Shapeshifting Antibiotics: Bullvalene Linked Vancomycin Dimers are Effective Against Multidrug-Resistant Gram-Positive Bacteria

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Abstract: The alarming rise in superbugs that are resistant to drugs of last resort, including vancomycin-resistant enterococci and staphylococci, has become a significant global health hazard. Here we report the click chemistry synthesis of an unprecedented class of shapeshifting vancomycin dimers (SVDs) that display potent activity against bacteria that are resistant to the parent drug, including the ESKAPE pathogens, vancomycin-resistant *Enterococcus* (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA) as well as vancomycin-resistant *S. aureus* (VRSA). The shapeshifting modality of the dimers is powered by a click-linked bullvalene core, hence exploiting the dynamic covalent rearrangements of the fluxional carbon cage and creating ligands with the capacity to inhibit bacterial cell wall biosynthesis. The new shapeshifting antibiotics are not disadvantaged by the common mechanism of vancomycin resistance resulting from the alteration of the C-terminal dipeptide with the corresponding D-Ala-D-Lac depsipeptide. Further, evidence suggests that the shapeshifting ligands destabilize the complex formed between the flippase MurJ and lipid II, inferring the potential for a new mode of action for polyvalent glycopeptides. The SVDs show little propensity for acquired resistance by enterococci, suggesting that this new class of shapeshifting antibiotic will display durable antimicrobial activity not prone to rapidly acquired clinical resistance.

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Introduction

Antibiotics are powerful tools for fighting life-threatening infections that have transformed human and animal health[1]. However, the threat of intractable antimicrobial resistance – partly a consequence of poor antibiotic stewardship – on healthcare and global economies has potentially catastrophic implications[2,3]; a situation that, in recent years, has been compounded by bacterial resistance emerging faster than new treatment options[4,5].

Ancient mechanisms of resistance are increasingly accumulating in pathogenic bacteria[6–8], and there is an acute need for renewed vigor and innovative strategies that will lead to new therapies for the treatment of multidrug-resistant (MDR) Gram-positive and Gram-negative bacterial infections[9,10]. The

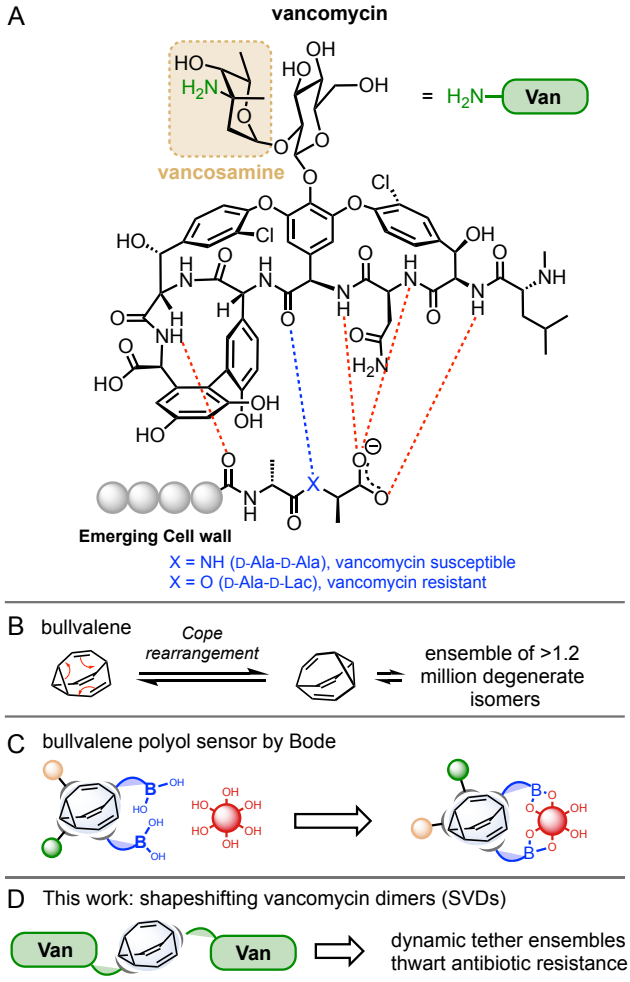


Figure 1. A) Vancomycin binding to lipid II. B) Bullvalene, the archetypal shapeshifting molecule. C) Bullvalene as a polyol sensing platform. D) Shapeshifting core tethered vancomycin dimer.

sustained development and flow of new[11–13] or re-engineered[14] antibiotics that overcome the forces of evolution and selection pressures responsible for bacterial resistance are necessary. Through innovation, creative design, and precision synthetic chemistry, antibiotics that are impervious to, or at least less prone to the development of resistance are within reach[15,16].

