Peripheral modifications of $[Ψ(CH_2NH)Tpg^4]v$ancomycin with added synergistic mechanisms of action provide durable and potent antibiotics

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Subsequent to binding pocket modifications designed to provide dual $\delta$-Ala-$\delta$-Ala/$\delta$-Ala-$\delta$-Lac binding that directly overcome the molecular basis of vancomycin resistance, peripheral structural changes have been explored to improve antimicrobial potency and provide additional synergistic mechanisms of action. A C-terminal peripheral modification, introducing a quaternary ammonium salt, is reported and was found to provide a binding pocket-modified vancomycin analog with a second mechanism of action that is independent of $\delta$-Ala-$\delta$-Ala/$\delta$-Ala-$\delta$-Lac binding. This modification, which induces cell wall permeability and is complementary to the glycopeptide inhibition of cell wall synthesis, was found to provide improvements in antimicrobial potency (200-fold) against vancomycin-resistant Enterococci (VRE). Furthermore, it is shown that this type of C-terminal modification may be combined with a second peripheral (4-chlorobiphenyl)methyl (CBP) addition to the vancomycin disaccharide to provide even more potent antimicrobial agents [VRE minimum inhibitory concentration (MIC) = 0.01–0.005 μg/mL] with activity that can be attributed to three independent and synergistic mechanisms of action, only one of which requires $\delta$-Ala-$\delta$-Ala/$\delta$-Ala-$\delta$-Lac binding. Finally, it is shown that such peripherally and binding pocket-modified vancomycin analogs display little propensity for acquired resistance by VRE and that their durability against such challenges as well as their antimicrobial potency follow now predictable trends (three > two > one mechanisms of action). Such antibiotics are expected to display durable antimicrobial activity not prone to rapidly acquired clinical resistance.

Recent years have seen a welcomed refocus on the need for new antibiotics to address the persistent threat of bacterial resistance (1–3). A number of actions have been advanced to address the challenges posed by bacterial resistance now emerging faster than new treatment options. These actions include providing new financial incentives to counter the declining economic interests in developing new antibiotics (4), revamping regulatory criteria for new drug approvals (5), improving the rate of diagnostic characterization of infecting organisms, enhancing nationwide resistance surveillance, encouraging work targeting mechanisms of resistance, and identifying new therapeutic targets for antibiotic discovery (6, 7). The suggested actions also champion antibiotic stewardship (8). Although sounding attractive, the effort to restrict antibiotic use seems counter to their importance, introduces guilt into even their most legitimate of uses, challenges the prevailing practices of initial empirical best guess therapy and prophylaxis deployment, and produces additional disincentives to antibiotic development. Although such initiatives highlight the pressing need for renewed antibiotic discovery and the fundamental importance of antibiotics in modern medicine (9), it has done little to define new approaches that directly address the underlying problem of evolutionarily driven and acquired resistance. The mechanisms of resistance are ancient and increasingly accumulating in pathogenic bacteria, which have now assimilated large elements of this bacterial resistance (10, 11). An additional and perhaps even more important question to ask is if new antibiotics can now be designed that overcome the forces of evolution and selection responsible for bacterial resistance, that are less prone or even impervious to resistance development, that avoid many of the common mechanisms of resistance, and that are more durable than ever before. As an alternative to championing the restricted use of antibiotics or conceding that bacteria will always outsmart us, can durable antibiotics be developed that are capable of continued or even more widespread use? Herein, we describe one such effort to create durable antibiotics by deliberate design that may directly counter such evolutionary forces. We identified the glycopeptide antibiotics as an antibiotic class already endowed with features that avoid many mechanisms of resistance. After introduction of designed structural changes that directly overcome the molecular basis of their only prevalent mechanism of resistance, we have explored the incorporation of peripheral structural modifications in the molecules that provide them with additional and now multiple synergistic mechanisms of action, thereby not only increasing their potency but also, creating prototype durable antibiotics.

In recent disclosures, we have discussed attributes of the glycopeptide antibiotics (12, 13) that have contributed to their sustained effectiveness in the clinic (14). Vancomycin (15), teicoplanin (16), and three recently approved semisynthetic derivatives, oritavancin (17), dalbavancin (18), and telavancin (19), are widely used to treat refractory bacterial infections, including methicillin-resistant Staphylococcus aureus (MRSA) (20). Vancomycin (1) (Fig. 1) (21) was disclosed in 1956 (15) and introduced into the clinic in 1958. After nearly 60 y of clinical use and even with the past use of glycopeptide antibiotics for agricultural livestock (avoparcin), resistant pathogens have only slowly emerged, and vancomycin remains an integral and

Significance

In a quest for antibiotics that may display durable clinical lifetimes, analogs of the glycopeptide antibiotics, including vancomycin, have been designed that not only directly overcome the molecular basis of existing vancomycin resistance but also contain two added peripheral modifications that endow them with two additional independent mechanisms of actions not found in the parent antibiotics. It is shown that such peripherally and binding pocket-modified vancomycin analogs display little propensity for acquired resistance by vancomycin-resistant Enterococci and that both their antimicrobial potencies and durability against such challenges follow trends (three > two > one mechanisms of action) that are now predictable.

