Communication

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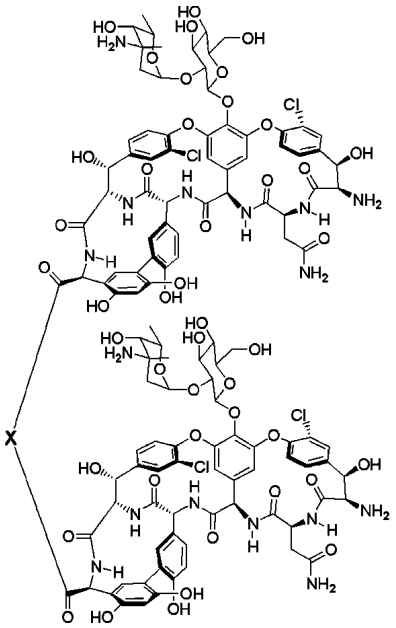

- Anti-VRE activity: 5.8-12 μg/mL
- No binding to Ac-Lys-D-Ala-D-Lac

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D-Ala-D-Lac Binding Is Not Required for the High Activity of Vancomycin Dimers against Vancomycin Resistant Enterococci

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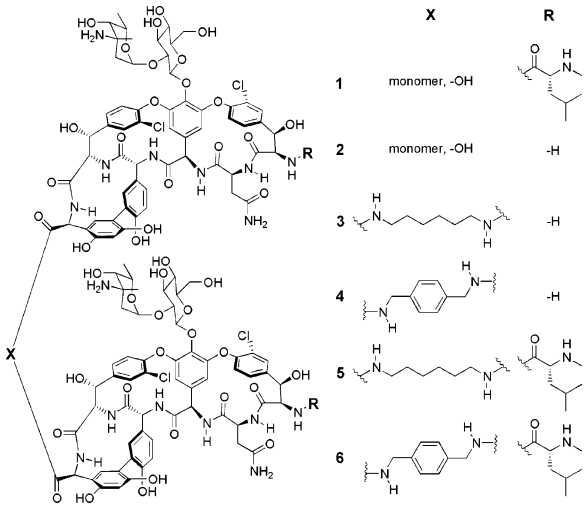
The emergence of bacterial resistance to the drug vancomycin poses a major public health threat and has prompted intensive efforts to develop analogues that overcome resistance.[1−4] Vancomycin is known to inhibit cell-wall biosynthesis and effect bacterial cell lysis by binding to the C-terminal L-Lys-D-Ala-D-Ala motif present in cell-wall precursors.[5] Resistance is typically conferred through alteration of this sequence to the depsipeptide L-Lys-D-Ala-D-Lac, leading to a $\sim 10^3$-fold loss of binding affinity.[6]

The preparation of dimers and oligomers of vancomycin is an important and extensively used approach to obtain glycopeptide analogues active against resistant strains.[3] These compounds have been designed on the principle that they should bind with greatly enhanced avidity through polyvalent interactions with D-Ala-D-Lac present at high density in cell-wall precursors of resistant bacteria.[7] Additionally, binding studies of vancomycin to di-*N*-acetyl-L-Lys-D-Ala-D-Ala suggest that cooperativity between ligand binding and dimerization could also contribute to their enhanced activity.[8]

In prior reports,[9] we have described the preparation and screening of libraries of synthetic vancomycin analogues resulting in the identification of moderately potent compounds against vancomycin resistant *Enterococcus faecium* (VRE, VanA phenotype) with minimum inhibitory concentrations (MIC) of 300 μg/mL. Dimers of these synthetic analogues showed 60-fold increased activity (MIC = 9−18 μg/mL) relative to the monomers. However, the activity of the dimers was maintained upon removal of the L-Lys-D-Ala-D-Lac peptide binding pocket,[9b] strongly indicating that the activity of these dimeric compounds is completely unrelated to L-Lys-D-Ala-D-Lac binding.