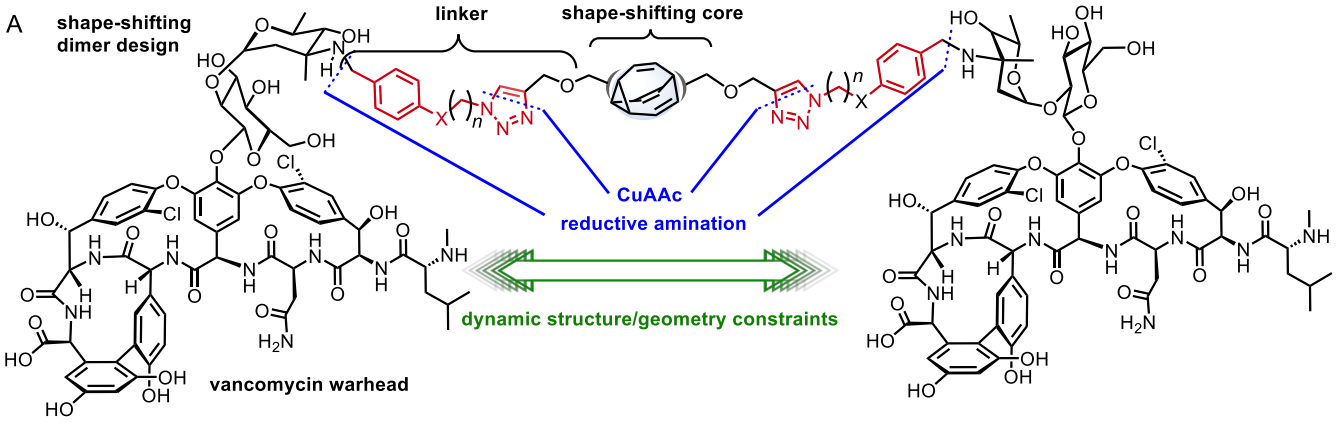
In this vein, the glycopeptide antibiotics, including vancomycin — forming a last line of defense in the fight against

serious infections — stand out as a class of antibiotic with much scope for structural modification that provides them with additional and now multiple synergistic mechanisms of action, as a means to reinvigorate activity against MDR pathogens[17,18].

Clinical resistance to vancomycin first emerged in enterococci (VanA and VanB, VRE) in 1987[19], and to *Staphylococcus aureus* (VRSA) in 2002[20], caused by the late-stage remodeling of the N-terminus of peptidoglycan precursors from D-Ala-D-Ala to D-Ala-D-Lac (Figure 1)[21]. This single-point modification to the cell wall precursors reduces vancomycin binding and derived antimicrobial activity by 1000-fold[22]. The chemical evolution of the privileged core structures of glycopeptides, achieved by means of complex total syntheses or semi-synthetic modification, has proven a profitable strategy for overcoming the molecular basis of resistance[18], with several recently approved drugs reaching the clinic[23].

The covalent tethering of glycopeptides, such as vancomycin, to create polyvalent assemblies is also known to harness improved activity against vancomycin-resistant bacteria[24–32]. While the reasons underpinning this phenomenon are not fully understood, for "head-to-tail"[29,30] and "back-to-back"[33,34] dimers, the hydrogen bonds at the dimer interface are governed by the same amide units responsible for binding to the terminal D-Ala-D-Ala binding site of vancomycin, albeit through a different network of hydrogen bonds[35]. This cooperative interaction results in the dimer having a greater affinity for the D-Ala-D-Ala ligand than the monomer, and the ligand-bound monomer has a higher propensity to dimerize than the free monomer[35].

