CHEMISTRY
A EUROPEAN JOURNAL

FULL PAPER

DOI: 10.1002/chem.201201211

Elucidation of the Active Conformation of Vancomycin Dimers with Antibacterial Activity against Vancomycin-Resistant Bacteria

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Abstract: Covalently linked vancomycin dimers have attracted a great deal of attention among researchers because of their enhanced antibacterial activity against vancomycin-resistant strains. However, the lack of a clear insight into the mechanisms of action of these dimers hampers rational optimization of their antibacterial potency. Here, we describe the synthesis and antibacterial activity of novel vancomycin dimers with a constrained molecular conformation achieved by two tethers between vancomycin units. Conformational restriction is a useful strategy for studying the relationship between the molecular topology and biological activity of compounds. In this study, two vancomycin units were linked at three distinct positions of the glycopeptide (vancosamine residue (V), C terminus (C), and N terminus (N)) to form two types of novel vancomycin cyclic dimers. Active NC-VV-linked dimers with a stable conformation as indicated by molecular mechanics calculations selectively suppressed the peptidoglycan polymerization reaction of vancomycin-resistant *Staphylococcus aureus* in vitro. In addition, double-disk diffusion tests indicated that the antibacterial activity of these dimers against vancomycin-resistant enterococci might arise from the inhibition of enzymes responsible for peptidoglycan polymerization. These findings provide a new insight into the biological targets of vancomycin dimers and the conformational requirements for efficient antibacterial activity against vancomycin-resistant strains.

Keywords: antibiotics · conformational restriction · dimers · structure–activity relationships · vancomycin

Introduction

Vancomycin-resistant enterococci (VREs)[1] and -*Staphylococcus aureus* (VRSA)[2] are major nosocomial pathogens worldwide, and as such there is an urgent need for novel antibiotics effective against VRE/VRSA infections.[3,4] As VRE and VRSA have a D-alanyl-D-lactate (D-Ala-D-Lac) terminal in their modified cell-wall precursors instead of a D-Ala-D-Ala terminal (vancomycin binding motif), vancomycin cannot bind to these modified precursors and is therefore not effective against VRE and VRSA.[5] Covalently linked vancomycin dimers have been extensively studied over the past decade for their antibacterial properties (Figure 1)[6–8] against vancomycin-resistant bacteria. As our recent studies have suggested that monomeric and dimeric analogues of vancomycin might have different inhibitory actions on cell-wall biosynthesis in vancomycin-resistant strains,[9,10] it is believed that further investigation of vancomycin dimers might lead to a new class of antibacterial agents. As the molecular mode of action of dimers is largely unknown, it would be of great interest to shed light on how the two vancomycin units cooperatively contribute to the optimal interaction with target molecules and induce antibacterial activity. All reported vancomycin dimers have a linker that joins two vancomycin units, and thus have considerable conformational flexibility. This makes it difficult to predict the geometry of each unit required for activity.

Conformational restriction is a useful strategy for studying the relationship between the molecular topology and biological activity of compounds. In general, dimeric ligands with carefully designed length and flexibility of the linker have enhanced affinity for target molecules. Indeed, flexibility of the linker allows the dimeric ligand to fit easily into the binding site during complex formation (Figure 2A).

However, it should be noted that the high degree of conformational freedom of flexible dimers in solution becomes highly constrained upon complex formation, thereby making

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/chem.201201211.

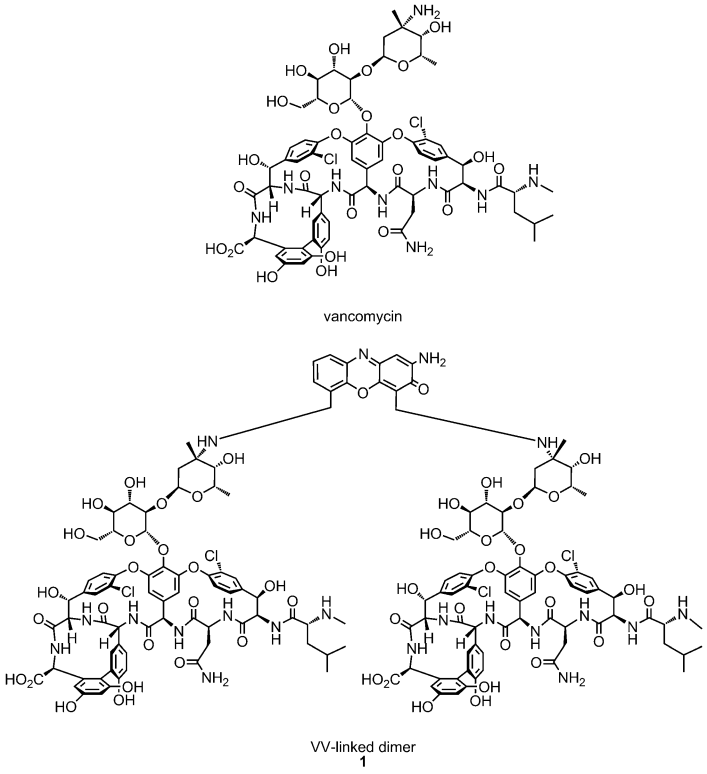


Figure 1. Structure of vancomycin and VV-linked vancomycin dimer **1**.[8] Vancomycin units are joined by a phenoxazone linker.

