# Novel Vancomycin Dimers with Activity against Vancomycin-Resistant Enterococci

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Vancomycin typifies the glycopeptide family of antibacterial agents, which exhibit an unusual, receptor-like mode of action: by binding with high affinity and specificity to the C-terminal L-lysyl-D-alanyl-D-alanine portion of peptidoglycan precursors, vancomycin prevents their incorporation into the polymeric bacterial cell wall.[1] Williams et al. have detailed a second molecular recognition function of glycopeptides, that of self-association into homodimers. They have demonstrated that glycopeptide dimerization can be highly favorable and cooperative with the binding of peptide ligands.[2] They have also provided evidence that self-association can lead to enhancements in *in vitro* antibacterial potency,[3] consistent with a model in which dimerization increases the intrinsic affinity of glycopeptides for their peptidoglycan precursor targets, and increases the overall avidity of interaction between self-associated, ditopic glycopeptide receptors and polyvalent ligands presented by the bacterial cell.[3,4] Interestingly, vancomycin self-associates in solution only weakly ($K_{dim}$ = 700 $M^{-1}$), suggesting that dimerization may not play a significant role in the biological action of this clinically important agent. This prompted us to examine the effects that *covalent* dimerization would have on the molecular recognition and antibacterial properties of vancomycin. We now report the synthesis of a series of novel bis(vancomycin)carboxamides and the discovery that some of these compounds exhibit promising *in vitro* activity against vancomycin-resistant enterococci and selectively enhanced affinity for depsipeptide ligands mimicking peptidoglycan precursors from these organisms.

Bis(vancomycin)carboxamides **1a−d** (Figure 1) were prepared by HBTU-mediated coupling of vancomycin (2.2 equiv) with 1,6-diaminohexane, cystamine, homocystamine, and triethylenetetramine, respectively.[5] The dimers were isolated in 44−68% yield by reversed-phase HPLC and characterized by high-field $^1$H NMR spectroscopy and liquid secondary ion (LSI) or electrospray (ES) mass spectrometry. As controls, the monomeric adducts of vancomycin with cystamine (**2b**) and triethylenetetramine (**2d**) were prepared by use of excess amine.

The *in vitro* antibacterial properties of vancomycin, **1a−d**, **2b**, and **2d** were determined by broth microdilution assays. It was found that both monomeric and dimeric vancomycin carboxamide derivatives retained activity against vancomycin-susceptible Gram-positive organisms, including staphylococci and enterococci (Table 1). Strikingly, *dimers **1a−c** display substantially enhanced in vitro potency against strains of enterococci which exhibit high-level resistance to vancomycin and other glycopeptides (vancomycin-resistant enterococci (VRE, VanA phenotype).*[6] The average minimum inhibitory concentration (MIC) displayed by bis(vancomycin)cystamide (**1b**) against VRE (19 μg/mL, 11 μM in vancomycin subunits) is more than a factor of 60 lower than that displayed by vancomycin against these strains and approximately 10-fold higher than the MICs displayed by vancomycin against susceptible enterococci. Dimerization appears to be required for significant activity against VRE, since **2b** and **2d** did not inhibit

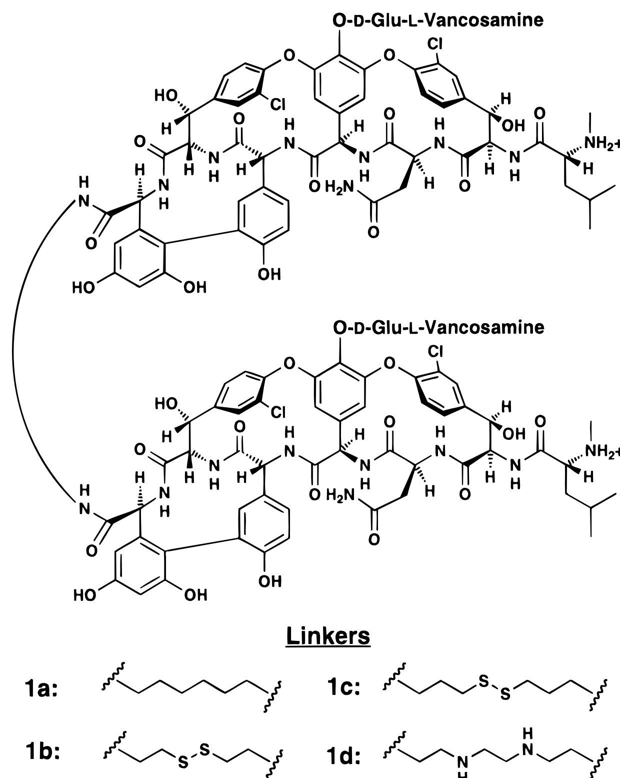


**Figure 1.** Bis(vancomycin)carboxamides **1a−d**.

**Table 1.** *In Vitro* Antibacterial Properties and Ligand-Binding Affinities of Monomeric and Dimeric Vancomycin Carboxamide Derivatives