Nicolaou and co-workers harnessed the power of template accelerated synthesis to discover highly potent vancomycin dimers[36]. Such dynamic combinatorial libraries rely on the high degree of reversibility of the chosen ligation reaction under a given set of conditions, but typically, systems often suffer from long equilibration times and the need for additional reagents both to mediate the reversible reactions and to freeze the equilibrium once adaptation has occurred[37]. Dynamic covalent unimolecular chemical systems offer the potential to overcome these limitations yet are a far less common strategy[37,38]. Bullvalene is the archetypal fluxional molecule which, through endless Cope rearrangements, achieves a state of total degeneracy whereby there are no permanent carbon-carbon



6a: X=CH_2, n=0, (20%)
6b: X=CH_2, n=1, (8%)
6c: X=O, n=2, (19%)
6d: X=O, n=3, (23%)
6e: X=O, n=4, (20%)
6f: X=O, n=5, (10%)
6g: X=O, n=6, (15%)
6h: X=O, n=7, (11%)
6i: X=O, n=8, (15%)
6j: X=O, n=9, (10%)
6k: X=O, n=10, (9%)

5a: X=CH_2, n=0, (45%)
5b: X=CH_2, n=1, (65%)
5c: X=O, n=2, (42%)
5d: X=O, n=3, (71%)
5e: X=O, n=4, (68%)
5f: X=O, n=5, (40%)
5g: X=O, n=6, (50%)
5h: X=O, n=7, (55%)
5i: X=O, n=8, (41%)
5j: X=O, n=9, (71%)
5k: X=O, n=10, (70%)

Figure 2. A) SVD design. B) Synthesis. C) Control ligands.

bonds (Figure 1B)[39–41]. Substituted derivatives represent unimolecular shapeshifting dynamic combinatorial libraries[42]. Bode *et al.* have developed this concept and demonstrated a self-sorting adaptive binding polyol sensor array (Figure 1C)[43].

However, the chemistry of bullvalene has been limited by difficult and/or lengthy synthetic access. Recently, one of our laboratories has introduced a range of new methods that provide easy and modular access to substituted bullvalenes[44]. Such derivatives have the potential to act as highly specific sensing molecules that can differentiate structurally similar biomolecules from one another. Furthermore, the vast number of permutations accessed by shapeshifting molecules can be considered self-contained adaptive systems[44,45] that respond to host-guest interactions — a feature, we posit, could offer potential in countering the evolutionary forces of drug resistance — particularly MDR bacteria.

Herein, we describe the click chemistry synthesis of a focused library of "back-to-back" vancomycin fused dimers connected through a fluxional bullvalene core that are circumspect to the resistance mechanisms typically associated with vancomycin-resistant bacterial strains (Figure 1D).

Results and Discussion

Synthesis: To explore the potential of shapeshifting antibiotics, we elected to focus on symmetrically disubstituted bullvalene derivatives. Our modular SVD design centered on three components: i) a shapeshifting bullvalene core, ii) a flexible linker, and iii) a vancomycin warhead connected through the vancosamine unit (Figure 2A).

Click reactions, being transformations with unprecedented levels of fidelity and versatility[46], are perfectly suited for the construction of the complex SVDs in the forward synthetic direction. First, a CuAAC[47–49] coupling of the bis-acetylene functionalized bullvalene core **3** with the aromatic azides (**4a–k**) would introduce the aldehyde functionalized linkers onto the bullvalene core (**5a–k**). The vancomycin warhead would then be clicked *via* reductive amination with the vancosamine unit[14].

The synthesis began from bis-methylenehydroxy-bicyclooctatetraene **1** (Figure 2B), by treatment with propargyl bromide and sodium hydride to afford the bis-propargyl ether **2**, which itself was then subjected to a photochemical induced di-π-methane rearrangement to give the bis-propargyl ether bullvalene ether **3**. Next, double CuAAC reaction of **3** with the azides (**4a–k**) gave a selection of aldehyde charged linkers of varied length in good yields. Reductive amination of the aldehydes **5a–k** with vancomycin and sodium cyanoborohydride resulted in complete consumption of starting material (**5**) by TLC, with the final SVDs **6a–k** isolated in moderate yields upon purification by preparative HPLC. Nevertheless, sufficient quantities of each of the target SVDs (**6a-k**) were available for screening the SVDs against drug-sensitive and resistant bacterial strains of *S. aureus* and enterococci. In addition, a set of covalently tethered dimeric controls, **7a–c**, with a central benzene unit rather than bullvalene were prepared through an analogous sequence (see SI for full details). The complex fluxional nature of the aldehydes **5a–k** and the SVDs **6a–k**, render structural characterization challenging (refer to SI for more information). Hence, we depend upon the guaranteed reliability of modular click chemistry, the resilience of vancomycin to reductive amination conditions, and high-resolution mass spectrometry for product identification[14].