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increasingly important antibiotic today. Clinical resistance was initially observed with vancomycin-resistant Enterococci (VRE; 1987) that was detected only after 30 y of clinical use (22) but now, also includes vancomycin-resistant S. aureus (VRSA; 2002) (23). Treatment options for the latter are limited and presently include antibiotics known to rapidly evoke resistance (24, 25). As a result, these latter antibiotics have been designated as reserve antibiotics to be deployed sparingly to preserve their effectiveness as drugs of last resort against intractable infections. Just as significantly, some VRE organisms, like MRSA, have also reached a stage where they are now resistant to most other common antibiotic classes (26). As a result and because they are already vancomycin-resistant, the CDC has now placed VRE on its serious threat list (27). 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It is not a protein or nucleic acid target subject to changes by a single genetic mutation that can result in resistance. The primary mechanism of action of vancomycin involves sequestration of this substrate (D-Ala-D-Ala) for a late-stage enzyme-catalyzed (transpeptidase) reaction used for cell wall maturation (29). This target is unique to bacteria and contributes to the selectivity of the antibiotic class for bacteria vs. their mammalian hosts. It is also an atypical biological target, being a substrate for an enzymatic reaction and a precursor to a structural component of the bacterial cell wall. It is not a protein or nucleic acid target subject to changes by a single genetic mutation that can result in resistance. The primary mechanism of action of vancomycin involves sequestration of this substrate (D-Ala-D-Ala) for a late-stage enzyme-catalyzed (transpeptidase) reaction used for cell wall maturation (29). 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action, only one of which is dependent on D-Ala-D-Ala/D-Ala-D-Lac binding (48).

Herein and along with studies that further clarify this second mechanism of action, an alternative peripheral modification that endows the pocket-modified vancomycin analogs with another different second mechanism of action is reported. This modification also provides similarly impressive improvements in antimicrobial potencies against vancomycin-resistant bacteria (VRE). Furthermore, we show that the two peripheral modifications may be combined with the pocket-modified vancomycins to provide even more potent antimicrobial agents with activity that can be attributed to express such effects, and act independent of mechanisms derived from transpeptidase or transglycosylase inhibition.

A select series of such vancomycin analogs was first prepared, including 10 and 11 not previously examined. For simplicity, they are referred to as C0 (9), C1 (10), cyclic C5 (11), and C14 (12) (51), denoting the terminal tertiary dimethylamine (9; C0) or the quaternary ammonium salts bearing a methyl (10; C1), C5 cyclic

**Results and Discussion**

These studies were conducted with the methylene pocket-modified vancomycin analog 4 ([Ψ(CH₂NH)Tpg₄]vancomycin) (48), presently the most readily available of our synthetic analogs prepared by total synthesis (50). Because it also exhibits the more modest dual D-Ala-D-Ala/D-Ala-D-Lac binding affinity and antimicrobial activity against vancomycin-resistant organisms of the two pocket-modified vancomycin analogs (4 vs. 3) (Fig. 1), the impact of alternative or multiple peripheral modifications was anticipated to be most easily quantitated. The alternative peripheral modification examined was C-terminal amidic functionalization with incorporation of either a basic amine capable of protonation or a quaternary ammonium salt. Such modifications have been shown to provide improved antimicrobial activity against vancomycin-resistant organisms and were found to act by disrupting bacterial cell wall membrane integrity, increasing cell permeability, and inducing membrane depolarization (51). Although inspired by the nonselective membrane disruption induced by quaternary ammonium salts, the studies herein provide one such modification that exhibits only a subset of such effects (membrane permeability) and acts by a more specific mechanism not resulting in cell lysis. It is a behavior not observed with the naturally occurring glycopeptide antibiotics or their more typical analogs, but the mechanism is one that may contribute to the activity of the semisynthetic drugs dalbavancin and telavancin (52). In vancomycin-resistant organisms, such modifications do not directly contribute to inhibition of cell wall biosynthesis, do not improve D-Ala-D-Lac binding needed to express such effects, and act independent of mechanisms derived from transpeptidase or transglycosylase inhibition.

<table>
<thead>
<tr>
<th>Antimicrobial Activity, MIC (µg/mL)</th>
<th>sensitive</th>
<th>MRSA</th>
<th>VanA</th>
<th>VanB</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus*</td>
<td>S. aureus*</td>
<td>E. faecalis*</td>
<td>E. faecium*</td>
<td>E. faecalis*</td>
</tr>
<tr>
<td>5, X = O</td>
<td>0.03</td>
<td>0.03</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>6, X = S</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>7, X = NH</td>
<td>0.03</td>
<td>0.06</td>
<td>0.005</td>
<td>0.005</td>
</tr>
<tr>
<td>8, X = H₂</td>
<td>0.5</td>
<td>0.25</td>
<td>0.13</td>
<td>0.06</td>
</tr>
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</table>

*MIC = Minimum inhibitory concentration. **ATCC 25923. ¹ATCC 43300. ²BM 4166. ³ATCC BAA-2317. ⁴ATCC 51299. ⁵not determined.

Fig. 2. Pocket-modified vancomycins that contain an additional peripheral CBP modification to the pendant disaccharide.

![Diagram of Antimicrobial Activity, MIC (µg/mL)](image_url)

![Diagram of Peripheral C-terminal modifications of vancomycin and the binding pocket-modified vancomycin analog 4.](image_url)

![Diagram of pocket-modified vancomycins that contain an additional peripheral CBP modification to the pendant disaccharide.](image_url)
C14H29

H

potent than the CBP derivative pocket analog Ala/D-Ala-D-Lac binding, this second additional peripheral modification that it alone imparts membrane permeability not found with derivatives. Although unanticipated, the most effective C-terminal modification of CBP derivatives. Subsequent studies show clearly that it alone imparts membrane permeability not found with derivatives lacking this particular C-terminal modification. For the pocket-modified CBP-vancomycin analog capable of dual d-Ala-d-Ala/d-Ala-d-Lac binding, this second additional peripheral modification with 18 produced a >10-fold increase in potency against VanA VRE relative to 8, lowering the MIC value for 18 to 0.01–0.005 μg/mL (Fig. 4). This vancomycin analog is >10-fold more potent than the CBP derivative 8, >1,000-fold more potent than the pocket analog 4, and a stunning >10,000-fold more potent than vancomycin itself. It is also >25-to 100-fold more potent than its comparison C1/CBP–vancomycin derivative 15 and >250-fold more active than either CBP–vancomycin (5) or C14–vancomycin (12).

