3.0 mL) in a 1-cm cell was titrated against a solution of **L** (8.5 mM). The fraction function **r** was defined as the ratio of the change in the absorbance at 280 nm to the maximal change ($\mathbf{r} = \Delta A^{280}/\Delta A_{max}^{280}$).

Table 1. Dissociation Constants of Complexes of Vancomycin (V) and Its Derivatives with $N^{\alpha,\epsilon}$-diacetyl-L-Lys-D-Ala-D-Ala

vancomycin and derivative	K_d (μM)	ref
V	0.67[a]	3
n-C_3H_7NHCO-**V**	1.0[b]	15
$NH_3^+(CH_2)_2$S-S$(CH_2)_2$NHCO-**V**	4.3[c]	20
V-CONH$(CH_2)_3$S-S$(CH_2)_3$NHCO-**V**	3.9[c]	20
V-CONH$(CH_2)_6$NHCO-**V**	1.5[c]	20
V-CONH$CH_2C_6H_4CH_2$NHCO-**V**	4.8[d]	1
R_tV_3	5.0[d,e]	this work

[a] UV difference spectroscopy in 20 mM sodium citrate buffer (pH 5.1). [b] UV difference spectroscopy in 20 mM sodium citrate buffer (pH 5.1). [c] Fluorescence competitive titration in 10 mM HEPES, 6 mM NaCl buffer (pH 7.0). [d] UV difference spectroscopy in 20 mM phosphate buffer (pH 6.9). [e] Estimated error is $\pm$1.5 μM.

Stability of the Complex of R_tV_3·$R'_tL'_3$. We tested the stability of the complex R_tV_3·$R'_tL'_3$ under the conditions used for HPLC experiments. We first injected a sample containing R_tV_3 (4.5 μM) into a C_{18} reverse-phase analytical column and eluted with a linear gradient starting from 85% of solvent A [0.1% trifluoroacetic acid (TFA) in water] and 15% solvent B (0.1% TFA in acetonitrile) to 70% A and 30% B in 45 min (Figure 3A). We observed a peak corresponding to R_tV_3. We then injected a sample containing a 1:1.1 mixture of R_tV_3 and $R'_tL'_3$ ([R_tV_3] $\approx$ 4.5 μM and [$R'_tL'_3$] $\approx$ 5 μM in 20 mM phosphate buffer at pH 7.0) under the same conditions. On injecting this mixture, we observed only the aggregate R_tV_3·$R'_tL'_3$ (Figure 3C); we did not observe free R_tV_3 (R_tV_3·$R'_tL'_3$ and R_tV_3 have clearly distinguishable retention times). The symmetry of the peak did not suggest dissociation or instability. This observation indicates, inter alia, that dissociation of this trivalent aggregate is slow relative to the time required for the HPLC experiment (~45 min).

A control experiment examined a mixture of R_tV_3 and the monomeric ligand **L** ([R_tV_3] $\approx$ 4.5 μM; [**L**] $\approx$ 19.1 mM in the

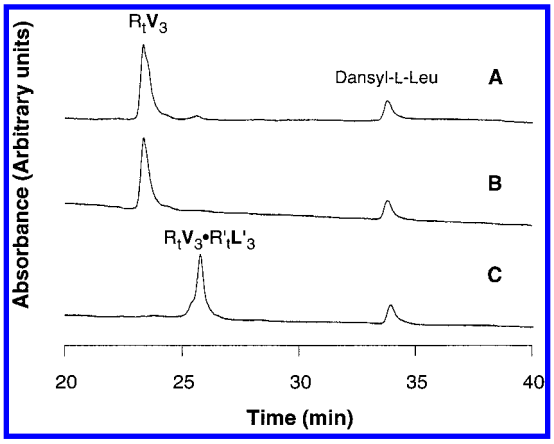


Figure 3. HPLC of aliquots of samples that contained (A) R_tV_3 (4.5 μM), (B) R_tV_3 (4.5 μM) + **L** (19.1 mM), and (C) R_tV_3 (4.5 μM) + $R'_tL'_3$ (4.5 μM). Dansyl-L-leucine (10 μM) was introduced into each sample as an internal standard. All analyses were carried out under the same conditions: Rainin analytical reverse-phase (RP) C_{18} column, linear elution gradient from 85% solvent A [0.1% trifluoroacetic acid (TFA) in water] and 15% solvent B (0.1% TFA in acetonitrile) to 70% A and 30% B, over 45 min. The absorbance was monitored at 280 nm.

same phosphate solution (Figure 3B); that is, a concentration that is approximately 3800 K_d^m) on HPLC using the same elution conditions; this experiment showed only R_tV_3. We infer that the 1:3 complex of R_tV_3•3**L** that was undoubtedly present at equilibrium dissociated completely during the passage through the column; the fact that the peak for R_tV_3 was sharp indicates that dissociation was fast under the conditions of the experiment.

We conclude that R_tV_3•$R'_tL'_3$ is stable enough to survive the HPLC experiment intact. We could therefore use HPLC to quantitate the relative quantities of $R'_tL'_3$, R_tV_3, and R_tV_3•$R'_tL'_3$ in a mixture of these species, and to estimate the trivalent dissociation constant.

Design of the Competitive Binding Assay. An attempt to determine the trivalent dissociation constant K_d^t by a direct titration of R_tV_3 with $R'_tL'_3$ (eq 8) was not successful; the

$$R_tV_3{\cdot}R'_tL'_3 \overset{K_d^t}{\rightleftharpoons} R_tV_3 + R'_tL'_3 \qquad (8)$$

reaction was stoichiometric even when R_tV_3 was at its minimum detectable concentration [in the μM range for the UV detector]. We thus turned to a competition assay to estimate K_d^t indirectly from the equilibrium constant K (eqs 9, 10).

$$R_tV_3{\cdot}3L + R'_tL'_3 \overset{K}{\rightleftharpoons} R_tV_3{\cdot}R'_tL'_3 + 3\mathbf{L} \qquad (9)$$

$$K = \frac{[R_tV_3R'_tL'_3]\,[L]^3}{[R_tV_3{\cdot}3\mathbf{L}][R'_tL'_3]} = \frac{(K_d^m)^3}{K_d^t} \qquad (10)$$

We allowed $R'_tL'_3$ to compete with the monovalent ligand **L** for R_tV_3.[22]

A competition assay is most easily interpreted if the concentration of **L** is such that all three of the binding sites of R_tV_3 are fully occupied in the absence of $R'_tL'_3$. The concentration of **L** required to saturate 99% of the three vancomycin binding sites, $[\mathbf{L}]_0^S$, is given by eq 11 (see Supporting Information for a detailed derivation). We defined θ as a measure of the progress of the displacement reaction as a function of the amount of $R'_tL'_3$ added; θ is the fraction of the total R_tV_3 present in the sample that is in the form of R_tV_3•$R'_tL'_3$ (eq 12).

$$[\mathbf{L}]_0^S = 300\,K_d^m + 3[R_tV_3]_0 \qquad (11)$$

$$\theta = \frac{[R_tV_3R'_tL'_3]}{[R_tV_3{\cdot}3\mathbf{L}] + [R_tV_3{\cdot}R'_tL'_3]} \qquad (12)$$

The competition assay depends on being able to establish three concentrations—$[R_tV_3{\cdot}R'_tL'_3]$, $[R'_tL'_3]$, and $[R_tV_3{\cdot}3\mathbf{L}]$—in a solution containing **L** at a fixed concentration $[\mathbf{L}]_0^S$, and $R'_tL'_3$ at a variable concentration. Under the conditions used in these experiments, the complex R_tV_3•3**L** dissociates rapidly:[23] the only peak observed in HPLC on injection of this complex is uncomplexed R_tV_3. The stability of the complex R_tV_3•$R'_tL'_3$, and the ability to separate these species on HPLC, allowed us to use an HPLC assay to establish their relevant concentrations at equilibrium in the solution. We established $[R_tV_3{\cdot}3\mathbf{L}]$ indirectly by measuring the concentration of one of the species, R_tV_3, derived from it by dissociation. On the basis of the HPLC experiments described previously, we assume that R_tV_3•3**L** (and any traces of R_tV_3•2**L** and R_tV_3•**L** present) are converted quantitatively to R_tV_3 on the HPLC column by rapid dissociation of **L**.[23]

Analysis of the Competitive Binding Assay and Estimation of K_d^t. For $K_d^m \approx 5.0$ μM, a concentration of **L** of ~1.5 mM in solution ensures that 99% of the binding sites of R_tV_3 are occupied by **L** (eq 11). We used a value of $[L]_0^S \approx 19.1$ mM (>10 times larger than 1.5 mM); because the binding of $R'_tL'_3$ to R_tV_3 was so tight, a high value of [**L**] was required to give a quantifiable concentration of $R'_tL'_3$ in solution at equilibrium in the system used in the competition experiments (described by eq 9). Aliquots of a stock solution of $R'_tL'_3$ (1.06 mM) were added to a solution of R_tV_3 (4.5 μM) and **L** (19.1 mM) in 20 mM pH 7.0 phosphate buffer. After each addition of $R'_tL'_3$, the solution was mixed by vortexing and allowed to equilibrate for about 40 min at room temperature (about 30 °C). A sample was removed and examined by HPLC, eluting with a linear gradient from 85% solvent A (0.1% TFA in water) and 15% solvent B (0.1% TFA in acetonitrile) to 70% A and 30% B over 45 min. Under these conditions, R_tV_3•3**L** dissociated into its components on injection and appeared as separate peaks for R_tV_3 and **L**. R_tV_3•$R'_tL'_3$ was stable and appeared as a well-defined peak. Additional aliquots of $R'_tL'_3$ were added until free R_tV_3 could no longer be detected by HPLC. Figure 4 shows the HPLC chromatograms of a representative titration.