To probe the dynamics of the shapeshifting ligands, a series of VT-NMR experiments were performed. For bullvalenes with this substitution pattern, there are 15 possible constitutional isomers[44,50]. For dipropargyl ether **3**, the populated isomers **A–F** were identified, with isomers **A** and **B** predominating (Figure 3A, also refer to SI). A network diagram showing the isomer

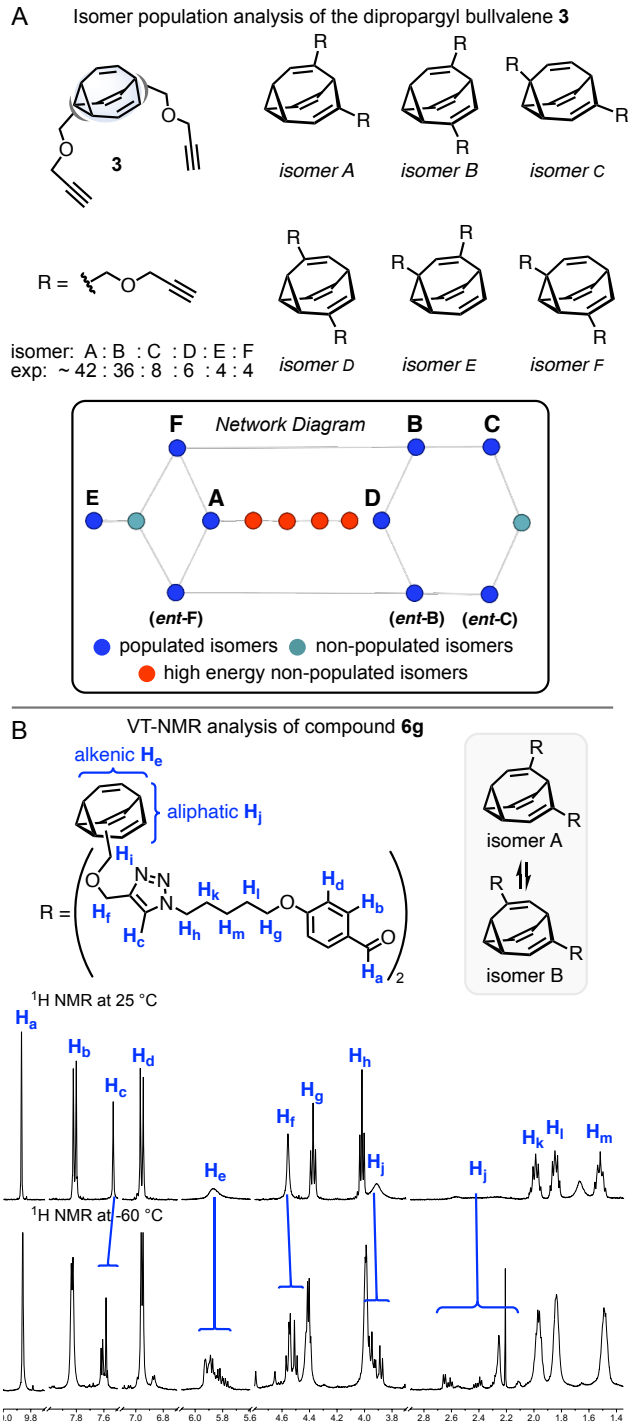
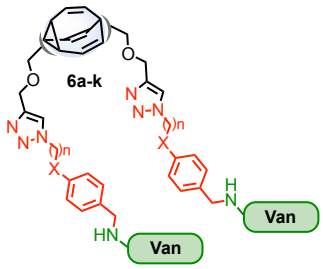


Figure 3. A) Isomer distribution and network analysis of dipropargyl bullvalene **3**. B) Room temperature and -60 °C ^{1}H NMR spectra of linker **5f**.

interconnections, with nodes representing isomers and edges transition structures. The isomers **B**, **C**, and **F** are chiral and will interconvert between enantiomers. Isomers with 1,2-adjacent substitution will be destabilized and are highlighted in red. The

relatively flat isomer distribution suggests that **3** and its derivatives should explore a relatively wide range of dynamic shape characteristics with varying distance and angle constraints. This notion is supported by NMR analysis of the aldehydes **5a–k,** revealing spectral features consistent with the isomer distribution of **3**. As an exemplar, the room temperature and -60 °C proton spectra of **5f** are shown in Figure 3B. The bullvalene core alkenic ($\mathbf{H_e}$) and aliphatic signals ($\mathbf{H_j}$), as well as the signals proximal to the bullvalene (methylenes $\mathbf{H_i}$, $\mathbf{H_f}$, and triazole proton $\mathbf{H_c}$), all show pronounced broadening at room temperature, which is resolved at -60 °C into complex signals sets. Diagnostic signals in the aliphatic region affirm the major isomers **A** and **B**, as well as unidentified minor isomers. Given the consistent isomer distribution within the series, it is feasible that the SVDs **6a–k**, reflect this trend, although detailed analysis was precluded due to the complexity of the fluxional system.