To clarify the contributing mechanisms responsible for the antimicrobial activity in vancomycin-resistant organisms (VanA VRE), the key analogs were examined in a range of assays, two of which defined the origin of their effects. One assay was used to establish inhibition of bacterial cell wall synthesis, and the second measured induced membrane permeability. The inhibition of bacterial cell wall synthesis was established in an assay that quantitates material cell wall synthesis was established in an assay that quantitates membrane permeability. The inhibition of bacterial cell wall synthesis was established in an assay that quantitates membrane permeability. The inhibition of bacterial cell wall synthesis was established in an assay that quantitates membrane permeability. The inhibition of bacterial cell wall synthesis was established in an assay that quantitates membrane permeability. 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tetracycline-pretreated VanA VRE for 30 min (37 °C) before the cultured bacteria were collected by centrifugation, washed, and resuspended in pH 7.2 buffer (5 mM Hepes:5 mM glucose; 1:1). The bacterial suspension was heated at 100 °C for 15 min to release cytosolic 19. The entire supernatant was analyzed by semi-preparative reverse-phase HPLC with a single injection, and the amount of 19 was quantitated with use of the calibration curve. The results of the evaluation of 5–18 in VanA vancomycin-resistant Enterococcus faecalis (BM 4166) and E. faecium (ATCC BAA-2317) are presented in Fig. 6 and represent the same strains used in the antimicrobial assays.

The effect of the compounds on cell membrane permeability was examined by measuring cytoplasmic membrane uptake of the fluorescent probe propidium iodide in the same VanA vancomycin-resistant E. faecalis (BM 4166) and E. faecium (ATCC BAA-2317) strains. This fluorescent probe only enters cells with permeabilized cell membranes and is detected by the emission of fluorescence on intracellular nucleic acid binding (54). Fresh midlog-phase VanA VRE in pH 7.2 buffer at 25 °C was preincubated with propidium iodide (10 μM) for 5 min before the test compounds were added and monitored for the fluorescence emission at 617 nm (excitation at 535 nm) over time, both before (5 min) and after (15 min) compound addition. Rapid and sustained increases in fluorescence intensity are observed immediately on addition of test compounds that induce bacterial cell membrane permeability.

Assessments of the compounds, identifying their contributing mechanisms of action in vancomycin-resistant VRE, were conducted with the two assays. The results are discussed below in sets defining first the role of the pocket modification found in 4 and then, the subsequent impact of the peripheral CBP modification to the vancomycin disaccharide found in 5–8. This summary is followed by the discussion of the results from the examination of the peripheral quaternary ammonium salt modifications found in 9–13, including their effects on both unmodified and pocket-modified vancomycin analogs. Finally, the effects of the two combined peripheral modifications in 14–18 are detailed.

Consistent with its inactivity and like the thioamide 2, vancomycin (1) does not effectively inhibit bacterial cell wall synthesis in VanA VRE and does not result in the significant accumulation of 19 in the assay (Fig. 6). In contrast, the pocket-modified analog 4 designed for dual D-Ala-D-Ala/D-Ala-D-Lac binding inhibits bacterial cell wall biosynthesis, resulting in the buildup of the precursor 19 in the assay at levels consistent with its relative model ligand binding affinities and antimicrobial activity. As anticipated, none of these compounds significantly impact membrane integrity, and none result in cytoplasmic membrane permeability as measured by propidium uptake (SI Appendix, Fig. S2). Thus, the antimicrobial activity of 4 correlates directly with its expected impact on bacterial cell wall biosynthesis, binding D-Ala-D-Lac and inhibiting cell wall maturation. Incorporation of the peripheral CBP modification in 5 (ineffective binding to D-Ala-D-Lac) and 6 (ineffective binding to either D-Ala-D-Ala or D-Ala-D-Lac) produced analogs with good activity against VanA VRE that was found to correlate with their ability to inhibit cell wall synthesis of VanA VRE, resulting in the accumulation of 19 in the assay (Fig. 6).

Neither compound impact...
membrane permeability (SI Appendix, Fig. S2). Compounds 5 and 6 exhibit nearly equivalent antimicrobial activity against VanA VRE, and both inhibit cell wall biosynthesis to a similar extent, but 6 is incapable of binding either D-Ala-D-Ala or D-Ala-D-Lac. Thus, this inhibition of bacterial cell wall synthesis is not derived from inhibition of transpeptidase-catalyzed cross-linking derived from D-Ala-d-Ala/d-Ala-d-Lac binding but rather, likely arises from direct inhibition of transglycosylase by the peripherally modified disaccharide. Previous studies of Kahne and coworkers (32, 33) and others (30, 31, 55) have shown such direct inhibition of transglycosylase by 5 and related CBP-bearing analogs. Finally, the potent pocket-modified vancomycin analog 8, containing the peripheral CBP modification, inhibits cell wall synthesis more effectively than 4, lacking the CBP modification and more potently than either 5 or 6, lacking a productive pocket modification. This behavior is the result of the combined effects of the two independent mechanisms of action, both of which impact cell wall biosynthesis but only one of which depends on D-Ala-d-Ala/d-Ala-d-Lac binding. We have interpreted these observations to represent inhibition of both transpeptidase-catalyzed cross-linking, requiring d-Ala-d-Ala/d-Ala-d-Lac binding, and the transglycosylase-catalyzed cell wall incorporation of Lipid II presumably by a direct enzyme interaction that does not require d-Ala-d-Ala/d-Ala-d-Lac binding.

The examination of the analogs that contain the peripheral C-terminal amides with quaternary ammonium salt modifications (9-13) was similarly revealing and clear. Despite the progressive increase in antimicrobial activity observed against VanA VRE with 9-12, little or no change in their ability to inhibit bacterial cell wall synthesis was observed, and they remained, like vancomycin itself, essentially inactive in this assay (Fig. 6). By contrast, the two vancomycin derivatives 11 and 12 that were active against VanA VRE produced pronounced, rapid cell membrane permeability immediately on their addition, whereas the inactive (9) and less active (10) variants did not when examined at 10 μM (Fig. 7). The less active compound 10 exhibited this induced permeability when examined at a higher concentration (100 μM) (SI Appendix, Fig. S3).