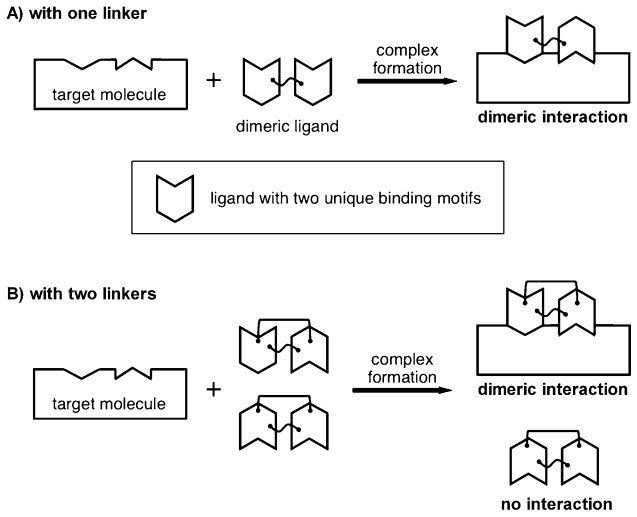


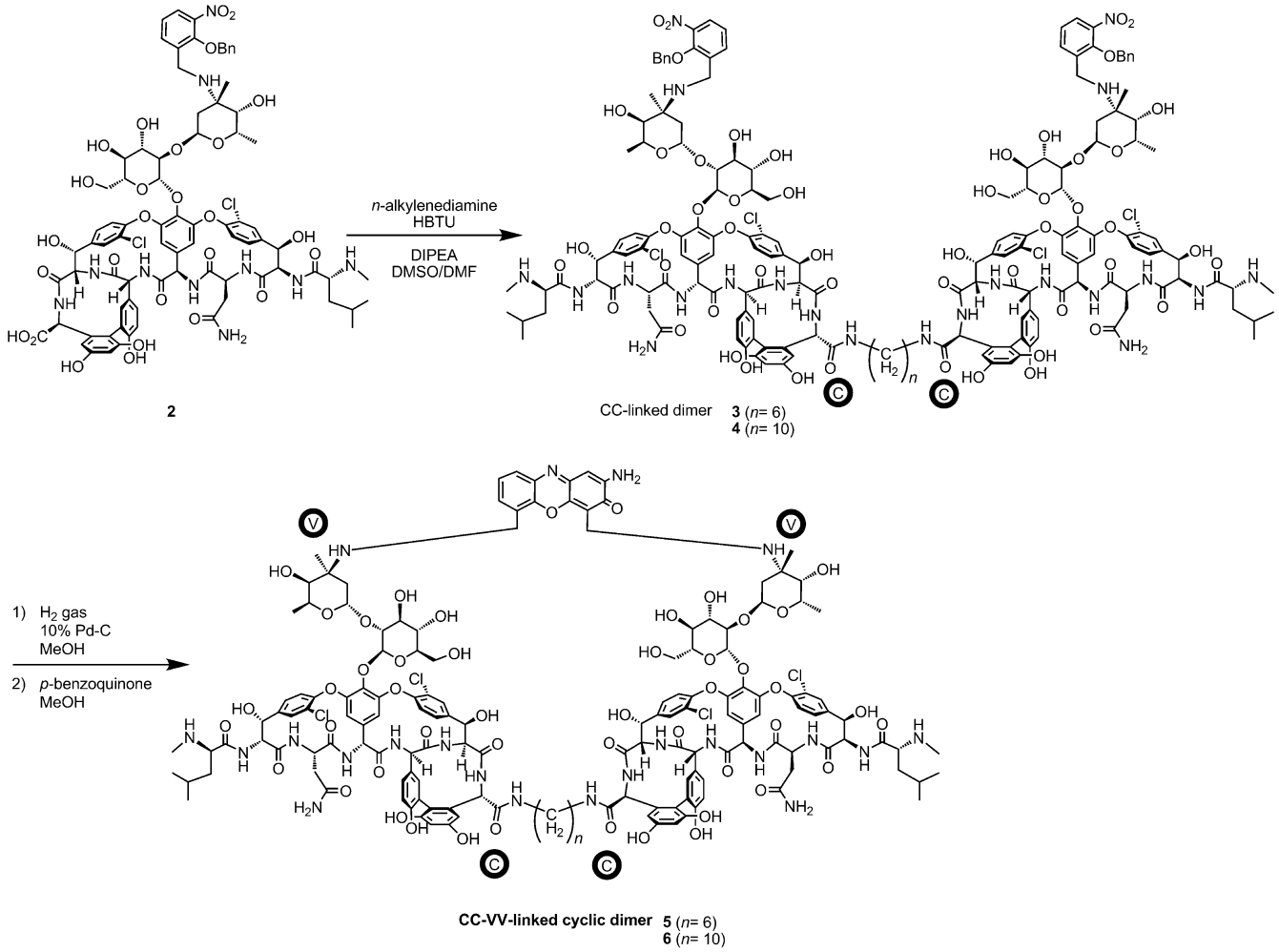
Figure 2. Interaction of dimeric ligand with a macromolecular target. A) A dimeric ligand with one linker can adjust its conformation during complex formation. B) Ligands with two linkers can form a complex with the target only when two units are properly fixed.

the dimer susceptible to a substantial entropic penalty upon binding.[11] Such an entropic penalty can be minimized by imposing appropriate structural constraints on the ligand (Figure 2B, top). However, constrained cyclic dimers with inappropriately folded conformation (Figure 2B, bottom) fail to bind to their target molecules. Thus, substantial effort is required to optimize the biological activity constrained dimers, especially when the target molecule is unknown or the detailed three-dimensional structure of the binding site is not available. Here, we describe the synthesis and antibacterial activity of novel vancomycin dimers with constrained molecular conformation achieved by two tethers between vancomycin units (cyclic dimers). These analogues provide important new information on the topological requirements for optimum dimer-target interaction and efficient antibacterial activity against vancomycin-resistant strains.

Results and Discussion

Synthesis of vancomycin constrained dimers: In this study, two vancomycin units were linked at three distinct positions of the glycopeptide (vancosamine residue (V), C terminus (C), and N terminus (N)) to form two types of novel vancomycin cyclic dimers (Schemes 1 and 2). To minimize the synthetic steps required, an extensive protection–deprotection strategy was avoided. However, macrocyclization of the dimers remained challenging. We have already shown that oxidative coupling of two *ortho*-aminophenols is a mild but powerful strategy to connect two vancomycin units by a phenoxazone linker without protecting their functional groups.[8] Thus, in this study, we first tethered two vancomycin units by an alkyl chain, and then constructed a more challenging second VV link by oxidative coupling. Synthesis of the CC-VV-linked dimers **5** and **6** commenced with reductive alkylation of the vancosamine nitrogen atom, which afforded compound **2**.[8] Two units of compound **2** were connected by means of the C6- or C10-*n*-alkylenediamine by using *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU).[12]

Palladium-catalyzed reduction of the CC-linked dimer **3** or **4** liberated two aminophenol moieties at the sugar residue. These aminophenols were connected at their terminals by oxidation using *p*-benzoquinone.[8] The resulting CC-VV-linked cyclic dimers **5** and **6** had a 56- and a 60-membered macrocycle, respectively, in the molecules. Synthesis of the NC-VV-linked compound **12** began with modification of **2** at the carboxylate terminal with *N*,*N*-dimethyl-1,3-propanediamine to give intermediate **7**. Reductive alkylation of **7** at the N-terminal by an *N*-Fmoc-protected (Fmoc = 9-fluorenylmethoxycarbonyl) aliphatic aldehyde, followed by deprotection of the Fmoc group by piperidine to give **8**. Condensation of **8** at its least-hindered primary amine with **2** by using (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP)[7] gave the NC-linked dimer **10**. Finally, the NC-VV-linked cyclic dimer **12** that contained a 56-membered macrocycle was successfully synthesized by means of a reduction–oxidation sequence as mentioned for the syntheses of **5** and **6**. Another NC-VV-linked dimer (compound **13**) was also prepared by a similar procedure. As the phenoxazone linker is asymmetrical, dimers **5**, **6**, **12**, and **13** were obtained as mixtures of diastereomeric isomers. These isomers could be separated only for **12** (Figure 3) and



Scheme 1. Synthesis of CC-VV-linked cyclic dimers (DIPEA = *N*,*N*-diisopropylethylamine).