Table 1. A) Antibacterial activity of vancomycin and the SVDs **6a–k** against drug-sensitive and resistant bacterial strains (N = 3). B) Extended panels against multiple strains of MRSA, VIE, and VRE; MSSA: methicillin-sensitive *S. aureus* (ATCC® 9144™); MRSA: methicillin-resistant *S. aureus* (ATCC® BAA-1720); VSE: vancomycin-sensitive *E. faecium* (ATCC® BAA-2127™); VIE: vancomycin-intermediate *E. gallinarum* (ATCC® 49608™); and VRE: vancomycin-resistant *E. faecium* (ATCC® 700221™); VSSA: SH1000; VRSA: vancomycin-resistant *S. aureus* (VRS1/HIP11714)



A

Compound	X	n	MSSA	MRSA	VSE	VIE	VRE	VSSA	VRSA
			MIC (μg/mL)						
Vancomycin	-	-	1-2	2	1	8-16	256	1.25	>200
6a	CH_2	1	4	2-4	1-2	2-4	32	-	-
6b	CH_2	2	8	4-8	2-4	0.5	16	-	-
6c	O	2	8	8-16	2-4	0.25-0.5	16	-	-
6d	O	3	8-16	8	2	1-2	16	-	>20
6e	O	4	8-16	4	2-4	2	32	10	>20
6f	O	5	16	8	4	2-4	16	5	>20
6g	O	6	8	8	2-4	2	32	-	>20
6h	O	7	8	16-32	2-4	1	4	-	-
6i	O	8	16	16	4	4	16-32	20	>20
6j	O	9	4-8	8	2	2-4	16	-	-
6k	O	10	4-8	8	2-4	4	>32	5	10

B

Compound	X	n	MRSA (10 strains)	VIE (5 strains)	VRE (10 strains)
			MIC (μg/mL)		
Vancomycin	-	-	1-4	8-16	>256
6d	O	3	8-16	1-4	16-32

Antibacterial activity: The antibacterial properties of the SVDs was assessed against drug-sensitive and resistant strains of *S. aureus* and *Enterococcus*, as reported in Table 1A. While all the dimers exhibited some degree of antibacterial activity, of note was the enhanced activity of the dimers against vancomycin-intermediate (VIE) and vancomycin-resistant (VRE) strains, with minimum inhibitory concentration (MIC) values up to 64-fold lower than that of vancomycin. Against VRSA, SVD **6k** was the most active with an MIC of 10 μg/mL. To assess whether this improved activity was maintained across various methicillin-resistant *S. aureus* (MRSA) and VRE strains, SVD **6d** was screened against extended panels of MRSA (ATCC® MP-3™) and VRE (ATCC® MP-1™) (Table 1b). While a similar potency against MRSA was observed relative to vancomycin, there was also a consistent increase in potency of SVD **6d**, relative to vancomycin, against VRE.

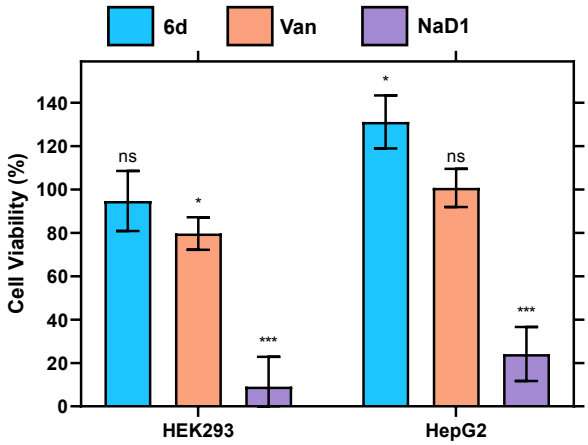


Figure 4. *In vitro* toxicity profiles assessed by an MTT viability assay. Viability of HEK293 cells and HepG2 cells treated with 500 μg/mL SVD **6d** (blue) relative to 500 μg/mL vancomycin (orange), and 50 μM of the cytotoxic peptide NaD1 (purple), normalized to a 1% (v/v) DMSO vehicle control. Error bars: S.D. (n = 3); ns p>0.05, * p < 0.05, *** p < 0.001.