Here, the antimicrobial activity against VanA VRE can be attributed to a mechanism independent of cell wall biosynthesis and independent of d-Ala-d-Ala/d-Ala-d-Lac binding. The antimicrobial activity correlates with disruption of the cell wall integrity as measured by its increased permeability. The incorporation of the most potent of these peripheral C-terminal modifications into the pocket-modified analog 4 with 13 (C14) further enhanced antimicrobial activity against VanA VRE 200-fold (Fig. 3).

This modification did not improve, diminish, or alter the inhibition of cell wall biosynthesis, where 4 and 13 were found to be equally active (Fig. 6). However, it did provide an analog that, unlike 4, produced pronounced cell membrane permeability immediately on addition (Fig. 7). Thus, compound 13 represents a pocket-modified vancomycin analog that displays potent and further improved activity against VanA VRE derived from two independent and synergistic mechanisms of action. One mechanism relies on the dual d-Ala-d-Ala/d-Ala-d-Lac binding like 4 and results in effective cell wall synthesis inhibition. The second mechanism is independent of this ligand binding property and derived from induced cell wall permeability. The two combined vancomycin modifications and the accompanying two synergistic mechanisms of action provide a vancomycin analog >1,000-fold more active than vancomycin against the most stringent vancomycin-resistant organisms, VanA VRE, displaying superb in vitro MICs (0.16 μg/mL). It represents now the second such example, complementing the observations made with 8 but with a different second mechanism of action introduced by a second alternative peripheral modification and generalizing the opportunities provided by such design principles.

The results of the examination of the analogs that incorporate the two peripheral modifications (14-17) and their combination with the pocket-modified vancomycin analog in 18 were even more revealing. In addition to showing that this may be successfully achieved, they highlight that it is not necessarily the most effective individual variants of the two peripheral modifications that combine to produce the desired effects but rather, that it is a combination that allows expression of the two independent mechanisms. As expected based on the CBP modification, 14-17 inhibit VanA VRE bacterial cell wall synthesis, and their relative activities are reflected in their functional activity in the antimicrobial assays (Fig. 6). The C14 and cyclic C5 quaternary ammonium salts actually diminish the inhibition of cell wall synthesis relative to CBP-vancomycin itself, and C0 was equally active, whereas C1 may have improved activity slightly (activity: 15 > 14 = 5 > 16 and 17). Even more revealing, their examination in the cell wall permeability assay indicates that only C1 combined with the CBP modification induced a pronounced, rapid, and potent cell membrane permeability (Fig. 8). By contrast, the combination of the peripheral CBP modification with the C14 and C5 quaternary ammonium salts was not productive, and none of the compounds exhibited the desired effects. Compound 18, which incorporates the redesigned pocket modification for dual d-Ala-d-Ala/d-Ala-d-Lac binding (blocks cell wall synthesis by ligand binding, including inhibition of transpeptidase-catalyzed cross-linking), the CBP disaccharide modification (blocks cell wall synthesis by direct transglycosylase inhibition without d-Ala-d-Ala/d-Ala-d-Lac binding), and the C1 quaternary ammonium salt C-terminal modification (induces membrane permeability), exhibited the most potent inhibition of cell wall synthesis in the assay of all compounds assessed (Fig. 6) as well as the most pronounced and potent induced cell membrane permeability of all compounds examined (Fig. 8). This behavior indicates that all three mechanisms of action are productively contributing to the expression of the antimicrobial activity of 18 against VanA VRE, resulting in its potent VanA VRE antimicrobial activity (MIC = 0.01-0.005 μg/mL). This compound represents an analog of vancomycin deliberately designed to overcome vancomycin resistance, which incorporates three structural modifications that impart three independent mechanisms of action. Only one mechanism depends on reengineered dual d-Ala-d-Ala/d-Ala-d-Lac ligand binding, and each of three mechanisms contributes to the expression of the antimicrobial activity.

These latter comparisons provided a direct correlation of the results of the permeability assay with the functional expression of antimicrobial activity where only C1 effectively expresses the functional behaviors. For us, this observation provided compelling evidence that the assay is an accurate readout of the correlated...
functional behaviors and that the underlying mechanistic interpretation of induced cell permeability is similarly accurate. In addition, it is remarkable that this effect is specific for C1 when combined with the CBP modification, suggesting that the mechanism responsible for induction of membrane permeability may involve specific interactions within the bacterial cell wall.

The results are summarized in Fig. 9 for the key analogs alongside their antimicrobial activity. Within this series, CBP-
vancomycin (5) is representative of the potency and characteristics of the clinically approved semisynthetic vancomycin analogs. For VanA VRE, the progression through the series from 1 to 4 to 8 or 13 and finally, to 18 represents vancomycin analogs with zero (1), one (4), two (8 and 13), and three (18) distinct and synergistic mechanisms of action that progressively provide increasingly potent antibacterial activity. This progressive improvement culminates in 18 (MICs = 0.005–0.01 μg/mL), with activity 25,000- to 50,000-fold more potent than vancomycin against VanA VRE. Notably, compound 18 is also 250- to 500-fold more potent than CBP–vancomycin (5), which is representative of the semisynthetic vancomycin analogs presently used in the clinic. Of special note, each structural modification and mechanism of action independently expresses its functional activity at the level of 2–30 μg/mL (1–15 μM) in both the antimicrobial and mechanistic assays, but each provides synergistic improvements in the functional antimicrobial activity when combined.