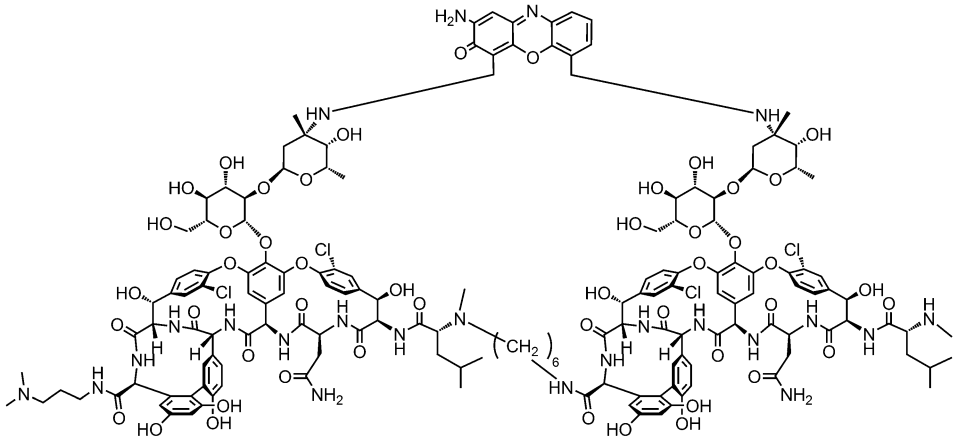
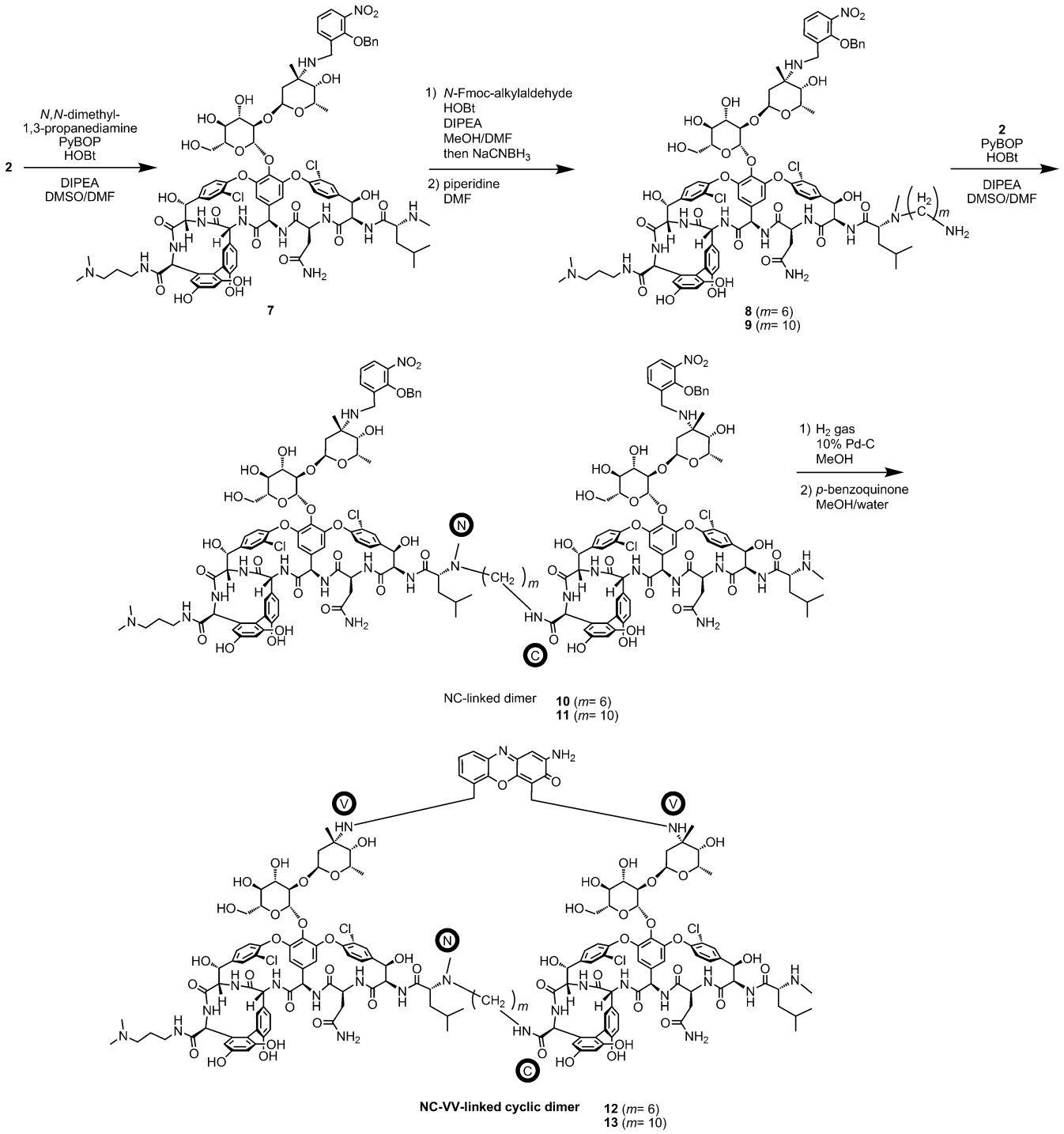


Figure 3. Isomer of the NC-VV-linked dimer **12**.

13 by high-performance liquid chromatography (HPLC). This is probably because dimers **5** and **6** have pseudo-*meso* symmetry and properties that resemble those of the diastereomers. As shown in the table below, diastereomers **12-1** and **12-2**; **13-1** and **13-2** exhibited similar antibacterial properties.

Activity of NC-VV dimers against VREs and VRSA: The antibacterial activity of the prepared constrained vancomycin dimers against selected strains is shown in Table 1. Because all the dimers listed in the Table 1 have a common phenoxazone linker, the observed differences in antibacterial activity are due to the second tethering by a polymethylene chain. The NC-VV-cyclic dimers **12** and **13** inhibited the growth of vancomycin-susceptible and -resistant strains with a potency similar to that of the VV-linked dimer **1**. However, the CC-VV-linked dimers **5** and **6** did not inhibit growth of vancomycin-resistant *E. faecium* or *S. aureus*, al-



Scheme 2. Synthesis of NC-VV-linked cyclic dimers (HOBt = 1-hydroxybenzotriazole).

though they had certain activity against vancomycin-susceptible strains. This finding emphasizes the importance of the conformational constraint in the loss of anti-VREs/VRSA activity of compound **5**. The constraint in **5** probably prohibited vancomycin units from being in the three-dimensional arrangement required for anti-VREs/VRSA activity. The moderate but slightly stronger anti-VREs/VRSA activity of **6** than that of **5** might be due to the longer linker length of **6**, which allows increased conformational freedom at the C terminals (a similar linker length/activity relationship was seen between vancosamine terminals; see Table S1 in the Supporting Information). As shown in Figure 2B, anti-VREs/VRSA activity requires simultaneous dimeric interaction of appropriately oriented ligands with the target molecule.