Since **L** is present in large excess relative to R_tV_3, we assume that the concentration of **L**, at equilibrium, is the same as its initial concentration, $[\mathbf{L}] \approx [\mathbf{L}]_0$. Analysis of the data requires a relationship between θ (eq 12) and $[R'_tL'_3]$. Equation 13,

$$\frac{\theta}{[R'_tL'_3]} = -\frac{K}{[\mathbf{L}]_0^3}\,\theta + \frac{K}{[\mathbf{L}]_0^3} \qquad (13)$$

obtained by combination of eqs 10 and 12, was used to characterize the binding: if the ratios of $\theta/[R'_tL'_3]$ are plotted versus θ, a straight line would be expected; both the slope and

(20) Sundram, U. N.; Griffin, J. H.; Nicas, T. I. *J. Am. Chem. Soc.* **1996**, *118*, 13107.
(21) Popieniek, P. H.; Pratt, R. F. *J. Am. Chem. Soc.* **1991**, *113*, 2264.
(22) Our analyses assume that the two intermediate species, $R'_tL'_3$•R_tV_3•2**L** and $R'_tL'_3$•R_tV_3•**L**, are present in very small amounts at equilibrium and can thus be neglected. This assumption leads to eq 13, which predicts a linear plot of $\theta/[R'_tL'_3]$ versus θ. The agreement of our experimental results with this prediction suggests that this assumption is valid under our experimental conditions.
(23) The value of the rate constant for dissociation of the monovalent complex of vancomycin with **L** is $k_{off} = 31\ s^{-1}$ (ref 21).

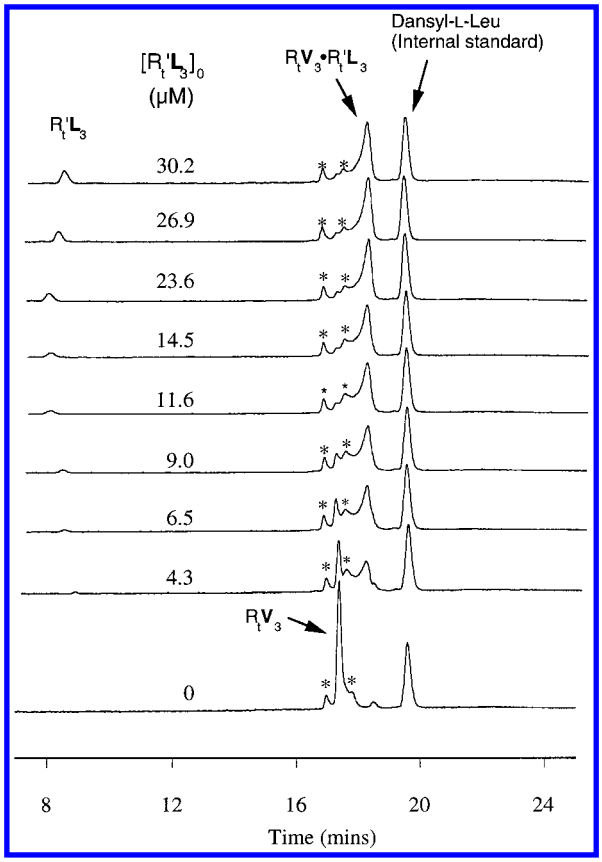


Figure 4. HPLC chromatograms of the titrated solution of R_tV_3 (4.5 μM) on addition of aliquots of $R'_tL'_3$ (1.06 mM) solution in the presence of **L** (19.1 mM). The sample of R_tV_3 used here contained small amounts of impurities, which are indicated by the symbols (*). The HPLC conditions were similar to those described in Figure 3, except that a Vydac analytical RP column was used with the same linear gradient, run over 30 min.

y-intercept of this line would afford the ratio of $K/[\mathbf{L}]^3$ and thus the value of K. The values of $[R'_tL'_3]$ were estimated from integration of the peaks for $R'_tL'_3$ on the HPLC; θ was calculated (eq 12) using the integrated intensities of $R_tV_3{\cdot}R'_tL'_3$ and R_tV_3. The Experimental Section details the procedure. The fact that the sum of the two integrals of the peaks of $R_tV_3{\cdot}R'_tL'_3$ and R_tV_3 during the titration was approximately constant indicates that no species other than those in eq 9 were present in significant amounts at equilibrium. The ratios of $\theta/[R'_tL'_3]$ were then plotted versus θ. As expected, the plot gave a straight line and yielded the value of K (Figure 5). We estimated K_d^t using K_d^m and K (eq 10); our estimate of K_d^t is $\sim 4 \pm 1 \times 10^{-17}$ M.

Exchange Reaction Between $R_tV_3{\cdot}R'_tL'_3$ and L. At first, the conclusion that R_tV_3 bound very tightly to $R'_tL'_3$ seemed at odds with the relatively fast equilibration we observed in solutions containing R_tV_3, $R'_tL'_3$, and **L**. We therefore turned to an examination of the rates of equilibrium of the species involved in this system. If we assume that k_{on} for association of R_tV_3 and $R'_tL'_3$ is 9 times that for association of **V** and **L**[21] (making a statistical correction of a factor of 9 for the number of receptor and ligand groups in the trimeric species), then k_{on} would be $\sim 2.5 \times 10^8$ M^{-1} s^{-1}, and k_{off} for $R_tV_3{\cdot}R'_tL'_3 = k_{on} K_d^t \approx 1 \times 10^{-8}$ s^{-1}. Even if we assume that the rate for association of R_tV_3 with $R'_tL'_3$ is diffusion controlled (which may be true for antibodies and certain other proteins but is probably not true for association of R_tV_3 and $R'_tL'_3$ by extension from the kinetic behavior of the monomeric species), and has a

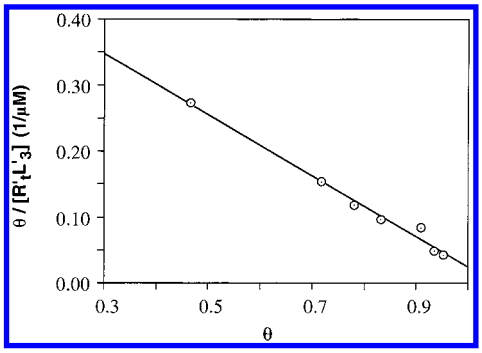


Figure 5. A plot of the ratios of $\theta/[R'_tL'_3]$ versus θ for the titration of R_tV_3 (4.5 μM) with $R'_tL'_3$ (0−35 μM) in the presence of **L** (19.1 mM). The line is a linear fit of the data to eq 13. The slope of the line yields a value of $K/[\mathbf{L}]^3 \approx 0.47$ μM^{-1}. The value of K_d^t for $R_tV_3{\cdot}R'_tL'_3$ is thus estimated as $\sim 4 \times 10^{-17}$ M (an average value of three repetitions; the error is within 25%).

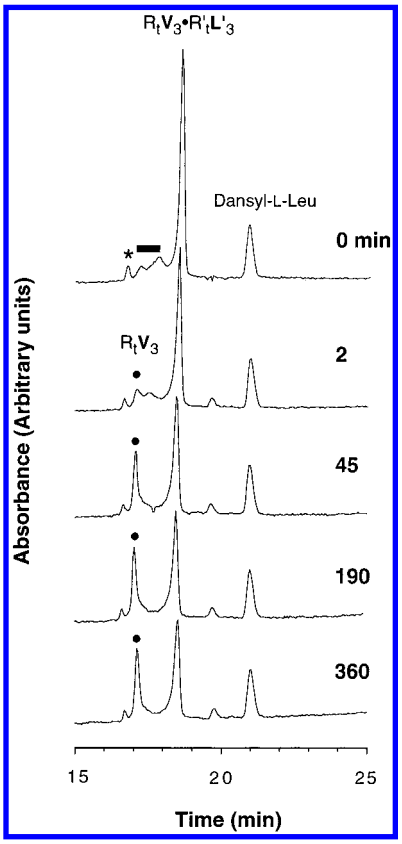


Figure 6. Representative HPLC traces demonstrating the exchange of R_tV_3 (3 μM) + $R'_tL'_3$ (22 μM) with **L** (86 mM) with formation of $R_tV_3{\cdot}3\mathbf{L}$. Exchange reactions were carried out by adding **L** to an equilibrated mixture of R_tV_3 and $R'_tL'_3$, and injecting the resulting mixture into the HPLC column after 0, 2, 45, 190, or 360 min. Symbols (•) indicate the retention time of $R_tV_3{\cdot}3\mathbf{L}$; this compound dissociates to free R_tV_3 on the column. The sample of R_tV_3 used here contained small amounts of impurities, which are indicated by the symbols (* and ■). The conditions were similar to those in Figure 3, except that a Vydac analytical RP column was used with the same linear gradient, run over 30 min.

value of $k_{on} \approx 10^9$ M^{-1} s^{-1}, the value of k_{off} would be $\sim 4 \times 10^{-8}$ s^{-1}. This value implies that dissociation of $R_tV_3{\cdot}R'_tL'_3$ would be too slow to be observed directly. This implication is consistent with our observation that the aggregate is stable for $>10^3$ s under HPLC conditions.