These results caused us to question the accepted mechanism for the antibacterial activity of covalently linked vancomycin dimers, which is proposed to occur through binding to L-Lys-D-Ala-D-Lac. Notably, the high activity of vancomycin analogues with hydrophobic appendages against resistant bacteria has recently been determined to be in large part due to mechanisms independent of L-Lys-D-Ala-D-Lac binding.[4] Herein, we report on the biological activity and binding properties of degraded vancomycin dimers that strongly indicate that L-Lys-D-Ala-D-Lac binding is not required for antibacterial activity.

We envisioned that, by preparing dimers that lack the ability to bind L-Lys-D-Ala-D-Lac, we could measure the degree to which alternative antibacterial mechanisms contribute to the activity of covalent vancomycin dimers. Desleucyl vancomycin (**2**) is not active against resistant or susceptible strains because the peptide binding pocket is damaged,[4a,10] and therefore it serves as an ideal replacement for vancomycin. We prepared two covalent tail-to-tail dimers of desleucyl vancomycin (**3** and **4**) by double-Edman degradation[12] of the corresponding intact dimers (**5**[3a] and **6**[3c]).[11]



The antibacterial activities of **1**−**6** were tested against VRE (VanA phenotype) in a broth microdilution assay (Table 1).[12] As expected, vancomycin (**1**) exhibited poor antibacterial activity with an MIC of 1200 μg/mL. Desleucyl vancomycin (**2**) was even less active. Consistent with previous reports by Griffin[3a] and Whitesides,[3f] the intact dimers **5** and **6**, respectively, showed large increases in potency over monomeric vancomycin (~800-fold). The corresponding damaged dimers **3** and **4** showed considerably enhanced activity against VRE over the monomer desleucyl vancomycin (**2**) (5.8−12 μg/mL vs >1200 μg/mL, respectively). Significantly, damaged dimers **3** and **4** are only 8- and 3.5-fold less active than the corresponding intact dimers **5** and **6**.

Vancomycin and corresponding dimers have been shown to bind very weakly to monomeric L-Lys-D-Ala-D-Lac peptides.[3a,f] Therefore, the affinities of **1**−**6** to the higher affinity model peptide, dansyl-Lys(Ac)-D-Ala-D-Ala, were measured and compared according to the methods of Pratt[13] (Table 1). Vancomycin (**1**) and the intact dimers (**5** and **6**) bound to the model peptide with low micromolar K_d values in agreement with the literature. Damaged dimers (**3** and **4**), on the other hand, did not bind to the same peptide at concentrations as high as 60 μM (Table 1).

To assess whether increased avidity through divalent binding to cell-surface L-Lys-D-Ala-D-Lac could possibly account for the high activity of our damaged dimers, we compared the binding properties of **4** and **6** against a dimeric model peptide ϵ-*N*-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$.[11] Addition of intact dimer **6** to solutions containing the peptide exhibited saturation at concentrations greater than 1.0 μM and linearity between 0.5 and 1.0 μM (Figure 1a). At lower concentrations, the large excess of ϵ-*N*-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$ relative to dimer **6** resulted in a complex titration profile indicative of the presence of multiple species, including 1:2 and 1:1 complexes of **6**:ϵ-*N*-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$.

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Table 1. Compound in Vitro Antibacterial Activity and Affinity for a Monomeric Model Peptide, dansyl-Lys(Ac)-D-Ala-D-Ala[13]

compound	MIC (μg/mL)[a]	K_d (μM)[b]
1	1200	1.3
2	>1200	>60
3	12	>60
4	5.8	>60
5	1.5	1.1
6	1.5	2.1

[a] Measured against *E. faecium* (VanA phenotype).[12] [b] Determined at peptide = 1 μM, 25 °C, in 10 mM HEPES, 6 mM NaCl, pH = 7.0.