Table 1. Antibacterial activity of the synthesized vancomycin dimers against selected strains.

Compound	Linker type	MIC [$\mu g mL^{-1}$][a] *S. aureus*[b] VS[g]	*E. faecium*[c] VS	*S. aureus*[d] VR[h] (VanA)	*E. faecium*[e] VR (VanA)	*E. faecium*[f] VR (VanB)
vancomycin	–	1	1	>64	>64	>64
1	VV	2	0.5	4	4	0.25
5	CC-VV	8	2	>64	>64	>64
6	CC-VV	4	1	16	32	8
12-1	NC-VV	2	1	2	4	2
12-2	NC-VV	2	1	4	8	8
13-1	NC-VV	2	2	8	8	2
13-2	NC-VV	4	2	8	8	4

[a] Minimum inhibitory concentrations were determined by the broth microdilution method. [b] RN4220. [c] SR16972. [d] HIP11983. [e] SR7940. [f] SR23598. [g] Vancomycin-susceptible. [h] Vancomycin-resistant.

Vancomycin dimers' inhibition of peptidoglycan synthesis by D-Ala-D-Lac-containing precursors: The molecular mechanism by which VREs or VRSA develop resistance to vancomycin is well documented.[13,14] The D-Ala-D-Ala terminal of bacterial cell-wall intermediates is replaced with D-Ala-D-Lac in these resistant strains. Because vancomycin binds weakly to the D-Ala-D-Lac terminal, it does not affect the cell-wall biosynthesis of VREs and VRSA.

Studies to elucidate the effects of dimers on bacterial cell-wall synthesis have been conducted only recently. In this context, we have previously reported the first experimental proof that covalently linked vancomycin dimers selectively suppress cell-wall synthesis in vancomycin-resistant bacteria by inhibiting peptidoglycan polymerization catalyzed by transglycosylase (penicillin-binding protein 2, or PBP2).[9,15,16] In this study, we shifted our attention to the efficacy of the constrained dimers (**5** and **12**). As shown in Table 2, the NC-VV dimers **12-1** and **12-2**, which exhibited potent antibacterial activity against VREs and VRSA, inhibited polymerization of peptidoglycan (Figure 4, PG step) more potently ((92 ± 38) and $(120\pm23)\ \mu g mL^{-1}$) than the VV-linked dimer **1** ($(400\pm220)\ \mu g mL^{-1}$). This enhanced inhibitory activity may be due to a reduced entropic cost of association between **12** and its target molecule as a result of conformational restriction. The CC-VV-linked dimer **5**, which exhibited antibacterial activity only against vancomycin susceptible strains, did not inhibit cell-wall synthesis in the resistant D-Ala-D-Lac model ($>950\ \mu g mL^{-1}$). However, dimer **5** strongly suppressed the peptidoglycan polymerization ($(9.4\pm1.6)\ \mu g mL^{-1}$) with a potency similar to that of vancomycin ($(9.6\pm1.3)\ \mu g mL^{-1}$) in D-Ala-D-Ala substrate. These findings underline the importance of molecular topology in vancomycin dimers' inhibition of peptidoglycan polymerization in bacterial resistant models.

Externally added D-Ala-D-Lac does not antagonize the anti-VRE activity of vancomycin dimers in the double-disk diffusion test: We next focused on whether the NC-VV dimer requires binding to the peptidoglycan precursor for anti-VRE/VRSA activity.

Vancomycin dimers were originally designed to potently bind D-Ala-D-Lac residues in cell-wall intermediates of resistant bacteria through dimeric interactions.[12,17,18] As described above, the vancomycin dimers prepared in this study showed good activity against VRE/VRSA. However, this antibacterial activity might not have necessarily originated from binding to the D-Ala-D-Lac residue. Accordingly, it is fair to assume that D-Ala-D-Lac is just a hypothetical target of covalently linked vancomycin dimers.[19]

To investigate whether the vancomycin dimers substantially enhanced the binding affinity for D-Ala-D-Lac, we carried out a double-disk diffusion test (Figure 5).

Disks that contained vancomycin, linezolid, compound **1**, and compound **12** were placed on a lawn of either vancomycin-susceptible *E. faecium* (SR16972) or vancomycin-resistant *E. faecium* (vanA, SR7940). The effect of D-Ala-D-Ala-containing tripeptide or D-Ala-D-Lac-containing tridepsipeptide on inhibition zones was evaluated. Competitive binding of the externally added tripeptide or depsipeptide with drugs can be visualized as a distortion in the usually circular inhibition zone, if bacterial cell-wall intermediates (D-Ala-D-Ala- or D-Ala-D-Lac terminal) are the binding target of the drugs. The antibacterial activity of linezolid is due to the inhibition

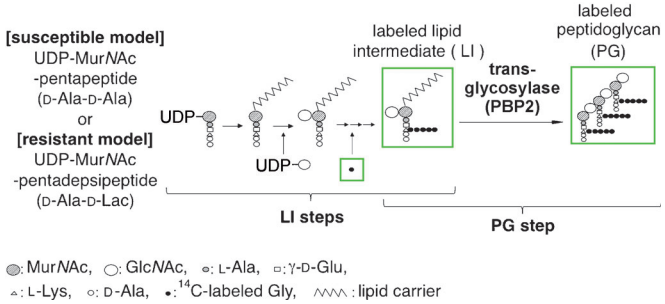


Figure 4. Late stage of bacterial peptidoglycan biosynthesis. Lipid intermediate (LI) synthetic steps catalyzed by a sequence of enzyme.

Table 2. Inhibitory activity of test compounds against cell-wall synthesis in an in vitro assay.

Compound	Linker type	IC_{80} [$\mu g mL^{-1}$][a] Susceptible model LI steps	Susceptible model PG step	Resistant model LI steps	Resistant model PG step
vancomycin	–	240 ± 8.2	9.6 ± 1.3	3900 ± 120	770 ± 240
1	VV	130 ± 57	6.9 ± 3.0	>1024	400 ± 220
5	CC-VV	>720	9.4 ± 1.6	>1024	>950
12-1	NC-VV	260 ± 8	12 ± 2	380 ± 120	92 ± 38
12-2	NC-VV	300 ± 46	17 ± 0.8	670 ± 110	120 ± 23

[a] 80% inhibitory concentration.