The exchange reaction between $R_tV_3{\cdot}R'_tL'_3$ and **L** proceeded surprisingly rapidly, however (equilibration was complete in

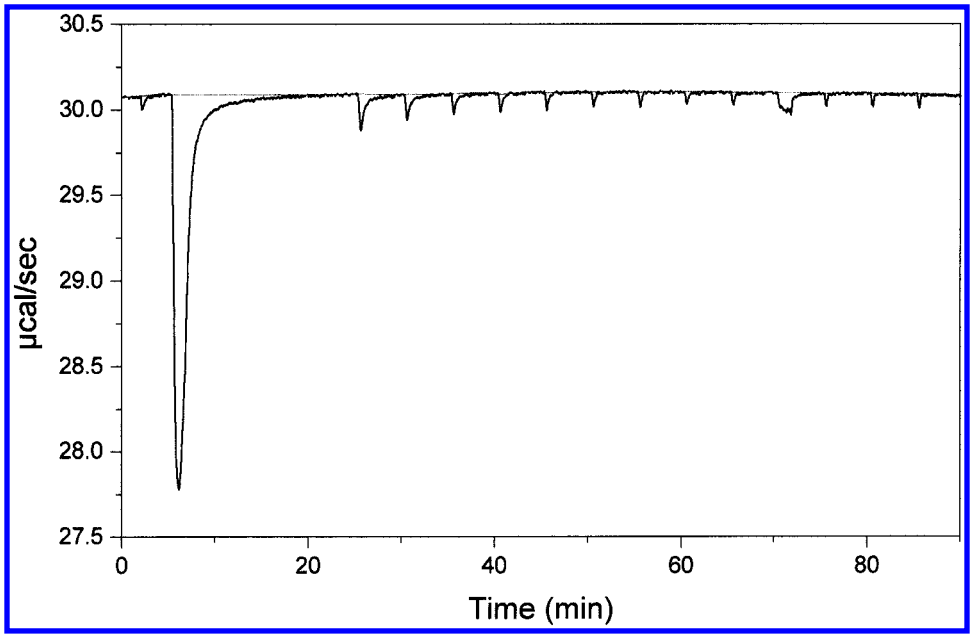


Figure 7. ITC titration of R_tV_3 by $R'_tL'_3$ (0.126 mM) in 5.0 mM phosphate buffer at 298 K. The injected volumes of the first, second, and twelfth injections were 0.5, 35, and 35 μL, respectively, and 3.0 μL for the remaining injections. The interval between the second and third injections was 20 min; the interval between the remaining injections was 5 min.

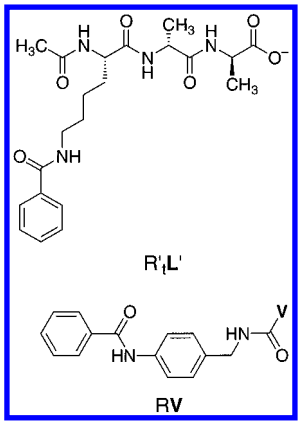
Table 2. Thermodynamic Parameters of Binding of Derivatives of Vancomycin to Derivatives of DADA at 298 K[a]

receptor	ligand	receptor/ ligand	valency	K_d (M)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$-T\Delta S°$ (kcal/mol)
R_tV_3	$R'_tL'_3$	1:1	tri	4×10^{-17}	−22	−40	18
			mono[b]		⟨−7.3⟩	⟨−13.3⟩	⟨6.0⟩
V[c]	**L**	1:1	mono	1.6×10^{-6}	−7.9	−12.0	4.1
R_tV_3	R'_tL'	1:3 [d]	mono	1.1×10^{-6}	−8.1	−12.4	4.3
R_tV_3	**L**	1:3 [d]	mono	2.7×10^{-6}	−7.6	−12.0	4.4
V	$R'_tL'_3$	3:1 [d]	mono	0.34×10^{-6}	−8.8	−17.5	8.7
R**V**	$R'_tL'_3$	3:1 [d]	mono	0.11×10^{-6}	−9.5	−17.8	8.3
R**V**	R'_tL'	1:1	mono	0.96×10^{-6}	−8.2	−21.0	13

[a] Values of $\Delta H°$ were determined by ITC titrations at pH 7.0 in 5.0 mM phosphate buffer at 298 K. Values of $\Delta G°$ were determined from K_d, measured in the same solution, except for that of $R_tV_3{\cdot}R'_tL'_3$. Values of $-T\Delta S°$ were calculated from $\Delta G° = \Delta H° - T\Delta S°$. Each titration was at least duplicated and differences are within 25% for K_d and 5% for $\Delta H°$. All thermodynamic values refer to the monovalent interaction between individual units of **V** and individual units of **L** or **L′**, except for those for binding of R_tV_3 to $R'_tL'_3$. [b] For convenience, the average values per **V·L** binding are also given for $R_tV_3{\cdot}R'_tL'_3$, and indicating by the label "mono" and brackets ⟨ ⟩. [c] Measured in 20 mM phosphate buffer at 25 °C by ITC; our values agree well with the literature values.[26] [d] In the cases of 1:3 or 3:1 binding, the values of K_d are K_d^m, the average dissociation constant per site (defined by eqs 4−7); these values are statistically corrected for the presence of multiple binding sites. Refer to Scheme 1 for structures of R_tV_3 and $R'_tL'_3$, and Scheme 4 for structures of R**V** and R'_tL'.

<45 min), as shown by HPLC during the exchange of $R_tV_3{\cdot}R'_tL'_3$ with excess **L** (Figure 6). We prepared the initial solution of $R_tV_3{\cdot}R'_tL'_3$ by mixing 3 μM of R_tV_3 and 22 μM of $R'_tL'_3$ in 20 mM phosphate solution. Examination of the sample by HPLC (using conditions similar to those used in the competition experiments) established that $R_tV_3{\cdot}R'_tL'_3$ had formed (Figure 6). Then **L** was added to this solution of $R_tV_3{\cdot}R'_tL'_3$ in quantities sufficient to make the concentration of **L** about 86 mM. At intervals, samples were withdrawn and examined on HPLC. Exchange of $R_tV_3{\cdot}R'_tL'_3$ with **L** yielded the complex of $R_tV_3{\cdot}3$**L**: this complex dissociated on the HPLC column to free R_tV_3 (as discussed previously). The integrated areas of the peaks for $R_tV_3{\cdot}R'_tL'_3$ and R_tV_3 (derived by dissociation of $R_tV_3{\cdot}3$**L**) were used to monitor the progress of the exchange reaction; dansyl-L-Leu was again used as an internal standard. Equilibration was complete within 45 min, and no further significant change in peak intensities occurred after that time. Approximately 60% of the R_tV_3 remained as $R_tV_3{\cdot}R'_tL'_3$ at equilibrium in a system containing **L** at a concentration of ~86 mM; this value of **L** is ~17 000 times greater than that of the dissociation constant for the complex of R_tV_3 and **L**, and confirmed that $R_tV_3{\cdot}R'_tL'_3$ was tightly associated.

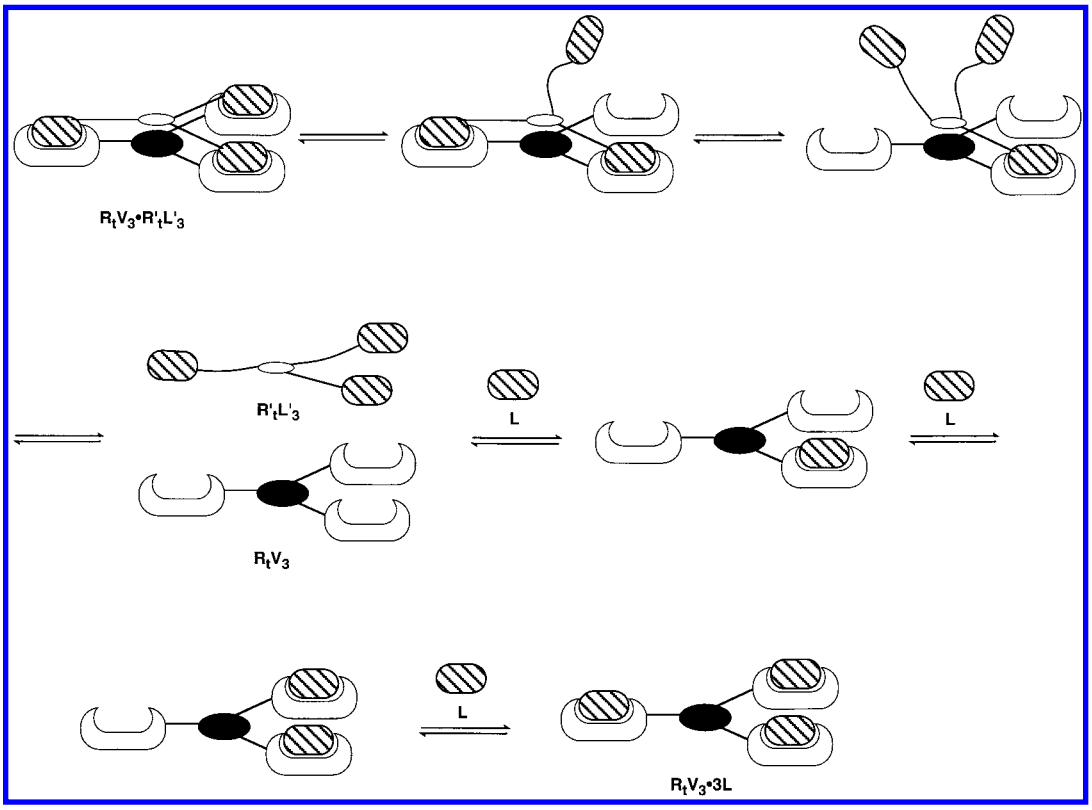
Scheme 4. Structures of the Monovalent Derivatives of DADA and **V**, R'_tL', and R**V**, Respectively