The discussion above focused on the identification of the contributing mechanisms of action and the demonstration that each independently improves antimicrobial activity potency. However, an additional and even more important feature of the expression of multiple independent mechanisms of action is its impact on the rate at which bacterial resistance may emerge. As a result, CBP–vancomycin (5; one mechanism of action), the peripherally C14-modified vancomycin analog (12; one mechanism of action), the peripherally CBP-modified pocket analog 8 (two mechanisms of action), the peripherally C14-modified pocket analog 13 (two mechanisms of action), and the pocket analog 18 that contains the two complementary peripheral C1 and CBP modifications (three mechanisms of action) were examined for their susceptibility to acquired resistance on sublethal (0.5x MIC) serial exposure to the same two VRE bacterial strains, monitoring MICs daily (Fig. 10).Distinct from most related studies enlisting MRSA or other vancomycin-sensitive bacterial strains, this study was conducted with the most stringent (VanA vs. VanB) vancomycin-resistant strains for which the mechanism of action associated with d-Ala-d-Ala binding is no longer effective. Consequently, it is not counted among the number of effective mechanisms of action imbedded in the compound structure. Notably, the antibiotic susceptibility profiles of the VanA VREs used herein indicate that they are resistant to a number of additional classes of antibiotics and on the verge of being classified as multidrug-resistant VRE, indicating that they have already assimilated a number of common resistance mechanisms (SI Appendix). Because the changes for 18 were so small throughout a typical 25-d study, the examination was extended to 50 d. These studies revealed that resistance to 5 and 12 emerged most rapidly and was pronounced (one mechanism). Changes in the potency of both 8 and 13 were much slower and more muted (two mechanisms; only two- to fourfold after 25 passages; 8 slower than 13). No change in susceptibility to 18 was observed after 25 d (three mechanisms), and little change in susceptibility to 18 was observed even after 50 daily passages (only two- to fourfold change in the MICs of 0.01 and 0.005 μg/mL in the two strains). Moreover, the magnitude of the changes in the MICs for the compounds acting by two or more mechanisms is sufficiently small to indicate that none experience a full loss of one of the contributing mechanisms. As such, each mechanism is rendered more robust when combined with structural

<table>
<thead>
<tr>
<th>select vancomycin analogs</th>
<th>D-Ala-D-Ala binding</th>
<th>D-Ala-D-Lac binding</th>
<th>cell wall synthesis inhibition with ligand binding</th>
<th>without ligand binding</th>
<th>disrupts cell wall integrity</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thioamide vancomycin (2)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>&gt;32</td>
</tr>
<tr>
<td>CBP-thioamide vancomycin (6)</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>4</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>250</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>2.5</td>
</tr>
<tr>
<td>C14-vancomycin (12)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>2</td>
</tr>
<tr>
<td>CBP C1-vancomycin (15)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>0.5</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>31</td>
</tr>
<tr>
<td>CBP-aminomethylene vancomycin (8)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>0.06</td>
</tr>
<tr>
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<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>0.16</td>
</tr>
<tr>
<td>CBP C1-aminomethylene vancomycin (18)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>0.005</td>
</tr>
</tbody>
</table>

Fig. 8. Examination of cell wall permeability induced by compounds 14–18 (10 μM added at 5 min) in VanA VRE (E. faecium ATCC BAA-2317).

Fig. 9. Summary of mechanisms of action of key vancomycin analogs and their individual and cumulative effect on VanA VRE antimicrobial activity.

modifications that provide one or two additional mechanisms of action. Thus, the durability of the antimicrobial activity in such challenges and the effectiveness of each individual mechanism of action as well as the compound potency were found to follow now predictable trends (three > two > one mechanisms of action). Most striking, resistance to daptomycin, linezolid, and tigecycline, each of which is now a frontline single-target antibiotic, arises much faster and is much more pronounced, highlighting the exceptional durability of the antimicrobial activity detailed for 8, 13, and especially 18. Finally, within the series examined, CBP–vancomycin (5) is representative of the expected behavior of the clinically approved semisynthetic vancomycin analogs.

The key compounds in the series were examined for in vitro toxicity that might result from the combined mechanisms of action, especially the introduction of structural modifications (quaternary ammonium salt) that might impact host as well as bacterial cell wall integrity. The compounds were examined for RBC hemolytic activity resulting from membrane lysis. No compound in the series, including 18, exhibited any hemolytic activity, even at concentrations >1,000-fold above their MICs (SI Appendix, Fig. S7). In addition, the mammalian cell toxicity of 5, 12, 13, 15, and 18 was established in cell growth inhibition assays against two mammalian cell lines, NIH/3T3 (ATCC CRL-1658; mouse embryonic fibroblast) and HepG2 (ATCC HB-8065; human liver cancer cell line). No growth inhibition (cytotoxic activity) was observed up to 100 μM, the highest dose tested. They were also found to be inactive (>100 μM; highest dose tested) against HCT116 (human colon cancer cell line). No growth inhibition (cytotoxic activity) was observed up to 100 μM, the highest dose tested. They were also found to be inactive (>100 μM; highest dose tested) against HCT116 (human colon cancer cell line). Finally, no correlation in activity with cLogP was found for the series of compounds studied, and none of the compounds (5, 12, 13, 15, 17, and 18) exhibited aggregation, higher-order complex formation, or micelle formation at concentrations up to 100 μM in PBS buffer (25 °C), indicating that such effects are not playing a role in the expression of the activity of the compounds. Compound 18 also failed to produce bacterial cell membrane depolarization in the same VanA vancomycin-resistant E. faecium (ATCC BAA-2317) as measured by fluorescence of a released membrane imbedded dye (DiSC3-5,3′-dipropylthiadicarbocyanine iodide) (SI Appendix, Fig. S4) (56). Because the C1 introduction is such a small and seemingly benign structural modification and because it induces membrane permeability without membrane depolarization or cell wall lysis, it suggests a more specific mechanism of action. The mechanism by which 15 and 18 exert their effects on membrane permeability is currently under investigation.