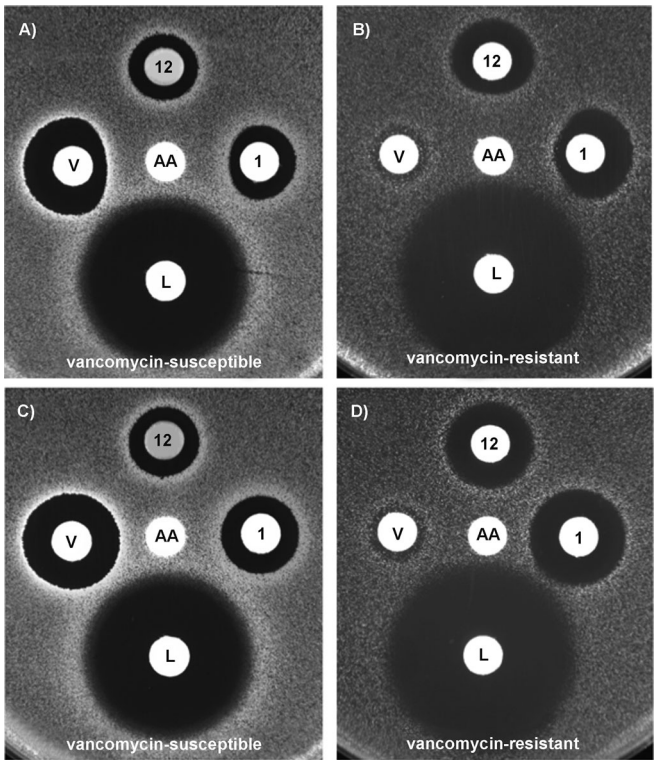


Figure 5. Double-disk diffusion test performed using vancomycin-susceptible *E. faecium* SR16972 (A and C) and vancomycin-resistant *E. faecium* SR7940 (B and D). Distortion of drugs-inhibition zones by D-Ala-D-Ala-containing tripeptide (Ac_2-L-Lys-D-Ala-D-Ala, 100 μg, A and B) and D-Ala-D-Lac-containing tridepsipeptide (Ac_2-L-Lys-D-Ala-D-Lac, 100 μg, C and D). Key: **V**, vancomycin (10 μg per disk); **L**, linezolid (10 μg per disk); **1**, vancomycin dimer **1** (50 μg per disk); **12**, vancomycin cyclic dimer **12**–**1** (80 μg per disk); **AA**, D-Ala-D-Ala-containing tripeptide; **AL**, D-Ala-D-Lac-containing tridepsipeptide. See Table 1 for the antibacterial activity of test compounds (**1**, **12**, and vancomycin) against *E. faecium* SR16972 and SR7940. In general, the size of the inhibition zone in the disk diffusion test does not always correlate with minimum inhibitory concentration (MIC) values obtained by the broth microdilution method because of differences in the physical properties of compounds.

of protein synthesis,[20] and thus it is not affected by the external addition of the tripeptide or the depsipeptide (used as negative control). Although all vancomycin derivatives showed clear distortion of the zones with external addition of the D-Ala-D-Ala-containing peptide, they did not show any detectable distortion with addition of the D-Ala-D-Lac-containing depsipeptide. If vancomycin dimers **1** and **12** exhibit anti-VRE activity by binding to the D-Ala-D-Lac terminal of bacterial cell-wall intermediates, the external addition of D-Ala-D-Lac-containing depsipeptide should have competitively inhibited this activity (Figure 5C,D). Overall, this double-disk diffusion test indicates that the binding target of vancomycin dimers is not the D-Ala-D-Lac terminal of the cell-wall intermediates of VRE/VRSA. As dimers **1** and **12** inhibit peptidoglycan polymerization catalyzed by transglycosylase (penicillin-binding protein 2 of *S. aureus*) without binding to the D-Ala-D-Lac-containing peptidoglycan precursor, a plausible anti-VRSA mechanism might be direct interaction of these dimers with the enzyme (see Figure 4).

Molecular topology of constrained dimer 12: A pharmacophore is a simple model that describes distances between important atoms or groups of atoms in a ligand that are required for it to interact with a specific biological macromolecule. Once the pharmacophore for a specific receptor is defined, it can be used to search chemical libraries for compounds that fit the pharmacophore. Although the binding target of NC-VV-linked dimer remains to be elucidated, molecular topology analysis of the constrained dimer could provide useful insight into the substructures involved in interactions with the target molecule in vancomycin-resistant bacteria.

To obtain information about the molecular topology of active dimers, a stable conformation of the cyclic dimer **12** was investigated in silico using MacroModel 9.1.[21] After drawing the structure of compound **12**, its potential energy was minimized and used for the subsequent conformational search. All conformers within 24 kcal mol^{-1} of the structure with the lowest energy could be classified into a group that was defined on the basis of the topology of the aglycon moieties of vancomycin units. The most stable generated conformation of cyclic dimer **12** is depicted in Figure 6.[22] In this conformer, two vancomycin units exist in a "back-to-back" configuration with an antiparallel β structure forming at the "back" of each unit. This constrained structure highlighted the importance of the back-to-back arrangement in the anti-

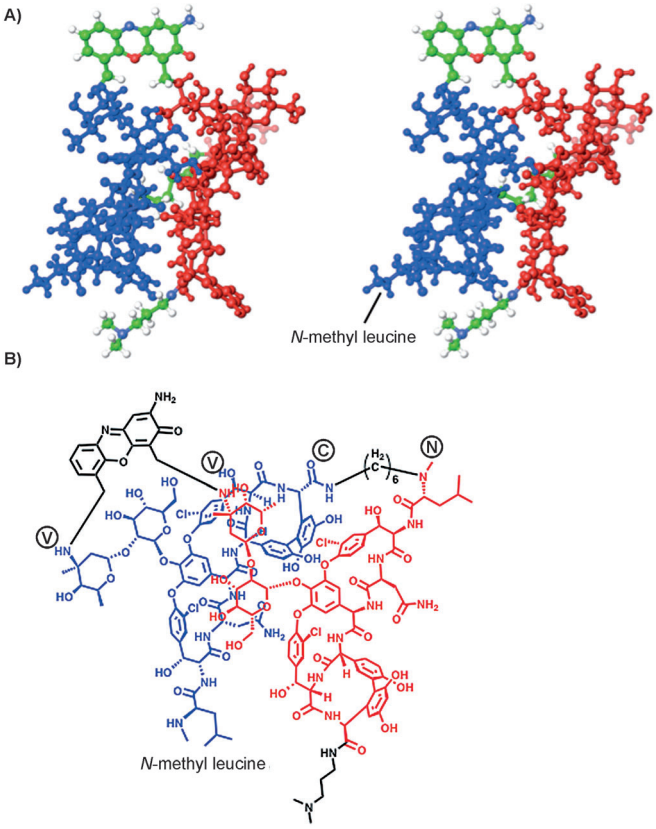


Figure 6. Stereoview of A) conformationally stable NC-VV-linked dimer **12**, and B) its chemical structure. Red and blue: two vancomycin units; green: phenoxazone bridge in the VV position, and alkyl chain.