There is an essential difference in kinetics between this trivalent system and biotin·avidin. Dissociation of biotin·avidin necessarily proceeds to completion in one step and is slow.[24]

(24) Green, N. M. *Biochem. J.* **1963**, *89*, 585.

Scheme 5. Mechanism 1



The presence of a large excess of biotin does not accelerate the dissociation of biotin·avidin. As we have demonstrated here, dissociation of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ is slow in isolation, but *rapid when carried out in the presence of excess monovalent ligand* ***L***. Dissociation of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ therefore proceeds by a mechanism different from that of the single-step dissociation of biotin·avidin. We discuss the kinetics of this dissociation in the Discussion section.

Estimation of $\Delta H°$ of the Binding of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$. A direct estimation of $\Delta H°$ for the association of $R_t\mathbf{V}_3$ with $R'_t\mathbf{L}'_3$ would separate $\Delta G°$ into enthalpic and entropic terms, and clarify the thermodynamic basis for the very high binding constant of this receptor/ligand pair. We have characterized the association of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$ using isothermal titration calorimetry (ITC).[25] ITC can be used to estimate dissociation constants (K_d) in the millimolar to nanomolar range. It allowed us to estimate both the enthalpy of binding, $\Delta H°$, and the dissociation constant K_d^m for the monovalent interactions, but only $\Delta H°$ for the interaction of $R_t\mathbf{V}_3$ with $R'_t\mathbf{L}'_3$. Experiments were carried out by adding aliquots of a solution of $R'_t\mathbf{L}'_3$ (~0.126 mM) in 5.0 mM phosphate buffer to a solution of $R_t\mathbf{V}_3$ (~3 μM) in 5.0 mM phosphate buffer in the cell of the calorimeter (Figure 7). By integration of the peaks, we estimated $\Delta H°$ to be −40 kcal/mol (Table 2). Titrations were repeated three times and the maximum difference among these values of $\Delta H°$ was less than 2 kcal/mol. The value of $T\Delta S°$ for binding of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$ is thus approximately −18 kcal/mol ($T\Delta S° = \Delta H° - \Delta G°$). The value of $\Delta H°$ for the binding of vancomycin to **L** has been reported as −12.8 kcal/mol.[26] The value of $\Delta H°$ of the trivalent system is thus approximately three times that for monovalent vancomycin and DADA, and the value of $T\Delta S°$ for the trivalent system is approximately 4.5 times that for the monovalent system.

We were also concerned about the effect of the interaction between the two linking groups in $R_t\mathbf{V}_3$ and $R'_t\mathbf{L}'_3$ on the binding in the trivalent system. We therefore synthesized two monovalent derivatives of DADA and **V**, $R'_t\mathbf{L}'$ (N^α-acetyl-N^ϵ-benzoyl-L-Lys-D-Ala-D-Ala) and R**V** ($C_6H_5CONHC_6H_4$-4-$CH_2NHCO\mathbf{V}$), respectively (Scheme 4), to simulate the influence of the R'_t group in $R'_t\mathbf{L}'_3$ and the R_t group in $R_t\mathbf{V}_3$. The ITC titrations characterized the interactions between these ligand/receptor pairs thermodynamically, and Table 2 summarizes the results.

All of the bindings examined in Table 2 are monovalent except that for the binding of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$. The parameters listed in this table thus referred to monovalent binding between **V** (or **V** unit in $R_t\mathbf{V}_3$) and its ligand except in the binding of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$. In particular, in the cases of 1:3 or 3:1 complexes, the dissociation constant (K_d) refers to a monovalent dissociation constant (K_d^m with units of molar), *not* a collective constant (a product of three monovalent binding constants, which is equal to $(K_d^m)^3$ and which has units of M^3; eqs 4−7). The results indicate that the value of K_d is essentially indifferent to substitution on either vancomycin or L-Lys-D-Ala-D-Ala groups, so long as the interaction is monovalent; only the trivalent interaction is exceptionally strong. In broad terms, the data suggest that the high free energy of association of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ originate primarily in the enthalpy of association of three **V** and **L** groups. The values of $\Delta H°$ of binding of **V**, or of its monomeric derivative R**V** to $R'_t\mathbf{L}'_3$ are, however, about 5 kcal/mol more negative than that of **V** to **L**. A discussion of these thermodynamic data follows in the next section.

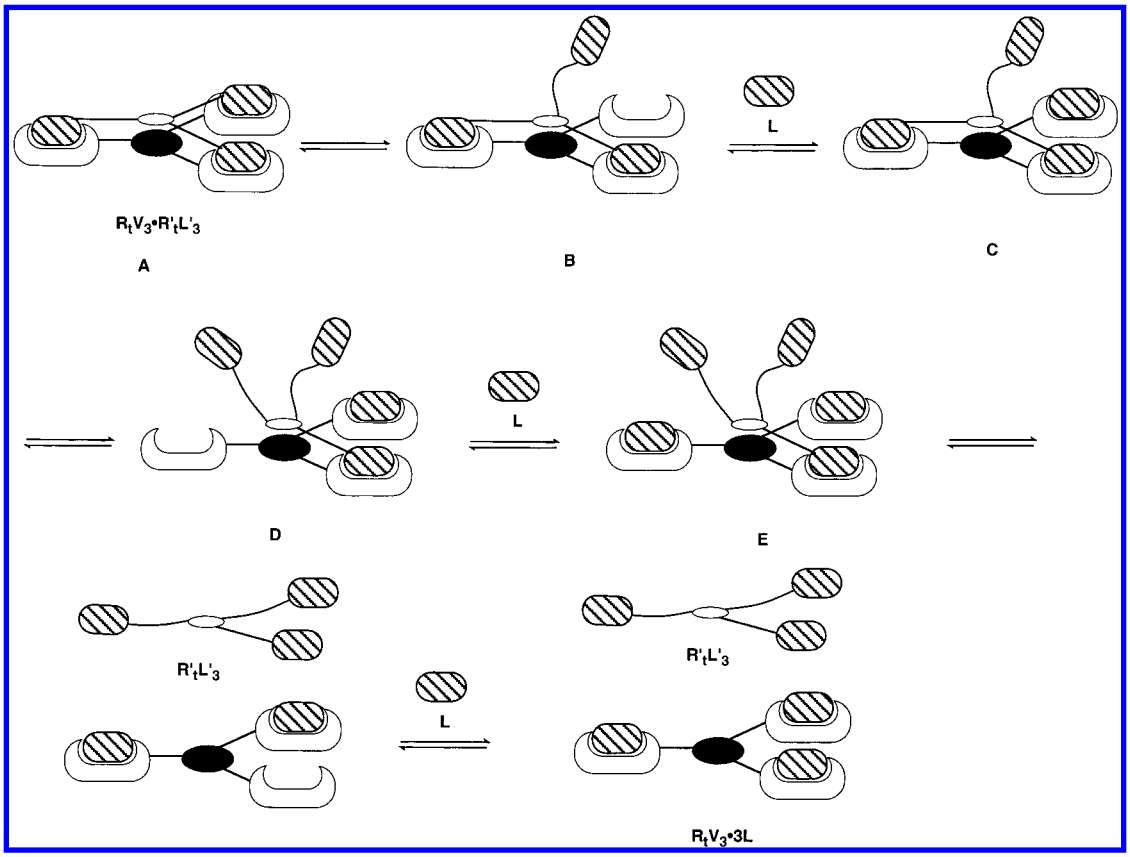
Discussion

The dissociation constant describing the dissociation of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ into $R_t\mathbf{V}_3 + R'_t\mathbf{L}'_3$ ($K_d^t \approx 4 \times 10^{-17}$ M) is 10^{-10} that of

(25) Wiseman, T.; Williston, S.; Brandt, J. F.; Lin, L.-N. *Anal. Biochem.* **1989**, *179*, 131.

(26) Cooper, A.; McAuley-Hecht, K. E. *Philos. Trans. R. Soc. London A* **1993**, *345*, 23.