Conclusions

Several programs have disclosed the development of antibiotic products that act by two mechanisms of action. These efforts have included the optimization of a single pharmacophore to independently bind two related targets (e.g., fluoroquinolones targeting both bacterial DNA gyrase and topoisomerase IV); the design of hybrids of two antimicrobial pharmacophores, including the covalent linkage of two antibiotics; the use of combinations of single-target antimicrobials to overcome or avoid resistance (e.g., combination drug treatment of multidrug-resistant TB); and the design of antibiotics that display additional drug target binding contacts to enhance the robustness of target engagement and decrease resistance susceptibility (57). Herein, we described a complementary approach, perhaps a subset of one of these approaches, to design durable antibiotics endowed with multiple synergistic mechanisms of action. This effort has provided prototype antibiotics with three independent mechanisms of action.

Summary of resistance development study against VRE, VanA E. faecium (ATCC BAA 2317)

<table>
<thead>
<tr>
<th>Compound</th>
<th>initial MIC</th>
<th>MIC² after 25 passages</th>
<th>fold-increase in MIC</th>
<th>MIC³ after 50 passages</th>
<th>fold-increase in MIC</th>
<th>number of distinct effective mechanisms⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBP–vancomycin (5)</td>
<td>2.5</td>
<td>40</td>
<td>16</td>
<td>160</td>
<td>64</td>
<td>1</td>
</tr>
<tr>
<td>C14–vancomycin (12)</td>
<td>2</td>
<td>16</td>
<td>8</td>
<td>256</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>C14-aminomethylene vancomycin (13)</td>
<td>0.16</td>
<td>0.64</td>
<td>4</td>
<td>2.5</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>CBP-aminomethylene vancomycin (8)</td>
<td>0.06</td>
<td>0.12</td>
<td>2</td>
<td>0.5</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>CBP C1-aminomethylene vancomycin (18)</td>
<td>0.005</td>
<td>0.005</td>
<td>0</td>
<td>0.02</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

⁵VanA E. faecium (ATCC BAA 2317), μg/mL. Number excludes mechanism of D-Ala-D-Ala binding ineffective against VanA VRE.

Fig. 10. Resistance acquisition on serial passaging of VanA VRE in the presence of 0.5x MIC levels of compound. One of two replicate experiments.
targeting VRE, for which vancomycin is ineffective. Because VRE is already vancomycin-resistant and because many have already reached a point where they are no longer susceptible to most other antibiotic classes, the CDC recently placed VRE on its serious threat list, and the WHO placed it fourth on its list of drug-resistant bacteria that pose the greatest threat to human health. The glycopeptide antibiotics constitute an antibiotic class already endowed with features that avoid many mechanisms of resistance (14). With an understanding of the molecular basis of bacterial resistance to the glycopeptide antibiotics, binding pocket modifications designed for dual ligand binding reinstated binding to the altered target D-Ala-D-Lac and maintained binding affinity for the unaltered target D-Ala-D-Ala. These modifications were found to reestablish antimicrobial activity against vancomycin-resistant organisms that use the altered d-Ala-d-Lac peptidoglycan precursor targets and remain active against vancomycin-sensitive bacteria that use only D-Ala-D-Ala precursors. There is reason to expect that these solutions to VanA and VanB VRE resistance alone may provide antibiotics with durable clinical lifetimes, perhaps approaching those of vancomycin itself (>50 y). Subsequent to these studies, we have explored the peripheral structural changes in the molecules that provide them with additional and now multiple synergistic mechanisms of action. Complementary to our initial disclosure with a carbohydrate CBP modification that produced a 100-fold increase in antimicrobial activity (48), a second peripheral modification at the C terminus of the pocket-modified analogs was detailed herein that enhances antimicrobial activity (200-fold) against VanA VRE by another additional mechanism of action (induced membrane permeability). These two peripheral modifications and their synergistic mechanisms of action were then combined with the pocket modification to provide a vancomycin analog endowed with three independent mechanisms of action, only one of which is dependent on D-Ala-D-Ala/D-Ala-D-Lac binding. This combination not only further increased the antimicrobial potency against VanA VRE (>6,000-fold) but also, reduced the susceptibility to resistance. Thus, the durability of the antimicrobial activity in a resistance challenge and the robustness of each individual mechanism of action as well as the compound potency were shown to follow now predictable trends (three > two > one mechanisms of action). Most striking, resistance to the front-line antibiotics daptomycin, linezolid, and tigecycline, some of which are regarded as durable by today’s standards, was found to arise much faster and more pronounced in the same resistance challenge, highlighting the exceptional durability of the antimicrobial activity detailed for 8, 13, and especially 18 (Fig. 11).

An important question that these results raise is presently what to do with conventional semisynthetic vancomycin analogs active against VanA VRE that incorporate a single peripheral modification and act by a single mechanism of action that is independent of D-Ala-D-Lac binding [e.g., CBP-vancomycin (5), oritavancin, and C14-vancomycin (12)]. Should their use be encouraged for VRE but at the risk of raising resistance to this otherwise effective approach for other challenging bacterial infections (e.g., MRSA)? The answer would seem to be to encourage their use for challenging vancomycin-sensitive bacterial infections (e.g., VSSA and MRSA), where they are not only more potent than vancomycin but also benefit from two independent mechanisms of action. Clinical resistance or loss in sensitivity to either mechanism would likely be slow to emerge and slower than for vancomycin itself. However, their use against vancomycin-resistant bacteria (e.g., VRE and VRSA), where they are less potent and where only a single and less durable mechanism of action remains operative, likely would more rapidly raise resistance, not only compromising its future use but also, potentially transferring that resistance to other organisms (e.g., MRSA).