| | MIC (μM)[a] | | | $K_d$[b] (μM) | |
|---|---|---|---|---|---|
| cmpd | *S. aureus* (mean)[c] | *Enterococcus* (mean)[d] | VRE (mean)[e] | Ac2KDADLac | Ac2KDADA |
| **Van** | 0.30 | 1.0 | 690 | 1800 ± 65 | 1.1 ± 0.4 |
| **1a** | 2.0 | 0.51 | 32 | 180 ± 20 | 1.5 ± 0.1 |
| **1b** | 0.48 | 0.50 | 11 | 440 ± 70 | 1.9 ± 0.8 |
| **1c** | 3.2 | 0.29 | 15 | 680 ± 160 | 3.9 ± 0.6 |
| **1d** | 0.46 | 2.9 | >69 | 1160 ± 380 | 2.9 ± 0.4 |
| **2b** | 0.08 | 1.4 | >67 | 2600 ± 120 | 4.3 ± 0.1 |
| **2d** | 0.40 | 2.8 | >60 | 3630 ± 230 | 8.4 ± 0.9 |

[a] Minimum concentration of derivative required to inhibit growth of bacterial cells in cation-adjusted Mueller−Hinton broth. For **1a−d**, MIC values refer to the concentrations of individual vancomycin subunits. [b] Dissociation constants determined by competitive titration with **AcK(F)DADA** in 10 mM HEPES, 6 mM NaCl, pH 7.0, buffer. For **1a−d**, $K_d$ values refer to the noncooperative association of individual vancomycin subunits to the ligands. [c] Average MIC values for 10 vancomycin-susceptible strains of *Staphylococcus aureus*. [d] Average MIC values for 4 vancomycin-susceptible strains of *Enterococcus faecium* and *Enterococcus faecalis*. [e] Average MIC values for 4 strains of *E. faecium* and *E. faecalis* exhibiting high-level resistance to vancomycin (VanA genotype confirmed by PCR).

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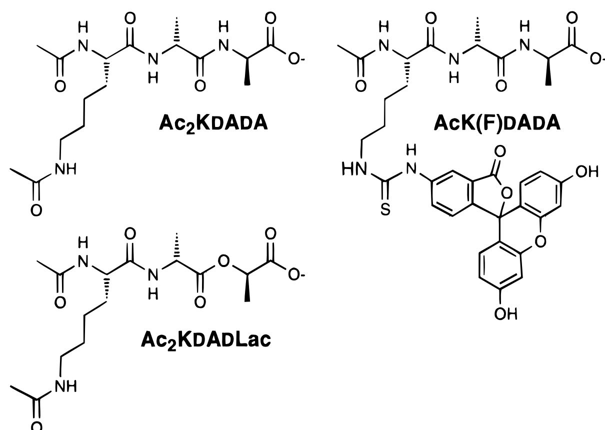


**Figure 2.** Ligands $N^{\alpha}$,$N'^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine (**Ac₂KDADA**), $N^{\alpha}$,$N'^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-lactate (**Ac₂KDADLac**), and $N^{\alpha}$-acetyl-$N'^{\epsilon}$-fluoresceinyl-L-lysyl-D-alanyl-D-alanine (**AcK(F)DADA**).

the growth of these organisms at the highest concentrations examined.[7] The level of activity displayed by **1a**−**d** against VRE varies with the structure of the linker. Dimers bearing disulfide linkages (**1b** and **1c**) exhibited the greatest potency, but the disulfide moiety is not requisite since bis(vancomycin)-hexamide (**1a**) is also active. Dimer **1d** did not inhibit the growth of VRE at 128 $\mu$g/mL (69 $\mu$M in vancomycin subunits). It may be of some relevance that, while **1a**−**c** were more potent than vancomycin toward vancomycin-susceptible enterococci, **1d** was less potent toward these organisms.