Scheme 6. Mechanism 2



V·**L** into **V** + **L** ($K_d^m \approx 1.6 \times 10^{-6}$ M).[2] The relationship between thermodynamic and kinetic characteristics of this dissociation is strikingly different from that of avidin·biotin: although the rate of dissociation of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ is slow (as is that of avidin·biotin), equilibration of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ with monomeric **L** to give $R_t\mathbf{V}_3$·3**L** is relatively fast, and monomeric **L** competes relatively effectively with trimeric $R'_t\mathbf{L}'_3$ for the receptor sites on $R_t\mathbf{V}_3$. We consider the issues of kinetics and thermodynamics separately.

Kinetics. The essential difference between $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ and biotin·avidin is that dissociation of the former, when carried out in the presence of excess **L**, proceeds in three steps, while the latter necessarily proceeds to completion in one step. We considered two plausible mechanisms for the exchange reaction between $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ and excess **L**.

A. Mechanism I. The first mechanism (Scheme 5) involves complete dissociation of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ into $R_t\mathbf{V}_3$ and $R'_t\mathbf{L}'_3$, followed by association of free $R_t\mathbf{V}_3$ with three equivalents of **L**. This mechanism is similar to that for dissociation of biotin·avidin, which proceeds to completion in one step. We presume that the rate-determining step in this mechanism would be the last one: that is, the step leading to complete dissociation of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ into $R_t\mathbf{V}_3$ + $R'_t\mathbf{L}'_3$. The rate of this process would be independent of [**L**], and would, as we have indicated, be very slow ($k_{off} \approx 10^{-8}$ s^{-1}, even if k_{on} were diffusion limited). This expectation is contrary to the experimental observation that, in the presence of excess **L**, exchange of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ with **L** is complete within 45 min. We therefore discard this mechanism.

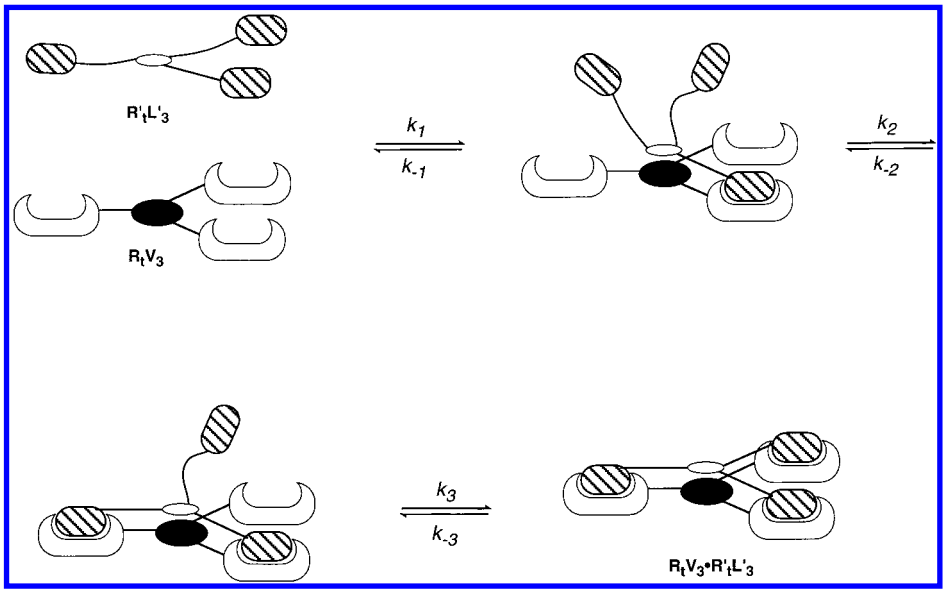
B. Mechanism II. The second mechanism (Scheme 6) involves a sequence of successive dissociation and association events occurring at the vancomycin binding sites of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$. After initial dissociation of DADA groups (A → B; C → D), the empty binding sites could associate with **L** (B → C; D → E) before the next step of dissociation. Association of **L** with each empty active site would inhibit rebinding of dissociated DADA groups on $R'_t\mathbf{L}'_3$. This mechanism predicts a fast exchange between $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ and **L** and is consistent with our experimental observations. We therefore infer that excess monovalent ligand **L** competes for the binding sites of $R_t\mathbf{V}_3$ on a site-by-site basis and thus accelerates the dissociation of the trivalent complex of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$.

C. Kinetics of Association of $R_t\mathbf{V}_3$ with $R'_t\mathbf{L}'_3$. Scheme 7 outlines the stepwise association of $R_t\mathbf{V}_3$ with $R'_t\mathbf{L}'_3$. So far, we have not been able to measure the rate constants for each kinetic step in this model experimentally. We thus applied rate constants obtained for the monovalent interaction between **V** and **L** to an estimate of theoretical values of the kinetic parameters in the binding of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$. This estimate should also provide insight into the kinetic origin of the high stability of $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$. We make three assumptions in our estimation. (i) The value of the rate constant for the first step in association of $R_t\mathbf{V}_3$ with $R'_t\mathbf{L}'_3$, k_1, is 9 times that of k_{on} for monomeric **V** and **L**: $k_1 = 9k_{on}$. (ii) The rate constant for dissociation of each **V**/DADA pair in $R_t\mathbf{V}_3$·$R'_t\mathbf{L}'_3$ is, after corrections for statistical factors, approximately the same as k_{off} for **V**·**L**, $k_{-1} = k_{-2}/2 = k_{-3}/3 = k_{off}$. (iii) The rate constants of the two intramolecular associations, after corrections for statistical factors, are approximately the same: $k_2/4 = k_3$. Combining these assumptions and the literature values of k_{on} and k_{off} for **V** and **L**, ($k_{on} \approx 2.8 \times 10^7$ M^{-1} s^{-1}; $k_{off} \approx 30$ s^{-1}), [21] we estimated k_2 and k_3 as follows

$$K_d^t = (k_{-1}k_{-2}k_{-3})/(k_1k_2k_3)$$
$$= (30 \times 2 \times 30 \times 3 \times 30)/[(9 \times 2.8 \times 10^7)k_2k_3]$$
$$\approx 4 \times 10^{-17}\ \text{M} \qquad (14)$$

With the assumption that $k_2/4 = k_3$, we estimated the rate

Scheme 7



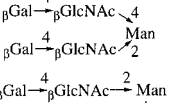
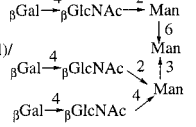
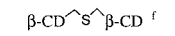
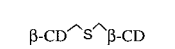
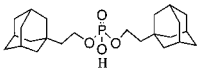
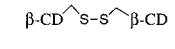
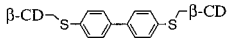
constants for the intramolecular association to be $k_2 = 8 \times 10^6$ s^{-1} and $k_3 = 2 \times 10^6$ s^{-1}. These large values of the rate constant for intramolecular association inside the complex suggest the kinetic origin of the high stability of $R_tV_3 \cdot R'_tL'_3$: fast intramolecular rebinding of partially dissociated **V**/DADA pairs in the complex. (This estimation is obviously highly approximate; it does not consider possible variations in the off-rate in each step of dissociation).

For a value of $k_2 = 8 \times 10^6$ s^{-1}, the value of effective molarity of the DADA group is approximately 290 mM ($k_2/k_1 = 8 \times 10^6$ $s^{-1}/2.8 \times 10^7$ M^{-1} s^{-1}), and a value of $k_3 = 2 \times 10^6$ s^{-1} corresponds to an effective molarity of about 70 mM. For comparison, Table 3 lists values of effective molarity for several intramolecular processes.[27−33] The values of effective molarity in our system, in general, are comparable to (slightly larger than) those for most biochemical systems, but they are smaller than those observed in systems involving smaller components (for example, the chelate effect in the coordination of small ligands with metal ions). This fact indicates that intramolecular binding in our system does not convey as much advantage as it does in chelation of metal ions. Our analysis of the entropic loss in this trivalent system suggests that the relatively small value of effective molarity that we observe here is due to loss in conformational entropy.

One interesting inference from this analysis is that exceptionally high values of effective molarity are not necessarily required to achieve very tight binding in the system $R_tV_3 + R'_tL'_3$. We would, thus, anticipate that a number of systems of receptors and ligands might give very tight binding in tri- or higher-valent form.