The approach used herein, which we suggest represents a case of durable antibiotic discovery by design, relied on the total synthesis of the candidate antibiotics (58–60) to obtain the previously inaccessible compounds. Although not highlighted in the preceding discussion, the total synthesis of the starting pocket-modified aglycon(s) (26 steps) (48), enzymatic installation of the disaccharide (2 steps) (58), and subsequent addition of the two peripheral modifications (2 steps) represent remarkable accomplishments in their own right. Finally, the work herein was conducted with the aminomethylene analog of vancomycin, in which the residue 4 amide carbonyl was removed. A more potent pocket-modified vancomycin analog is the residue 4 amidine (3 vs. 4), which exhibits antimicrobial activity against both vancomycin-resistant and -sensitive bacteria equipotent with the activity that vancomycin displays against vancomycin-sensitive bacteria. Incorporation of such peripheral changes on 3 or 7, providing all three independent mechanisms of action, would be expected to further improve on the already stunning potency of 18 (ca. 30-fold) while displaying the outstanding durability of 18.

Materials and Methods

Synthesis of Vancomycin Analogs. Full details of the synthesis, purification, and characterization of all compounds reported herein, including copies of the 1H NMR of all tested compounds, are provided in SI Appendix. All reagents were obtained from commercial sources unless noted otherwise.

Bacterial Cell Growth Inhibition Assays. Full details of the bacterial cell growth inhibition assays with vancomycin-resistant E. faecalis (VanA VRE; BM166), E. faecium (VanA VRE; ATCC BAA-2317), and vancomycin-resistant E. faecalis (VanB VRE; strain ATCC 51299) are provided in SI Appendix. Compounds were tested in duplicate (n = 2–18 times) at serially diluted concentrations, and the MIC values reported represent the average of 4–36 determinations.

Cell Wall Biosynthesis Inhibition Assay. Full details of the cell wall biosynthesis assay are provided in SI Appendix. Values reported in Fig. 6 are the average of four measurements (SD ± 10%).

Bacterial Cell Wall Permeability Assay. Full details of the permeability assay are provided in SI Appendix. Results shown in Figs. 7–9 are one of two to four replicate experiments, each performed at the same time.

Resistance Development Study. Full details of the study are provided in SI Appendix. Results shown in Fig. 10 are one of two replicate experiments.

SI Appendix. Full experimental details and copies of 1H NMR spectra are provided. The supplementary data associated with this article can be found in SI Appendix.
18. Bugg TDH, et al. (1991) Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: Biosynthesis of a depsipeptide peptidoglycan precursor by vanco-
23. Baltz RH, Miao V, Wrigley SK (2005) Natural products to drugs: Daptomycin and re-
Peripheral Modifications of \( \Psi[\text{CH}_2\text{NH}] \text{Tpg}^4 \) Vancomycin with Added Synergistic Mechanisms of Action Provide Durable and Potent Antibiotics

Akinori Okano, Nicholas A. Isley and Dale L. Boger

Department of Chemistry and the Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037

For compounds 9-18: Rapidly exchanging amide NH signals and OH signals are absent in \(^1\text{H} \) NMR spectra taken in CD\(_3\)OD or D\(_2\)O and often broadened in \(^1\text{H} \) NMR spectra taken in DMSO-\( d_6 \). This leads to a lower proton count in the \(^1\text{H} \) NMR spectra listings and in the presented spectra.

**Compound C14:** A solution of \( \text{S1} \) (300 mg, 1.5 mmol) in anhydrous EtOH (4 mL) was treated with \( \text{C14} \)H\(_2\)Br (0.74 g, 3.0 mmol) at 25 °C and the reaction mixture was stirred at 70 °C for 2 h. The reaction mixture was cooled to 25 °C and the solvent was removed under a stream of N\(_2\). The residue was purified by flash chromatography (SiO\(_2 \), 5–15% MeOH/CH\(_2\)Cl\(_2 \)) to afford the corresponding ammonium salt as a yellow oil. This oil was treated with conc. HCl/MeOH = 1/5 (2 mL) at 25 °C and the mixture was stirred at 25 °C for 3 h. The solvent and HCl were removed under a stream of N\(_2\) to afford \( \text{C14} \) (271 mg, 61%, 2 steps) as a white solid identical in all respects with authentic material (\(^1\text{H} \) NMR, D\(_2\)O).

**Compound Cyclic C5:** A solution of \( \text{S2} \) (300 mg, 2.4 mmol) in anhydrous THF (3 mL) was treated with Boc\(_2\)O (510 mg, 2.3 mmol) at 25 °C and the reaction mixture was stirred at 25
°C for 5 min. The solvent was removed under a stream of N\textsubscript{2} to afford the crude Boc protected amine as a colorless oil. This oil was dissolved in anhydrous CH\textsubscript{2}Cl\textsubscript{2} (2 mL) and treated with MeI (3.3 g, 23.4 mmol) at 25 °C and the reaction mixture was stirred at 60 °C for 10 min. The reaction mixture was cooled to 25 °C and the solvent and MeI were removed under a stream of N\textsubscript{2}. The residue was purified by flash chromatography (SiO\textsubscript{2}, 5–20% MeOH/CH\textsubscript{2}Cl\textsubscript{2}) to afford the corresponding ammonium salt as a yellow oil. This oil was treated with TFA/CH\textsubscript{2}Cl\textsubscript{2} (1/1, 2 mL) at 25 °C and the reaction mixture was stirred at 25 °C for 1 h. The solvent and TFA were removed under a stream of N\textsubscript{2} to afford Cyclic C5 (154 mg, 46%, 3 steps) as a yellow oil: \textsuperscript{1}H NMR (D\textsubscript{2}O, 600 MHz, 298 K) δ 3.50–3.39 (m, 4H), 3.38–3.34 (m, 2H), 2.97 (s, 3H), 2.94 (t, 2H, J = 7.2 Hz), 2.15–2.05 (m, 6H); ESI-TOF HRMS m/z 143.1547 (M + H\textsuperscript{+}, C\textsubscript{8}H\textsubscript{19}N\textsubscript{2} requires 143.1548).