In vitro toxicity: Vancomycin and SVD **6d** were assessed for their cytotoxicity *in vitro* using the MTT viability assay with the cytotoxic peptide NaD1 employed as a positive control (Figure 4). At the highest dose tested of vancomycin (2-fold greater than the MIC), a significant decrease in the viability of the HEK293 cell line was observed, consistent with reports of nephrotoxicity[51,52]. Vancomycin has previously been associated with acute kidney injury, a result of the larger doses and longer duration of treatment needed to curb the increasing incidence of vancomycin-resistant strains of *S. aureus* and *Enterococcus*. Therefore, it was necessary to evaluate whether the modifications and dimerization of vancomycin reduced the toxic effects. At the same concentration as vancomycin, the SVD **6d** resulted in no significant decrease in viability for either cell line, indicating that the analog is not cytotoxic to HEK293 and HepG2 cell lines, relative to the vehicle control. Further, given the difference in potency between vancomycin and SVD **6d**, the highest dose of the dimer tested equates to 10-fold higher than the effective antibacterial dose, and as such, SVD **6d** may provide a wider therapeutic window than vancomycin.

In vivo infection model: To further explore the potential of the SVDs as novel antibiotics, we assessed their efficacy in an *in vivo* infection model. Specifically, the larvae of *Galleria mellonella* were used, which is a well-established model to study bacterial

virulence and for assessing antibiotic efficacy (Figure 5)[53,54]. When challenged with vancomycin at 20 mg/kg, a standard clinical dose, *G. mellonella* larval survival rate was not significantly reduced, with 75% survival at day 7. Similarly, the larvae tolerated treatment with SVD **6d** with 70% survival, indicating minimum toxicity in the *G. mellonella* model. When challenged with VRE without treatment, larval survival was reduced to 10% by day 7. The treatment with vancomycin increased survival rates to only 40%, whereas treatment with SVD **6d** retained larval survival at 70%, the same as the SVD treatment alone. This indicates that SVD **6d** can successfully treat a VRE infection in the *G. mellonella* model.

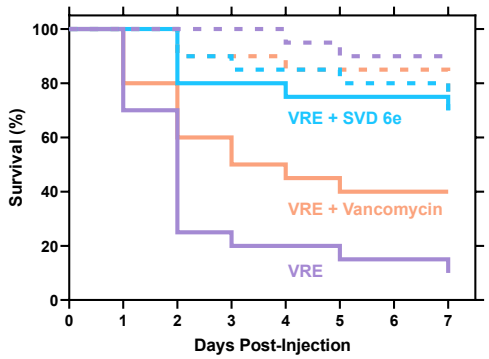


Figure 5. *In vivo* infection model using *Galleria mellonella*. Tolerance of the *G. mellonella* larvae to 20 mg/kg SVD **6d** (blue dash), 20 mg/kg vancomycin (orange dash) and 1% (v/v) DMSO vehicle control (purple dash), compared to the survival of the larvae following infection with VRE (ATCC® 51575™) (purple solid) and treatment with either 20 mg/kg SVD **6d** (blue solid) or 20 mg/kg vancomycin (orange solid) (N = 20).

Resistance studies: We envisioned that the incorporation of the fluxional bullvalene core would minimize the development of acquired resistance compared to dimers that lack the intrinsic shapeshifting characteristic. Our hypothesis is that such a dynamic core would favor the most preferred binding interactions on the cell wall, which we posit may confer reduced propensity for resistance relative to the parent drug. To validate this hypothesis, we replaced the bullvalene core of SVD **6d** with aryl linkers, *ortho* **7a**, *meta* **7b,** and *para* **7c** substituted (Figure 2C; see SI), and assessed the propensity of a drug-sensitive strain of *E. faecium* (VSE) (ATCC® BAA-2127™) to develop resistance to the three control analogs (**7a-c**), SVD **6d** and vancomycin (Figure 6).