Thermodynamics. The free energy of trivalent binding of $R_tV_3 + R'_tL'_3$ is approximately three times that of monvalent binding of **V** to **L** (Table 2): that is, $\Delta G°_t \approx 3\Delta G°_m \approx 3\Delta H°_m - 3T\Delta S°_m$. Calorimetric measurements indicated that $\Delta H°$ for association of R_tV_3 and $R'_tL'_3$ (∼−40 kcal/mol) is approximately three times that for **V** and **L** ($\Delta H° = -12.8$ kcal/mol). The value of $-T\Delta S°$ for association of the two trivalent species is thus 18 kcal/mol; this value is approximately 4.5 times that of **V** and **L** ($T\Delta S° = -4.1$ kcal/mol) (Table 2). In the simplest analysis, we envision two limiting cases for association of $R_tV_3 + R'_tL'_3$ (Scheme 2). In one, after the first **V** and **L′** group interact, the other two pairs of **V** and **L′** groups are correctly

Table 3. Effective Molarities for Some Oligovalent Association Reactions

Receptor	Ligand	Effective molarity [a] (M)	ref
R_tV_3	$R'_tL'_3$	0.07; 0.29	This study
Dimeric **V**	Dimeric DADA	0.02	1
Dimeric **V**	AcKDADA	0.04 [b]	28
Non-covalent dimer of Ristocetin A	*N*-decanoyl-DADA	0.01 [c]	29
Mammalian hepatic lectin for Galactose (Gal)/ *N*-acetylgalactosamine (GlcNAc)		0.03	30
Mammalian hepatic lectin for Galactose (Gal)/ *N*-acetylgalactosamine (GlcNAc)		0.01 [d]	30
Rabbit γG anti-DNP antibody [e]	bacteriophage conjugated with DNP	0.01	31
	cholesterol	0.02	32
		0.01	32, 33
	"	0.02	33
	"	0.002	33
EDTA [g]	Cu^{2+}	126	34
"	Zn^{2+}	8.7×10^3	34
"	Cd^{2+}	7.8×10^6	34

[a] Calculated from the reported values of K_d. [b] Presented at a self-assembly monolayer on gold. [c] In the presence of sodium dodecylsulphate. [d] Assuming that the values of effective molarity for the two intramolecular associations are the same in this trivalent binding. [e] DNP: 2,4-dinitrophenyl group. [f] β-CD: β-cyclodextrin. [g] EDTA: ethylenediaminetetraacetate.

(27) Rao, J.; Yan, L.; Xu, B.; Whitesides, G. M. *J. Am. Chem. Soc.* **1999**, *121*, 2629.
(28) Westwell, M. S.; Bardsley, B.; Dancer, R. J.; Try, A. C.; Williams, D. H. *J. Chem. Soc., Chem. Commun.* **1996**, 589.
(29) Lee, Y. C.; Townsend, R. R.; Hardy, M. R.; Lönngren, J.; Arnarp, J.; Haraldsson, M.; Lönn, H. *J. Biol. Chem.* **1983**, *258*, 199.
(30) Hornick, C. L.; Karush, F. *Isr. J. Med. Sci.* **1969**, *5*, 163.
(31) Breslow, R.; Zhang, B. *J. Am. Chem. Soc.* **1996**, *118*, 8495.
(32) Zhang, B.; Breslow, R. *J. Am. Chem. Soc.* **1993**, *115*, 9353.
(33) Irving, H.; Williams, R. J. P.; Ferrett, D. J.; Williams, A. E. *J. Chem. Soc.* **1954**, 3494.

positioned to interact with negligible further loss in entropy; in this case, $\Delta G^\circ_t \approx 3\ \Delta H^\circ_m - T\Delta S^\circ_m + 2T\Delta S^\circ_{trans+rot,m}$ ($T\Delta S^\circ_{trans+rot,m}$ is the translational and rotational entropy for the association of **V** and **L**). In the second, the linkers are so long and flexible that after interaction of the first **V** and **L′** pair, there is large entropic penalty (conformational entropy loss) that prevents any further intramolecular association. In practice, interactions involving the linkers and hubs can make ΔH°_t different from $3\ \Delta H^\circ_m$, and can also disguise any simple relationship between $T\Delta S^\circ_t$ and $T\Delta S^\circ_m$.

We wish to understand the thermodynamic origins of these values. This task would require separating the enthalpic and entropic contributions of each binding event to the free energy of the association of the two trivalent species. Unfortunately, we do not have the information required to carry out this separation, and given the fact that we do not observe intermediates in association or dissociation in HPLC analyses, we are unlikely to be able to obtain this information. Instead, we have inferred the enthalpic and entropic contributions of certain binding events to the ΔG° of trivalent binding from semiquantitative analyses of ΔH° and $T\Delta S^\circ$.

ΔH°. To analyze the value of ΔH°_t, we need first to decide on the appropriate reference value to which ΔH°_t should be compared: that is, should the reference value for the "comparable" monomeric interaction, ΔH°_m, be considered the value for **V** and **L** or for these species with linkers attached? And if the latter, what types of linkers? Our instinct is that the most appropriate structural comparisons for processes involving $R_t\mathbf{V}_3$ and $R'_t\mathbf{L}'_3$ will be derivatives of **V** and **L** that have linking groups attached, but it is difficult to justify this instinct analytically: unfortunately, we cannot draw a clear conclusion concerning the influence of structures that are, or mimic, the linking groups on the values of ΔH°_m. Table 2 contains values of enthalpy that range from that for **V** + **L** ($\Delta H^\circ_m \approx -12$ kcal/mol; two examples) to substantially larger ($\Delta H^\circ_m \approx -17.5$ to -21 kcal/mol; three examples). Thus, we can conclude only that the average value of ΔH° for association of one **V** and one **L′** group in $R_t\mathbf{V}_3$ and $R'_t\mathbf{L}'_3$ is approximately equal to or larger than (i.e., energetically less favorable than) ΔH° for reference monomers.

We have considered three explanations for the differences in ΔH° for the compounds in Table 2: (i) experimental error, (ii) linker−vancomycin interactions, and (iii) dimerization of vancomycin. We discuss each in turn.

Experimental Error. A troublesome aspect of the data in Table 2 is the observation that, with the exception of $R_t\mathbf{V}_3 + R'_t\mathbf{L}'_3$, ΔG° varies by less than 1.5 kcal/mol, while ΔH° (and of course $T\Delta S^\circ$) varies by 9 kcal/mol. The appearance of apparent example of enthalpy/entropy compensation is always a warning, although Williams and others have made a convincing case for the existence of compensating relationships in circumstances in which tighter binding (and a more negative enthalpy of binding) is accompanied with loss in conformational mobility (and a greater loss in entropy). In the data in Table 2, the historically most common and plausible origin of an artifactual enthalpy/entropy compensation—an inaccurate decomposition of ΔG° into ΔH° and $T\Delta S^\circ$ based on the temperature dependence of ΔG°—is eliminated since ΔG° and ΔH° were obtained using independent experimental techniques (UV or HPLC for ΔG°; ITC for ΔH°). The values were also consistent over three replications. We therefore believe that they are accurate within the stated uncertainties, and that, as usual, the trends are even more reliable than the numerical data.

Having said that we are confident that the data are largely free of artifact, we point out that they are not simple: we do not see an obvious, qualitative explanation for the trends. For example, for most of the data, increasing the molecular surface of the molecule containing the DADA group increases ΔH°, but the value for $R_t\mathbf{V}_3 + R'_t\mathbf{L}'_3$ is an exception. We therefore believe, but cannot prove, that increases in contacting surface and changes in entropy, probably due to restrictions on conformational mobility, compensate.

Linker−Vancomycin Interactions. We examined possible contributions of the linker groups to the enthalpy of binding. The values of ΔH° for binding of $R_t\mathbf{V}_3$ to monovalent ligands (**L** or $R'_t\mathbf{L}'$) are similar to that for the binding of **V** to **L**. This result, by itself, seems to imply that the contribution of the linker groups to ΔH° is small. The value of ΔH° for binding of $R'_t\mathbf{L}'_3$ to R**V** (a monovalent vancomycin containing an R group that was designed to simulate the structure of R_t) is, however, about 5 kcal/mol more negative than that for **V** to **L** (Table 2). This result seems to conflict with the previous implication that the influence of the linker groups on the value of ΔH° is small. Moreover, the value of ΔH° for binding of $R'_t\mathbf{L}'_3$ to **V** was, surprisingly, still approximately 5 kcal/mol more negative than that for binding of **V** to **L**, although only $R'_t\mathbf{L}'_3$ bears a linker group in this binding pair. We do not to see a simple, consistent relationship between linker groups and their contributions to binding enthalpies that makes it clear what the appropriate reference compound is against which to compare the thermodynamic values for binding of $R_t\mathbf{V}_3$ to $R'_t\mathbf{L}'_3$.

Dimerization of Vancomycin. Vancomycin has a strong tendency to form a noncovalent dimer.[16−18] This tendency to dimerize is enhanced by ligand binding.[34] The pattern of dimerization identified by Williams is head-to-tail, and this geometry is not possible for the vancomycin groups of $R_t\mathbf{V}_3$. It is more plausible that it might play a role for the combination of **V** (or R**V**) interacting with $R'_t\mathbf{L}'_3$, but we have, at present, no direct evidence for or against dimerization of vancomycin as a process that influences the thermodynamics of this system.

***T*ΔS°.** There is an approximately 18 kcal/mol loss of entropy on association of $R_t\mathbf{V}_3$ with $R'_t\mathbf{L}'_3$. As we discussed previously (Scheme 7), the trivalent binding process takes place in three steps: a bimolecular association as the first binding event, then two intramolecular binding steps. The problem in any analysis of $T\Delta S^\circ$ for the trimeric interaction is, however, the same as that mentioned for ΔH°_m: that is, deciding on an appropriate reference value of $T\Delta S^\circ_m$.