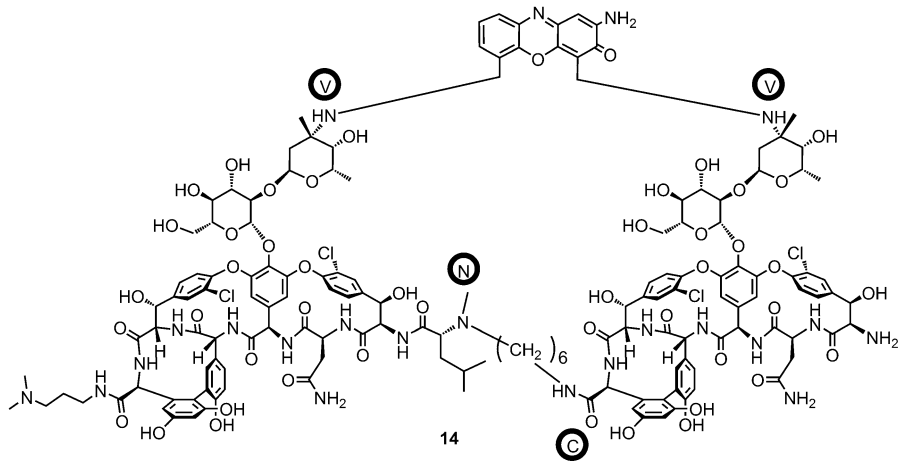


Figure 7. *Des-N*-methyl leucyl-NC-VV-linked cyclic dimer **14**.

bacterial activity **12**. The conformation of **12** also suggests that the VV-linked dimer **1** (with one tether) is in the back-to-back conformation, which is a target-bound state. This interpretation can be supported by the fact that vancomycin and the related glycopeptide antibiotics (without tethering) have a propensity to form a back-to-back dimer through hydrogen bonds between aglycons in an antiparallel arrangement in the crystal (Figure S1 in the Supporting information).[23,24] In the back-to-back conformation of **12**, leucine residues and carboxylate terminals of vancomycin are proximate to each other. This proximity might favor simultaneous binding (Figure 2B) of an N-terminal and a C-terminal of vancomycin units with the target molecule in vancomycin-resistant bacteria.

To gain further insight into the dimer–target interaction, an *N*-methyl leucine residue of dimer **12** was removed by Edman degradation to give **14** (Figure 7). This removal significantly diminished the antibacterial activity against vancomycin-resistant strains (minimum inhibitory concentration (MIC) for *S. aureus* HIP11983 $>64\ \mu g\,mL^{-1}$; see Table S2 in the Supporting information). Collectively, these findings stress the involvement of the *N*-methyl leucine residue at the uncrosslinked terminal in the dimer–target interaction required for anti-VRSA/VRE activity.

Conclusion

By using a mild phenoxazone-based macrocyclization, we have prepared conformationally constrained vancomycin dimers that exhibit excellent inhibitory activity on the peptidoglycan synthesis of vancomycin-resistant bacteria. Evaluations of this antibacterial activity along with molecular mechanics calculations allowed us to identify the active conformation of vancomycin dimers necessary to ensure activity against vancomycin-resistant stains. We have also showed in this study that the potent antibacterial activity of vancomycin dimers is likely to arise from their action on transglycosylase enzymes. These findings would promote the rational molecular design of better antibacterial agents.

Experimental Section

General methods: Reagents and solvents were used as purchased from commercial suppliers. All reactions were carried out at room temperature unless otherwise noted. Mass spectra were obtained using a JEOL LMS-700 mass spectrometer (FAB MS) and a Bruker Daltonics microTOF-TSfocus (LC-ESI-TOF MS). LC was performed using an Agilent 1100 Series, with an Imtakt UNISON UK $3C_8$ column (150×2 mm) under the following conditions: flow rate of $0.2\ mL\,min^{-1}$, 65% to 95% acetonitrile/water containing 0.1% formic acid, duration of 5 min, temperature of 40°C, and UV detection at $\lambda=$ 214, 254, 280, and 190–400 nm. Monoisotopic mass was used for calculation of exact mass (C 12.0000, H 1.0078, O 15.9949, N 14.0031, Cl 35.9689). NMR spectra were recorded using a JEOL ECA600 at 600 MHz, Varian Unity INOVA 500 at 500 MHz, and an INOVA 600 instrument at 600 MHz. The ^{1}H NMR spectra are relative to the residual solvent peak of tetramethylsilane ($\delta=0$ ppm), $CHCl_3$ ($\delta=7.26$ ppm), CHD_2OD ($\delta=3.31$ ppm), $(CHD_2)(CD_3)SO$ ($\delta=2.5$ ppm), and water ($\delta=$ 3.33 ppm in [D_6]DMSO); ^{13}C NMR spectra are relative to the residual solvent peak of $CHCl_3$ ($\delta=77.16$ ppm). TLC was performed using Merck silica gel 60 F254 precoated plates (0.25 mm). Flash chromatography was carried out using 60–230 mesh silica gel (Kanto Chemical, silica gel 60N). Analytical reversed-phase HPLC was performed using a 150×4.6 mm column (Nacalai Tesque, Cosmosil $5C_{18}$-AR-II) under the following conditions: flow rate of $1.0\ mL\,min^{-1}$, 15 to 100% acetonitrile/water containing 0.1% trifluoroacetic acid (TFA), duration of 10 min, temperature of 30°C, and UV detection at $\lambda=280$ nm.