To probe the basis for the observed antibacterial activities, we determined and compared the affinities of vancomycin and the monomeric and dimeric carboxamide derivatives for $N^{\alpha}$,$N'^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-alanine (**Ac₂KDADA**) and $N^{\alpha}$,$N'^{\epsilon}$-diacetyl-L-lysyl-D-alanyl-D-lactate (**Ac₂KDADLac**, Figure 2). These ligands mimic the C-terminal segments of peptidoglycan precursors from vancomycin-susceptible Gram-positive organisms and vancomycin-resistant enterococci, respectively.[8] Dissociation constants were determined by fluorescence-based competition binding assays in which **Ac₂KDADA** and **Ac₂KDADLac** compete with binding of $N^{\alpha}$-acetyl-$N'^{\epsilon}$-fluoresceinyl-L-lysyl-D-alanyl-D-alanine (**AcK(F)DADA**).[9,10] Representative data for **1b** are shown in Figure 3. The data for bis(vancomycin)carboxamides were well fit by a model in which ligand binding to the two receptor sites was noncooperative, affording the dissociation constants collected in Table 1. Relative to vancomycin, **1a**−**d** bind with *reduced* affinity to the peptide **Ac₂KDADA** but with *increased* affinity to the depsipeptide **Ac₂KDADLac**. The monomeric adducts **2b** and **2d** bind less well to both **Ac₂KDADA** and **Ac₂KDADLac**. These results are consistent with the observation that **1a**−**c** but not **2b** or **2d** exhibit enhanced *in vitro* antibacterial potency against VRE. Dimer **1d**, which displays no activity against VRE at 69 $\mu$M, displays only a slight increase in affinity for **Ac₂KDADLac**.

The observed increases in affinity for **Ac₂KDADLac** do not quantitatively account for the antibacterial activity of **1a**−**c**, since the measured dissociation constants for this ligand are 5.6- to 45-fold greater than the MIC values. This suggests that either **1a**−**c** bind more tightly to the full-length depsipeptide-containing peptidoglycan precursors found in resistant enterococci than to **Ac₂KDADLac** or that additional effects contribute to the antibacterial potency of these compounds. One such effect could derive from divalent interactions between bis(vancomycin)carboxamides and peptidoglycan precursors. However, the divalent nature of **1a**−**c** does not appear to provide a substantial advantage against vancomycin-susceptible organisms. Dimers **1a**−**c** are joined in head-to-head fashion by flexible tethers that are too short to allow interactions between the vancomycin subunits in the back-to-back, head-to-tail orientation observed with self-associated glycopeptides.[2,11] Therefore, bis(vancomycin)carboxamides and self-associated glycopeptides may engage in different types of divalent interactions with ligands presented by the bacterial cell.

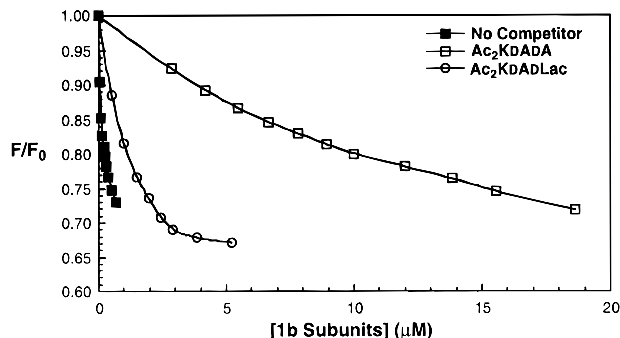


**Figure 3.** Fluorescence titration of **AcK(F)DADA** (0.010−0.050 $\mu$M) with **1b** in the absence of competitor ligand and in the presence of competitors **Ac₂KDADA** (300 $\mu$M) and **Ac₂KDADLac** (4000 $\mu$M). Titrations were carried out at 25 °C in 10 mM HEPES, 6 mM NaCl, pH 7.0.

Previous work at Lilly has shown that *N*-alkylation of glycopeptide amino sugar residues can increase antibacterial potency against both vancomycin-susceptible and -resistant strains.[12] In covalent dimerization, we have discovered a different semisynthetic modification which can increase affinity for depsipeptide ligands and return significant *in vitro* potency against VRE. These effects warrant further study given the gravity of the emerging antibiotic resistance problem,[13] which includes the anticipated spread of high-level vancomycin resistance from enterococci to staphylococci.[14]

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**Supporting Information Available:** Experimental procedures for the preparation of mono- and dimeric vancomycin derivatives, $^{1}$H NMR and MS data for compounds **1a**−**d** and **2b**,**d**, and methods for the determination of antibacterial activities and binding constants (5 pages). See any current masthead page for ordering and Internet access instructions.

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(7) We have observed that vancomycin−cysteamide (obtained by reduction of **1b** or **2b** with dithiothreitol) exhibits activity against VRE (mean MIC = 91 $\mu$g/mL). However, we believe that this activity is due to *in situ* oxidation of vancomycin−cysteamide to **1b**, which occurs with a half-life of less than 24 h.

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