We have analyzed the value of $T\Delta S^\circ$ for association of $R_t\mathbf{V}_3 + R'_t\mathbf{L}'_3$ assuming, for explicitness, that the association of **V** to **L** provides an appropriate reference value. For the first binding event, we assume that the loss in entropy is mainly associated with translational and rotational terms. The effect of molecular size on the loss of translational and rotational entropy is relatively small ($\leq$15% for an increase in molecular weight of a factor of 3 [12]). For binding of **V** to **L**, $T\Delta S^\circ$ is ~ -4.1 kcal/mol. We thus assume the value of $T\Delta S^\circ$ for the first binding step of association of $R_t\mathbf{V}_3 + R'_t\mathbf{L}'_3$ to be ~ -4.7 kcal/mol (based on this 15% correction). We expected the next two steps to be less entropically unfavorable than the first, since they take place intramolecularly. The total value of $T\Delta S^\circ$ for these two steps is, however, a surprisingly negative number: ~ -13.3 kcal/mol (estimated by subtracting $T\Delta S^\circ$ for the first binding from the total $T\Delta S^\circ$ for the trivalent binding). If this value is correct, where does it come from? A review of the contributors to the entropic loss of binding of **V** to **L** suggests clues to its origin.

(34) Williams, D. H.; Maguire, A. J.; Tsuzuki, W.; Westwell, M. S. *Science* **1998**, *280*, 711.

Williams et al. have carefully examined the entropic loss of binding of *N*-Ac-D-Ala-D-Ala to vancomycin and ristocetin A (a vancomycin group antibiotic).[12] In this semiquantitative analysis, they dissected $T\Delta S°$ for binding of **V** to *N*-Ac-D-Ala-D-Ala into three major terms (eq 15): loss of translation and rotation due to a bimolecular association ($T\Delta S°_{trans+rot} \approx -17$ kcal/mol), the freezing out of four rotors of the peptide ($T\Delta S°_{rotors} \approx -5$ kcal/mol), and the release of water ($T\Delta S°_{solv} \approx 19$ kcal/mol). The predicted value of $T\Delta S°$ is close to the experimental value in this system ($\sim T\Delta S° \approx -4.1$ kcal/mol).

$$T\Delta S°_{m} = T\Delta S°_{trans+rot} + T\Delta S°_{rotors} + T\Delta S°_{solv} \quad (15)$$
$$\approx -17 - 5 + 19 \approx -3 \text{ kcal/mol}$$

Using analogous reasoning, we estimated the entropic loss in the two intramolecular binding steps for $R_tV_3 + R'_tL'_3$. We estimate the total entropic loss for the two intramolecular binding steps by eq 16, where $T\Delta S°_{conf}$ defines the additional conformational entropy loss in the two intramolecular binding steps. As estimated above, the value of $T\Delta S°_{second+third}$ for the second and third binding steps is approximately -13.3 kcal/mol. We therefore estimate $T\Delta S°$ conf ~ -41 kcal/mol (eq 17).

$$T\Delta S°_{second+third} = 2\,T\Delta S°_{rotors} + 2\,T\Delta S°_{solv} + T\Delta S°_{conf} \quad (16)$$
$$\approx 2 \times (-5) + 2 \times 19 + T\Delta S°_{conf}$$
$$\approx -13.3 \text{ kcal/mol} \quad (17)$$

This large loss of conformational entropy can partly be rationalized in terms of structures of R_tV_3 and $R'_tL'_3$. Although R_tV_3 is fairly rigid, it still contains nine rotors that are frozen upon complexation to $R'_tL'_3$ (Scheme 1). $R'_tL'_3$ is a more flexible molecule, and its complexation to R_tV_3 freezes, or partly freezes, additional 18 rotors (Scheme 1). Page and Jencks estimated a value of $\sim 1.2-1.5$ kcal/mol per rotor frozen upon complexation.[35] The entropic penalty from freezing out these rotors, (assuming that all are free in the unassociated state, and frozen in the complex), thus would range between 32 and 40 kcal/mol. This range is reasonably close to the value of our estimate of $T\Delta S°_{conf}$ in the two intramolecular binding steps, given the high degree of approximation in this estimation (the entropic loss per rotor frozen obviously is not the same for the cases considered by Page and Jencks and those considered here, nor are all the rotors in R_tV_3 and $R'_tL'_3$ the same).

In summary, our analysis of $T\Delta S°$ for the trivalent binding of R_tV_3 and $R'_tL'_3$ is compatible with (but does not demand) a scheme in which the loss in conformational entropy in two intramolecular bindings offsets the gain in $T\Delta S°_{tran+rot}$ from linking three monomers as trimers. The observation that overall $T\Delta S°$ for the trivalent binding appears to be slightly more than three times of that for binding of **V** to **L** is thus, in this analysis, a coincidence.

Conclusions

Polyvalency aims to enhance binding through entropic advantage. The trivalent system we have designed, based on vancomycin and DADA, demonstrates the successful application of this approach, and clarifies some of its principles: this system is the tightest binding one (among relatively low molecular weight organic species) of which we know. Our current understanding of thermodynamics of this trivalent system shows that, as expected, $\Delta H°$ for the trimer originates primarily in the enthalpy of association of three **V** and **L** groups, and the gains in $T\Delta S°_{tran+rot}$ that come from trivalency are coincidentally largely offset by the loss in conformational entropy of the linking groups on binding.

Aside from its demonstration of a large binding constant, the work carries a prescription for systems in which trivalency might give even larger binding constants. The features in these systems would include (i) rigid receptors and ligands, (ii) conformational stiff linkers, correctly designed to allow receptors and ligands to interact without enthalpic strain and with minimal loss in entropy due to freezing of conformational and rotational terms, and (iii) a large hydrophobic component to binding to give a large positive contribution from $T\Delta S$ from release of water.

Experimental Section

General Methods. The ^{1}H NMR spectra were recorded at 400 MHz. Chemical shifts are reported in parts per million downfield from tetramethylsilane. Vancomycin hydrochloride was purchased from Sigma and used without further purification. Amino acids were purchased from Sigma (St. Louis, Missouri), except for D-Ala-*O*-tBu from BACHEM Bioscience (King of Prussia, Pennsylvania). The peptide coupling reagent HBTU was purchased from Applied Biosystems (Atlanta, Georgia). Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were dried overnight over silica gel and 4 Å molecular sieves, respectively, followed by distillation under reduced pressure. Diisopropylethylamine (DIEA) was distilled from ninhydrin. The monomeric peptide ligand diacetyl-L-lysyl-D-alanyl-D-alanine (**L**) was available from a previous study.[1]

***tert*-Butyl 4-Aminobenzylamine Carboxylate.** To a solution of 4-aminobenzylamine (3.66 g, 30 mmol) in 60 mL of acetone solution was slowly added di-*tert*-butyl dicarbonate (2.84 g, 15 mmol) in 10 mL of acetone and 6 mL of triethylamine. The reaction was stirred at room temperature for 30 min, and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography (eluting with 2:3 hexane/ethyl acetate) to give 2.67 g (12 mmol, 80%) of the product as a white solid: $R_f = 0.43$ (1:1 hexane/ethyl acetate); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.45 (s, 9 H, CCH_3), 3.65 (s, 2 H, NH_2), 4.18 (d, $J = 5.4$ Hz, 2 H, CH_2NH), 4.73 (b, 1 H, NHCO), 6.64 (d, $J = 6.4$ Hz, 2 H, C_6H_4), 7.07 (d, $J = 8.1$ Hz, 2 H, C_6H_4); HRMS−FAB (M + Na^+) calcd for $C_{12}H_{18}N_2O_2Na$ 245.1266, found 245.1253.

1,3,5-Benzene Tris(*N*-(*tert*-butyloxycarbonyl)-4′-aminomethylphenyl-carboxamide). To a solution of *tert*-butyl 4-aminobenzylamine carboxylate (1.01 g, 4.5 mmol) in 50 mL of dry CH_2Cl_2 was added 1.0 mL of DIEA and 1,3,5-benzene tris(carbonyl chloride) (0.40 g, 1.5 mmol) at 0 °C under stirring. After 2 h, the reaction mixture was poured into 5 mL of saturated aqueous NaCl solution and extracted with CH_2Cl_2. The solvent was removed by rotary evaporation, and the residue was purified by flash chromatography (eluting with 100:3 CH_2Cl_2/methanol) to give 1.01 g (1.2 mmol, 82%) of the product as a white powder: $R_f = 0.50$ (20:1 CH_2Cl_2/methanol); ^{1}H NMR (DMSO-d_6) δ 1.39 (s, 27 H, CCH_3), 4.10 (d, $J = 5.96$ Hz 6 H, CH_2), 7.24 (d, $J = 8.3$ Hz, 3 H, C_6H_4), 7.36 (t, $J = 6.1$ Hz, 6 H, CH_2NH), 7.72 (d, $J = 8.2$ Hz, 6 H, C_6H_4), 8.67 (s, 3 H, C_6H_3), 10.55 (s, 3 H, CONH); HRMS−FAB (M + Na^+) calcd for $C_{45}H_{54}N_6O_9Na$ 845.3850, found 845.3834.