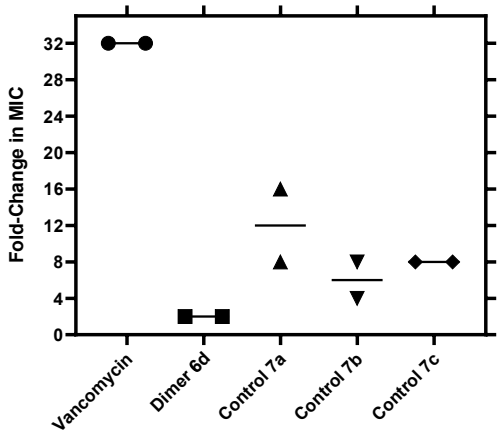


Figure 6. Propensity of *Enterococcus faecium* to develop resistance. *E. faecium* (ATCC® BAA-2127™) was exposed to increasing concentrations of vancomycin, SVD **6d**, control **7a**, control **7b** and control **7c**, up to 4 × MIC. Data is plotted as the fold-change in MIC value relative to a DMSO vehicle control (N = 2).

As anticipated, the serial passaging of VSE in the presence of vancomycin resulted in bacteria with a >32-fold increase in MIC, indicative of the emergence of a vancomycin-resistant strain of *Enterococcus.* Passaging with the control analogs resulted in bacteria with a 4- to 16-fold increase in MICs, signifying the emergence of early-stage resistance. Contrariwise, the passaging with SVD **6d** did not invoke significant resistance development in the bacterial strain, with MICs only increasing by 2-fold. The evidence suggests that the incorporation of a dynamic linker seems to minimize the propensity for bacteria to develop resistance. Furthermore, previous studies have predominantly focused on monomeric vancomycin analogs with modifications at the C-terminus and within the target binding pocket[16,55,56]. While these monomers demonstrated a significantly reduced resistance development propensity, our results, to the best of our knowledge, represent the first reported evidence for time-limited resistance development to tethered vancomycin dimers.

Lipid II model binding assays: The mechanisms behind the enhanced activity of vancomycin dimers are not fully understood. Ellman and co-workers, using covalent tail-to-tail dimers of vancomycin and the corresponding dimers of damaged vancomycin (the latter unable to bind to Lys-D-Ala-D-Ala or Lys-D-Ala-D-Lac), demonstrated that Lys-D-Ala-D-Lac binding is not required for the high activity of their vancomycin dimers against VRE, suggesting an alternate mode of action involving disruption of the function of proteins critical for VRE cell wall biosynthesis[25,57,58].

To explore the mode of binding of the back-to-back SVDs, we employed microscale thermophoresis (MST) binding studies with vancomycin and SVD **6d** using the fluorescently labeled tripeptides acetyl-Lys-D-Ala-D-Ala and acetyl-Lys-D-Ala-D-Lac to determine dissociation constants (K_D) (Table 2). Vancomycin was found to bind to the acetyl-Lys-D-Ala-D-Ala tripeptide with a K_D of 1.0 ± 0.3 μM, which is in agreement with reported K_D values for vancomycin binding to model peptides[57]. On the other hand, no binding was observed with the acetyl-Lys-D-Ala-D-Lac tripeptide[25], correlating with the poor activity against VRE. The SVD **6d** bound to acetyl-Lys-D-Ala-D-Ala tripeptide with a K_D of 11 ± 1.9 μM, which is of the order of magnitude reported for other dimeric vancomycin species[25]. However, we also observed binding of **6d** to the acetyl-Lys-D-Ala-D-Lac, albeit with relatively lower affinity and a K_D of 25 ± 4.1 μM. The similar affinity in binding supports the hypothesis that the bullvalene core allows for multiple binding conformations, resulting in antibacterial activity against not only vancomycin-sensitive but vancomycin-resistant enterococci.

Table 2. Binding affinities of vancomycin and SVD **6d** for acetyl-Lys-D-Ala-D-Ala and acetyl-Lys-D-Ala-D-Lac, defined as the dissociation constant (K_D) (N = 3, ± S.D.); n.b.: no binding.

Compound	KD (μM)	
	acetyl-Lys-D-Ala-D-Ala	acetyl-Lys-D-Ala-D-Lac
Vancomycin	1.0 ± 0.3	n.b.
6d	11 ± 1.9	25 ± 4.1