Compound 5: Compound **3** (59 mg, 0.017 mmol) in methanol (3 mL) was reduced for 26 h with hydrogen in the presence of 10% Pd/C catalyst (60 mg) to give the corresponding aminophenol intermediate. The Pd/C catalyst was removed by filtration by using a membrane filter (Millipore, pore size 1.0 μm, JAWP04700) and rinsed with methanol (12 mL) at 0°C (ice–water bath). A solution of *p*-benzoquinone (11 mg, 0.098 mmol) in methanol (3 mL) was added to the filtrate, and the reaction solution was stirred at 0°C for an additional 1.5 h in the dark. The resulting crude sample was purified by reverse-phase HPLC (Nacalai Tesque, Cosmosil $5C_{18}$ AR-II-waters, 250×10 mm, acetonitrile/water containing 0.1% TFA, flow rate $4\ mL\,min^{-1}$) and lyophilized overnight to yield compound **5** as an orange solid (4.1 mg, 1.27 μmol, 7.5% yield, >99% purity). $t_R=$ 3.4 min; ^{1}H NMR (600 MHz, [D_6]DMSO and one drop of D_2O, 40°C): $\delta=9.01$ (s, 1H), 8.65 (s, 1H), 8.59 (s, 1H), 8.54 (s, 1H), 8.44 (s, 1H), 8.41 (s, 1H), 7.88 (s, 1H), 7.84 (d, $J=7.2$ Hz, 1H), 7.74 (s, 1H), 7.68 (d, $J=$ 7.2 Hz, 1H), 7.62–7.51 (comp, 4H), 7.49 (d, $J=7.6$ Hz, 2H), 7.37 (d, $J=$ 7.9 Hz, 1H), 7.33 (d, $J=7.9$ Hz, 1H), 7.28 (t, $J=8.6$ Hz, 1H), 7.21 (t, $J=$ 8.6 Hz, 1H), 7.12 (d, $J=7.9$ Hz, 2H), 6.76 (t, $J=8.6$ Hz, 2H), 6.72 (t, $J=$ 8.6 Hz, 2H), 6.36 (s, 2H), 6.25 (s, 1H), 6.22 (s, 1H), 5.73–5.7 (comp, 2H), 5.64 (t, $J=11$ Hz, 1H), 5.48 (q, $J=6.5$ Hz, 1H), 5.25 (s, 1H), 5.22 (s, 1H), 5.17 (s, 1H), 4.98 (s, 1H), 4.9 (s, 1H), 4.67 (s, 2H), 4.46 (s, 2H), 4.39 (s, 1H), 4.34 (t, $J=4.4$ Hz, 1H), 4.28 (s, 1H), 4.22 (s, 2H), 3.3 (t, $J=8.6$ Hz, 2H), 3.16 (s, 3H), 2.64 (s, 2H), 2.62 (s, 1H), 2.28 (s, 3H), 2.15 (d, $J=$ 9.7 Hz, 1H), 1.87 (d, $J=13$ Hz, 1H), 1.69–1.57 (comp, 8H), 1.49 (m, 1H), 1.4 (s, 1H), 1,32–1.06 (comp, 8H), 0.91 (d, $J=4.9$ Hz, 6H), 0.87 ppm (d, $J=5.5$ Hz, 6H); HRMS (LC-ESI): *m/z* calcd for $C_{152}H_{172}Cl_4N_{22}O_{48}$: 1606.5224 $[M+2H]^{2+}$; found: 1606.5167.

Compound 12: Compound **10** (0.40 g, 0.11 mmol) was dissolved in methanol (18 mL). Pd/C catalyst (0.30 g) was added under an argon atmosphere, which was subsequently replaced by a hydrogen atmosphere. The mixture was then reduced for 18 h in the dark to give the corresponding aminophenol intermediate. The palladium catalyst was removed by filtration by use of a Celite filter (Kanto Chemical, Hyflo Super-Cel), rinsed with water (82 mL), and the resulting filtrate was treated with a freshly prepared solution of *p*-benzoquinone (36 mg, 0.33 mmol) in methanol (10 mL). The reaction mixture was stirred at room temperature for 70 min in the dark, at which point methanol was removed under vacuum, and the aqueous solution was frozen at −78 °C (dry ice–methanol bath) for 2 h, and then lyophilized overnight. The resulting crude solid was purified by reverse-phase column chromatography (Yamazen, Ultra Pack ODS-S-50B, 300 mm × 26 mm, 50 μm particle size, 15, 20, 25, 30, 35, 50, 80% methanol/water containing 0.1% TFA), and then lyophilized overnight to yield compound **12-1** and **12-2** (TFA salts) as a red orange solids (**12-1**: 38 mg, 11% yield, 95% purity, $t_R = 2.9$ min; **12-2**: 45 mg, 13% yield, 94% purity, $t_R = 3.1$ min). The counterion was changed as described below. Toyo Pearl DEAE-650 M (2 mL, 40–90 μm particle size) was first preactivated by the washing the resin with 0.1 M aqueous HCl (2 mL), distilled water, 0.5 M aqueous NaOH (4 mL), distilled water, 50% aqueous methanol (10 mL), methanol (20 mL), distilled water, 0.1 M acetic acid (8 mL), and distilled water (20 mL). A solution of compound **12-1** and **12-2** TFA salts (30 mg each) in methanol was then uploaded onto the resin and eluted with methanol. Aqueous HCl (0.9 mL, 0.1 M) was added dropwise into the resulting solution, and the mixture was concentrated under vacuum to remove methanol and lyophilized to yield compound **12-1** and **12-2** (HCl salts) as a red solid. Compound **12-1** (22 mg, 6.67 μmol, 96% purity): $t_R = 3.8$ min; ^{1}H NMR (600 MHz, CD_3OD, 20 °C): $\delta = 9.43$ (s, 1H), 9.42 (s, 1H), 9.37 (s, 1H), 9.03 (s, 2H), 8.66 (s, 1H), 8.61 (s, 1H), 8.56 (s, 1H), 8.44 (s, 1H), 7.88 (t, $J = 7.8$ Hz, 2H), 7.85 (comp, 8H), 7.75–7.52 (comp, 28H), 7.47 (d, $J = 8.4$ Hz, 1H), 7.39–7.37 (comp, 4H), 7.34 (d, $J = 8.4$ Hz, 2H), 7.31 (d, $J = 9$ Hz, 2H), 7.13–7.06 (comp, 8H), 7.04 (t, $J = 7.8$ Hz, 2H), 6.92 (d, $J = 7.8$ Hz, 1H), 6.87 (t, $J = 8.4$ Hz, 4H), 6.71 (d, 7.8 Hz, 2H), 6.64–6.56 (comp, 10H), 6.51 (d, $J = 8.4$ Hz, 2H), 6.43–6.42 (comp, 5H), 6.4 (s, 2H), 6.39 (s, 1H), 6.32 (s, 2H), 6.28 (s, 2H), 6.26 (s, 1H), 6.23 (comp, 4H), 6.04 (d, $J = 7.8$ Hz, 1H), 5.76 (s, 1H), 5.7 (s, 1H), 5.59 (s, 4H), 5.53 (s, 2H), 5.51 (s, 1H), 5.5 (s, 2H), 5.46 (s, 2H), 5.44 (s, 4H), 5.4–5.36 (comp, 8H), 5.34 (d, $J = 7.8$ Hz, 1H), 5.24 (s, 2H), 5.19 (d, $J = 4.8$ Hz, 1H), 4.81 (d, $J = 9$ Hz, 1H), 4.68 (t, $J = 6$ Hz, 2H), 4.63 (s, 1H), 4.59 (dd, $J = 10.9$, 4.3 Hz, 2H), 4.52 (t, $J = 6$ Hz, 2H), 4.35 (d, $J = 11$ Hz, 1H), 4.3 (d, $J = 11$ Hz, 1H), 4.24–4.13 (comp, 10H), 4.07–3.99 (comp, 4H), 3.96–3.93 (comp, 2H), 3.91–3.81 (comp, 6H), 3.74 (t, $J = 8.4$ Hz, 2H), 3.69 (t, $J = 8.4$ Hz, 2H), 3.65–3.55 (comp, 6H), 3.49–3.32 (comp, 10H), 3.22–3.18 (comp, 8H), 3.11 (s, 3H), 3.07 (s, 3H), 3.01 (s, 3H), 2.96 (s, 6H), 2.93 (d, $J = 9$ Hz, 6H), 2.88 (s, 3H), 2.81 (s, 3H), 2.78 (s, 3H), 2.7 (s, 1H), 2.68 (s, 1H), 2.43–2.31 (comp, 4H), 2.26–2.21 (comp, 4H), 2.09 (s, 2H), 2.04 (br, 10H), 1.91 (s, 3H), 1.88–1.84 (comp, 3H), 1.8 (s, 3H), 1.74 (d, $J = 8.4$ Hz, 6H), 1.67 (d, $J = 6$ Hz, 3H), 1.61 (m, 3H), 1.49–1.39 (comp, 8H), 1.29 (d, $J = 4.8$ Hz, 3H), 1.25 (d, $J = 6$ Hz, 6H), 1.11 (d, $J = 6$ Hz, 3H), 1.02 (d, $J = 6$ Hz, 3H), 0.98 (d, $J = 4.8$ Hz, 6H), 0.89 (d, $J = 5.4$ Hz, 3H), 0.82 ppm (d, $J = 5.4$ Hz, 3H); HRMS (FAB): m/z calcd for $C_{157}H_{182}Cl_4N_{23}O_{48}$: 3297.1262 $[M+H]^+$; found: 3297.1284. Compound **12-2** (19 mg, 5.76 μmol, 98% purity): $t_R = 3.9$ min; ^{1}H NMR (600 MHz, CD_3OD, 20 °C): $\delta = 9.48$ (s, 2H), 7.84 (s, 6H), 7.76–7.63 (comp, 12H), 7.55 (t, $J = 7.8$ Hz, 2H), 7.49 (d, $J = 7.8$ Hz, 1H), 7.35 (s, 2H), 7.26 (d, $J = 7.8$ Hz, 1H), 7.14–7.04 (comp, 10H), 7 (d, $J = 7.8$ Hz, 1H), 6.88–6.81 (comp, 6H), 6.62 (s, 4H), 6.59 (d, $J = 6$ Hz, 4H), 6.51 (s, 1H), 6.47 (d, $J = 10.2$ Hz, 4H), 6.41 (s, 3H), 6.35–6.29 (comp, 6H), 6.23 (s, 2H), 5.73 (s, 4H), 5.54–5.36 (comp, 22H), 5.25 (s, 1H), 5.19 (s, 2H), 4.36 (s, 2H), 4.24–4.07 (comp, 6H), 4.02 (s, 2H), 3.98–3.84 (comp, 2H), 3.74 (d, $J = 8.4$ Hz, 2H), 3.65–3.59 (comp, 6H), 3.41 (s, 6H), 3.4 (s, 3H), 3.18 (s, 3H), 3.04 (s, 3H), 2.99 (s, 1H), 2.95 (br, 2H), 2.92 (s, 6H), 2.88 (s, 3H), 2.78 (s, 6H), 2.2 (s, 1H), 2.16 (s, 3H), 2.1 (s, 2H), 2.04–2.01 (comp, 3H), 1.85 (s, 3H), 1.81 (s, 3H), 1.76–1.7 (comp, 4H), 1.57 (s, 2H), 1.47 (br, 4H), 1.28 (br, 4H), 1.1 (s, 3H), 1.03 (s, 3H), 0.97 (s, 6H), 0.89 (d, $J = 6$ Hz, 3H), 0.82 ppm (s, 3H); HRMS (FAB): m/z calcd for $C_{157}H_{182}Cl_4N_{23}O_{48}$: 3297.1262 $[M+H]^+$; found: 3297.1316.