1,3,5-Benzene Tris(*N*-4′-aminomethylphenyl-carboxamide). A solution of 1,3,5-Benzene Tris(*N*-(*tert*-butyloxycarbonyl)-4′-aminomethyl-phenyl-carboxamide) (170 mg, 0.21 mmol) in 5 mL of 1:1 CH_2Cl_2/TFA was stirred at room temperature for 1 h. After the solvent had been removed by rotary evaporation, we recovered 170 mg of a white powder (95%) as tris(trifluoroacetate) salt: ^{1}H NMR (DMSO-d_6) δ 4.02 (q, 6 H, CH_2), 7.46 (d, $J = 8.6$ Hz, 6 H, C_6H_4), 7.83 (d, $J = 8.6$ Hz, 6 H, C_6H_4), 8.21 (bs, 9 H, NH_3^+), 8.70 (s, 3 H, C_6H_3), 10.71 (s, 3 H, CONH); HRMS−FAB (M + H^+) calcd for $C_{30}H_{31}N_6O_3$ 523.2458, found 523.2464.

R_tV_3. To a solution of 100 mg of vancomycin hydrochloride (67 μmol), in 0.5 mL of dry DMSO, were added 0.5 mL of dry DMF and 1,3,5-benzene tris(*N*-4′-aminomethylphenyl-carboxamide) (19 mg, 22 μmol). The mixture was cooled to 0 °C, and 38 mg (100 μmol) of HBTU was added, followed by 26 mg (206 μmol) of DIEA. The

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

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 130 mg of crude product. Preparative reverse-phase HPLC purification and lyophilization afforded a white foam as the R_tV_3 hexa(trifluoroacetate). The ^{1}H NMR spectrum showed the resonances expected for vancomycin and the hub units [dry DMSO-d_6, δ 4.45 (d, CH_2NH), 7.28 (d, ArH), 7.74 (d, ArH), 8.70 (s, ArH), 10.60 (s, CONH)]. The ESIMS exhibited an ion at *m*/*z* 4818.8 consistent with the calculated average molecular weight of 4817.4 for the parent ion (M+H$^+$), $C_{228}H_{250}N_{33}O_{72}Cl_6$.

R′$_t$L′$_3$. A suspension of 86 mg of N^{α}-Ac-L-Lys-D-Ala-D-Ala-O-tBu (0.22 mmol) in CH_2Cl_2 was cooled to 0 °C. To this suspension was added 20 mg of 1,3,5-benzene tris(carbonyl chloride) (0.075 mmol) in 2 mL of CH_2Cl_2 in portions. The ice bath was removed, and the solution was stirred under argon for 2 h, and a fine white precipitate formed. Filtration afforded 25 mg of product as white flaky solid (0.019 mmol). The crude *tert*-butyl ester-protected peptide was then stirred in a 1:1 mixture of TFA/CH_2Cl_2 for 1 h to cleave *tert*-butyl groups. After removal of the solvent and TFA by rotary evaporation, the final residue was purified by reverse phase HPLC and afforded the desired R′$_t$L′$_3$. ^{1}H NMR (DMSO-d_6) δ 1.16 (d, 9 H, CHCH_3), 1.26 (d, 9 H, CHCH_3), 1.22−1.36 (m, 6 H, CH_2), 1.43−1.64 (m, 12 H, CH_2), 1.81 (s, 9 H, COCH_3), 3.24 (d, 6 H, CH_2NH), 4.13−4.19 (m, 6 H, COC*H*NH), 4.28 (m, 3 H, C*H*CH$_3$), 8.03 (d, 3 H, NH), 8.08 (d, 3 H, NH), 8.15 (d, 3 H, NH), 8.34 (s, 3 H, ArH), 8.65 (t, 3 H, NH); ESIMS exhibited an ion at *m*/*z* 1148.7 consistent with the calculated *m*/*z* of 1147 for $C_{51}H_{79}N_{12}O_{18}$ (M + H$^+$).

RV. To a solution of 100 mg of vancomycin hydrochloride (67 μmol), in 0.5 mL of dry DMSO, were added 0.5 mL of dry DMF and 4-aminomethylphenyl-carboxamide trifloroacetate salt (51 mg, 135 μmol). The mixture was cooled to 0 °C, and 31 mg (82 μmol) of HBTU was added, followed by 26 mg (206 μmol) of 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. The residue after removal of the solvent was subject to preparative reverse-phase HPLC. Lyophilization of collected fractions afforded a white foam as R**V** di-(trifluoroacetate). The ^{1}H NMR spectrum showed the resonances expected for vancomycin and the R unit [dry DMSO-d_6, δ 4.47 (d, CH_2NH), 7.25 (d, ArH), 7.50−7.58 (m, ArH), 7.70 (d, ArH), 7.94 (d, ArH), 8.42 (t, CONH), 10.23 (s, CONH)]. The ESIMS exhibited an ion at *m*/*z* 1658.9 consistent with the calculated average molecular weight of 1658.5 for the parent ion (M + H$^+$), $C_{80}H_{88}N_{11}O_{24}Cl_2$.

R′$_t$L′. A suspension of 98 mg of N^{α}-Ac-L-Lys-D-Ala-D-Ala-O−Benzyl ester (0.19 mmol) in CH_2Cl_2 was cooled to 0 °C. To this suspension was added 30 μL of benzoyl chloride (0.26 mmol) and 100 μL of triethylamine (1.1 mmol). The ice bath was removed, and the solution was stirred under argon for 2 h. The solvent was removed by rotary evaporation, and the residue was purified by flash chromatography (eluting with 10:1 CH_2Cl_2/methanol) to give a white powder: R_f = 0.45 (10:1 CH_2Cl_2/ methanol). Deprotection of the benzyl ester by hydrogenation afforded the desired free peptide R′$_t$L′ (70 mg; 85% yield). ^{1}H NMR (DMSO-d_6) δ 1.15 (d, 3 H, CHCH_3), 1.26 (d, 3 H, CHCH_3), 1.12−1.36 (m, 3 H, CH$_2$), 1.43−1.60 (m, 3 H, CH$_2$), 1.81 (s, 3 H, COCH_3), 3.19−3.24 (m, 2 H, CH_2NH), 4.12−4.20 (m, 2 H, COC*H*NH), 4.26−4.29 (m, 1 H, COC*H*NH), 7.41−7.48 (m, 3 H, ArH), 7.81 (d, 2 H, ArH), 8.02 (d, 1 H, NH), 8.06 (d, 1 H, NH), 8.15 (d, 1 H, NH), 8.43 (t, 1 H, NH); HR−FABMS (M + H$^+$) calcd for $C_{21}H_{31}N_4O_6$ 435.2244, found 435.2221.

UV Spectrophotometry. The binding constant for association of R_tV_3 with **L** was determined by UV difference spectrophotometry, using the method described previously.[3] The data were fit to a simple 1:1 binding model, assuming that the association of **L** with each site of R_tV_3 is an independent event with the same affinity. A nonlinear least-squares curve fitting routine was used in extracting the binding constant.

High-Pressure Liquid Chromatography. HPLC was performed using a Waters 600E or a Rainin Dynamax Model SD-300 HPLC chromatography system with a UV detector using C18 columns from Rainin or Vydac. A 4.6 mm i.d. column was used for analysis and a 21.4 mm i.d. column was used for preparative separations. Linear gradients of 0.1% TFA in acetonitrile and 0.1% TFA in water were used in HPLC elution. The flow rate for the analytical study was 1.0 mL/min, and 7.5 mL/min for the preparative separation. The injection volume for analytical separation was 20 μL. The parameters in eq 12 were determined as follows. Values of [**L**] were assumed to be constant since **L** was in excess in the experiments. Values of [R′$_t$L′$_3$] and [R_tV_3·R′$_t$L′$_3$] were estimated from the integrals of their peaks on HPLC. Values of [R_tV_3·3**L**] were estimated from integrals of R_tV_3 because R_tV_3·3**L** dissociated to R_tV_3 on the column. Values of θ were thus calculated using eq 12. Integrations were performed using Baseline 810, a program provided by Waters, by plotting chromatograms, estimating baselines, and integrating areas of peaks.

Isothermal Titration Calorimetry. Calorimetry experiments were carried out using a MicroCal MCS titration calorimeter as described in detail elsewhere.[25] Briefly, titrations were performed in 5.0 mM phosphate buffer at pH 7.0, and typically consisted of 3- or 5-μL of ligand solution in a schedule of 20 to 30 injections spaced at intervals of 4−5 min with a syringe stirring speed of 500 rpm. The calibrated feedback current (μcal/s) was recorded as a function of time and was used to determine the heat released following an injection by integration. Heats were nomarlized to give the enthalpy of binding ($\Delta H°$, kcal/mol). For experiments in which binding constants were determined, the data were analyzed by a single-set-of-sites binding model with MicroCal Origin software. Concentrations of R′$_t$L′$_3$ were determined by amino acid analysis.

Acknowledgment. This work was supported in part by the National Science Foundation NSF CHE-9801358, the National Institutes of Health (NIH) Grants GM30367, and GM53210 (to R.M.W.). J.R. thanks Eli Lilly (1996), Hoffman-La Roche (1997), and Glaxo Wellcome (1998) for Predoctoral Fellowships. NMR instrumentation was supported by National Institutes of Health Grant 1 S10 RR4870 and National Science Foundation Grant CHE-88-14019. FAB-Mass spectra were obtained by Dr. Andrew Tyler at Harvard University. ESI-Mass spectra of R_tV_3 and R′$_t$L′$_3$ and concentrations of R′$_t$L′$_3$ solution were obtained by the Harvard MicroChem Facilities.

Supporting Information Available: Complete derivation of eq 11 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA992648L