To explore the effects of the dynamic nature of bullvalene ring in our SVD **6d**, we employed a novel native mass spectrometry-based assay where vancomycin has been shown to

form a ternary complex through binding lipid II within a lipid II:MurJ complex[59]. MurJ is the key flippase protein that transports lipid II across the cytoplasmic membrane. To determine the differences between vancomycin and SVD **6d** actions, we first considered detecting if there are any possible interactions with MurJ directly. For this, we added 3 μM of each of these compounds to MurJ, and no binding was observed in the respective mass spectra (bottom spectra, Figure 7a, 7b). Next, we made MurJ:lipid II complex by incubating 5 μM MurJ with 3 μM lipid II, and as expected, the spectra indicate lipid II binding to MurJ (middle spectra, Figure 7a, 7b). We then attempted to form the ternary complexes by incubating this MurJ:lipid II complex with 7 μM of vancomycin and SVD **6d**. While the expected ternary complex was formed with vancomycin, complete loss of lipid II binding from MurJ was observed with SVD **6d**, suggesting that ligand disruption of the complex had occurred (top spectra, Figure 7a, 7b). A plausible explanation for the action of **6d** may include: (i) the SVD **6d** interacts with other regions of lipid II such as the MurNAc, GlcNaC or pyrophosphate group, with high affinity; (ii) the SVD **6d**:lipid II complex is too large to accommodate lipid II binding to MurJ , and/or (iii) the lipophilic linker of SVD **6d** participates in binding to lipid II, in which case, the interaction with C_{55}PP tail may cause dissociation. Collectively, our data indicates that there may exist a new mode of action for the SVD **6d**, and further advanced studies are required to unravel the complexity of this system.

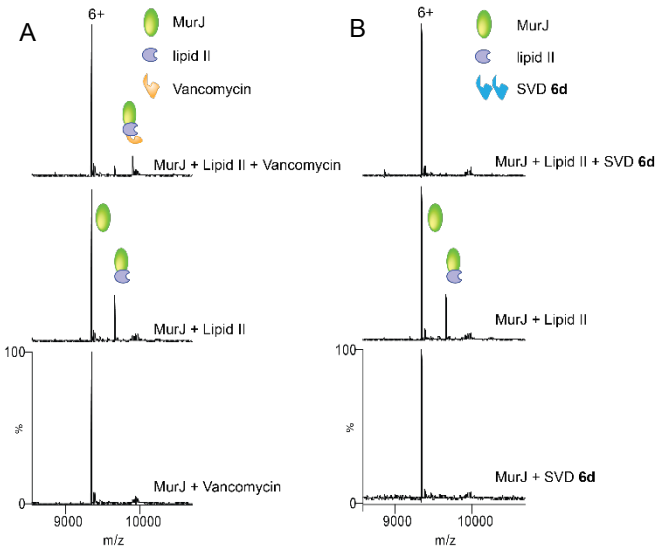


Figure 7. Native mass spectrometry suggests additional modes of action for SVD **6d**. (a) mass spectra of vancomycin with MurJ (bottom), lipid II with MurJ (middle), and vancomycin with MurJ:lipid II complex (top). Ternary complex of MurJ:lipid II:vancomycin is observed (top spectra). (b) mass spectra of SVD **6d** with MurJ (bottom), lipid II with MurJ (middle), and SVD **6d** with MurJ;lipid II complex (top). No ternary complex and complete loss of lipid II binding from MurJ (top spectra). Charge state 6+ is shown in both cases.

Conclusion

The development of vancomycin derivatives that act by multiple mechanisms of action and decrease resistance susceptibility have been previously disclosed[16,60,61]. Three semisynthetic vancomycin analogues[62], telavancin (2009), dalbavancin (2014), and oritavancin (2015) have made it to the clinic for the treatment of MRSA infection[18]. By taking advantage of the fluxional shapeshifting properties of substituted bullvalene, we have developed a conceptually new style of covalently linked vancomycin dimers as novel antibiotics. This effort has provided prototype antibiotics with independent mechanisms of action targeting VRE (and VRSA), for which vancomycin is ineffective. Given the extreme threat of drug-resistant bacteria, the CDC has placed VRE on its serious threat list[63], and the WHO[64] placed it fourth on its list of drug-resistant bacteria that pose the greatest threat to human health. While the glycopeptide and other antibiotics have been endowed with features that avoid many mechanisms of resistance[65], the potential benefits of molecular shapeshifting have, until now, been overlooked. The resilience of shapeshifting vancomycin dimers (SVDs) to the onset of antibiotic resistance and strong binding to acetyl-Lys-D-Ala-D-Lac render this molecular class of ligand attractive for further development. We posit that the observed enhancements arise through dynamic adaptive binding interactions imparted by the shapeshifting bullvalene core, and through destabilization of the complex formed between the flippase MurJ and lipid II. The effect of the linker chain length also appears to play a major part in the activity against bacterial species as demonstrated, for example, by the potency of **6d** and **6k** against VRS and VRSA species, respectively. We believe this work showcases the potential of shapeshifting hydrocarbons in drug discovery and sets the stage for further studies.

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