Semiquantitative enzyme inhibition assay: The inhibitory activity of the synthetic compounds against enzyme reactions involved in bacterial cell-wall biosynthesis in *S. aureus* was evaluated according to the procedure of Miura et al.[10]

Double-disk diffusion test: After preincubation at 37 °C overnight in brain–heart infusion broth (BHIB), the suspension of the test strains (*E. faecium* SR16972 or SR7940) was diluted to an optical density of 0.1 at 660 nm (OD_{660}) and incubated at 37 °C for 3 h (SR16972) or 6 h (SR7940). The bacterial suspension in exponential growth phase was then diluted to an OD_{660} of 0.01. A portion of this suspension was spread on a brain-heart infusion agar (BHIA) plate. After air-drying, paper disks (Advantech, 6 mm in diameter, thin type) that contained test compounds were put on the plate and incubated at 37 °C for 24 h.

Molecular mechanics calculations: Conformational analysis of the compounds was performed by molecular mechanics calculations with MacroModel 9.1.[21] A three-dimensional structure of the cyclic dimer **12** was built using the crystal structure of vancomycin obtained from the Protein Data Bank (ID: 1fvm).[24] The potential energy of the cyclic compound was minimized in the OPLS_2005 force field with water solvation until a 0.05 gradient convergence criterion was met. In the minimized structure, two vancomycin units located away from each other were in a conformation recognized as "extended" topology. The global minimum conformation of **12** was calculated by the Low-Mode Conformational Search[25] using the OPLS_2005 force field. Any structure with energy greater than 24 kcal mol^{-1} above the current global minimum was discarded. The maximum number of minimization with truncated Newton conjugate gradient (TNCG) method was 500. This process generated 4973 conformers that lay greater than 24 kcal mol^{-1} above the global minimum. All conformers could be classified into a group defined based on the topology of the aglycon moieties of vancomycin units. Molecules in this group folded their two vancomycin units in a back-to-back manner as shown in Figure 6A. This conformation was stabilized in "folded" topology by eight hydrogen bonds between two vancomycin units like the self-associated state of noncovalently formed vancomycin dimer (Figure S1 in the Supporting Information).[23]

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research, MEXT (nos. 17035039, 18032010, and 21310136), Japan; by the Uehara Memorial Foundation; and by the Mochida Foundation. We are grateful to Prof. Makoto Sasaki (Tohoku University) for use of LC-MS to provide high- and low-resolution mass spectra.

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Received: April 10, 2012
Published online: ■■ ▮▮, 0000

Vancomycin

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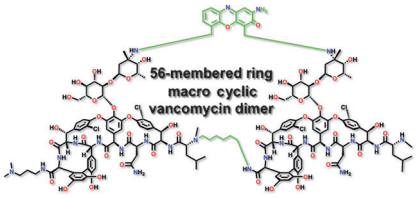
Elucidation of the Active Conformation of Vancomycin Dimers with Antibacterial Activity against Vancomycin-Resistant Bacteria



Squashing superbugs: Conformationally constrained vancomycin dimers that inhibit the peptidoglycan synthesis of vancomycin-resistant bacteria were prepared (see scheme). The potent antibacterial activity of the dimers was suggested to arise from their direct action on transglycosylase enzymes.