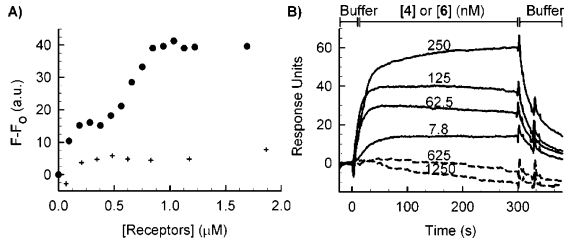

Figure 1. (a) Fluorescence titration of dimeric model peptide ϵ-*N*-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$ with compounds **4** (+) and **6** (●). Determined at 25 °C, peptide = 1 μM, in 10 mM HEPES, 6 mM NaCl, pH = 7.0. (b) SPR sensorgrams of Ac-Lys-D-Ala-D-Ala immobilized surfaces eluted by indicated concentrations of **4** (dashed lines) or **6** (solid lines). Determined at 25 °C, in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P-20.

The K_d is <0.5 μM, based upon the linearity from 0.5 to 1 μM (stoichiometric binding), which is in agreement with literature reports for binding interactions between a dimeric vancomycin and a dimeric L-Lys-D-Ala-D-Ala.[3c] In contrast, titrations performed with damaged dimer **4** resulted in minimal fluorescence intensity changes up to 2 μM (Figure 1a). Further addition of damaged dimer **4** (60 μM) did not result in fluorescence enhancement of the dimeric peptide **4**, indicating that the K_d is >60 μM.[11]

To better model interactions that occur at the cell-wall surface, the binding of **4** and **6** to both surface-immobilized L-Lys-D-Ala-D-Ala and L-Lys-D-Ala-D-Lac[3k] was evaluated using surface plasmon resonance spectroscopy (SPR).[14] The SPR sensorgrams obtained by eluting dimer **6** over the surface-immobilized L-Lys-D-Ala-D-Ala chip represented binding at concentrations as low as 7.8 nM (Figure 1b). A Scatchard plot[11] derived from data obtained at varying concentrations deviated from linearity, indicating the presence of divalent and monovalent binding. These were separately fit to approximate the K_d values of 5 and 245 nM, for divalent and monovalent binding, respectively, consistent with previously reported values.[3f] The sensorgrams obtained by eluting **4** over the chip surface, however, were featureless and indicated the lack of specific binding even at 80−160-fold higher concentrations (Figure 1b).[15] Similarly, absolutely no binding interactions were detected between **4** and surface-immobilized L-Lys-D-Ala-D-Lac.[16]

In summary, we have shown that the covalent tail-to-tail dimers of vancomycin and the corresponding dimers of damaged vancomycin have similarly high antibacterial activity against VRE. We confirmed the literature reports that the vancomycin dimer **6** binds tightly to dimers of L-Lys-D-Ala-D-Ala and to surface-immobilized L-Lys-D-Ala-D-Ala. No binding, however, could be detected between the damaged dimer **4** and dimers of L-Lys-D-Ala-D-Ala or surface-immobilized L-Lys-D-Ala-D-Ala at relevant concentrations. Furthermore, absolutely no binding was detected between **4** and surface-immobilized L-Lys-D-Ala-D-Lac. Clearly, the in vitro antibacterial activity of the damaged dimers cannot be due to a L-Lys-D-Ala-D-Lac binding mechanism. The close structural resemblance and similar activities between the damaged and intact dimers further argues that the antibacterial activity of the intact vancomycin dimers is also likely to be due primarily to mechanisms that do not involve L-Lys-D-Ala-D-Lac binding. We have not yet determined the alternative mechanisms of action. However, it is possible that these dimers bind to and disrupt the function of proteins critical for VRE cell-wall biosynthesis as has been demonstrated for other vancomycin derivatives active against VRE.[4,17]

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Supporting Information Available: Experimental procedures and characterization data for **3**−**6**, ϵ-*N*-succinyl-(dansyl-Lys-D-Ala-D-Ala)$_2$, methods for fluorescence and SPR experiments (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) Soluble carboxymethyl-dextran (0.5 mg/mL) was added to suppress nonspecific adsorption, allowing the measurement of specific binding of **4** to chip surfaces at higher concentrations. Even under these conditions, only modest binding was detected for **4** (25 μM) to Ac-Lys-D-Ala-D-Ala surfaces, and no binding was observed for **4** (25 μM) to Ac-Lys-D-Ala-D-Lac surfaces (see Supporting Information).
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