**Compound 9:** A solution of 1 (2.0 mg, 1.4 μmol) in DMF/DMSO (1/1, 60 μL) was treated with C0 (1 M in DMF/DMSO = 1/1, 7.0 μL, 7.0 μmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 41.2 μL, 41.2 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 27.6 μL, 27.6 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H\textsubscript{2}O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 1–40% MeCN/H\textsubscript{2}O–0.07% TFA gradient over 40 min, 3 mL/min, t\textsubscript{R} = 20.8 min) to afford 9 (1.4 mg, 64%) as a white amorphous solid identical in all respects with authentic material (\textsuperscript{1}H NMR, D\textsubscript{2}O).\textsuperscript{51}
**Compound 10**: A solution of 1 (1.5 mg, 1.0 μmol) in DMF/DMSO (1/1, 100 μL) was treated with 10 (1 M in DMF/DMSO = 1/1, 5.2 μL, 5.2 μmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 31.2 μL, 31.2 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 20.8 μL, 20.8 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H₂O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 1–40% MeCN/H₂O–0.07% TFA gradient over 40 min, 3 mL/min, tᵣ = 20.6 min) to afford 10 (1.1 mg, 68%) as a white film: ¹H NMR (DMSO-d₆, 600 MHz, 298 K) δ 9.43 (br s, 1H), 9.02 (s, 1H), 8.69 (s, 1H), 8.56 (s, 1H), 8.20 (s, 1H), 7.82 (s, 1H), 7.76 (s, 1H), 7.73 (s, 1H), 7.58 (d, 2H, J = 8.4 Hz), 7.47 (d, 2H, J = 9.0 Hz), 7.37 (d, 1H, J = 8.4 Hz), 7.31 (d, 1H, J = 8.4 Hz), 7.19 (d, 1H, J = 8.4 Hz), 7.02 (br s, 1H), 6.88 (d, 1H, J = 10.8 Hz), 6.78 (d, 1H, J = 8.4 Hz), 6.71 (d, 1H, J = 8.4 Hz), 6.39 (s, 1H), 6.22 (s, 1H), 5.76 (s, 1H), 5.57 (s, 1H), 5.36 (s, 1H), 5.26 (d, 1H, J = 7.8 Hz), 5.23 (s, 1H), 5.20 (s, 1H), 4.96 (s, 1H), 4.69 (d, 1H, J = 7.8 Hz), 4.48 (s, 1H), 4.28 (s, 1H), 4.25 (d, 1H, J = 5.4 Hz), 3.96 (s, 1H), 3.69 (s, 1H), 3.67 (s, 1H), 3.60–3.40 (m, 5H), 3.27 (s, 1H), 3.25–3.10 (m, 4H), 3.09–3.05 (m, 2H), 3.00 (s, 9H), 2.69 (s, 6H), 2.66 (s, 1H), 2.25–2.10 (m, 1H), 1.91 (d, 1H, J = 11.4 Hz), 1.85 (s, 2H), 1.74 (d, 1H, J = 13.2 Hz), 1.69–1.64 (m, 1H), 1.59–1.51 (m, 2H), 1.30 (s, 3H), 1.07 (d, 3H, J = 6.0 Hz), 0.90 (d, 3H, J = 6.0 Hz), 0.85 (d, 3H, J = 6.0 Hz); ESI-TOF HRMS m/z 774.2867 ([M + 2H]⁺², C₇₂H₉₀Cl₂N₁₁O₂₃ requires 774.2861).
**Compound 11:** A solution of 1 (1.8 mg, 1.1 µmol) in DMF/DMSO (1/1, 50 µL) was treated with **Cyclic C5** (1 M in DMF/DMSO = 1/1, 5.5 µL, 5.5 µmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 32.7 µL, 32.7 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 21.8 µL, 21.8 µmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H₂O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10 × 150 mm, 1–40% MeCN/H₂O–0.07% TFA gradient over 40 min, 3 mL/min, tR = 20.4 min) to afford 11 (1.2 mg, 61%) as a white film: 

1H NMR (DMSO-d6, 600 MHz, 298 K) δ 9.14 (br s, 2H), 8.99 (s, 1H), 8.80–8.65 (m, 1H), 8.56 (s, 1H), 8.18 (s, 1H), 7.85–7.81 (m, 1H), 7.67 (s, 3H), 7.60–7.45 (m, 4H), 7.35 (dd, 1H, J = 6.6, 3.0 Hz), 7.30 (s, 1H), 7.25 (d, 1H, J = 8.4 Hz), 7.20 (d, 1H, J = 7.8 Hz), 7.08 (br s, 1H), 6.85 (d, 1H, J = 11.4 Hz), 6.78 (d, 1H, J = 8.4 Hz), 6.70 (d, 1H, J = 8.4 Hz), 6.38 (d, 1H, J = 2.4 Hz), 6.22 (d, 1H, J = 2.4 Hz), 5.77 (d, 1H, J = 7.8 Hz), 5.58 (d, 1H, J = 13.2 Hz), 5.35–5.15 (m, 6H), 4.93 (br s, 1H), 4.69 (t, 1H, J = 5.4 Hz), 4.49 (d, 2H, J = 5.4 Hz), 4.27 (d, 2H, J = 5.4 Hz), 4.00–3.90 (m, 1H), 3.35–3.25 (m, 3H), 3.24–3.15 (m, 3H), 3.11–3.02 (m, 1H), 2.93 (s, 3H), 2.67–2.60 (m, 4H), 2.54 (s, 1H), 2.20–2.00 (m, 5H), 1.95–1.83 (m, 3H), 1.80–1.50 (m, 4H), 1.29 (d, 3H, J = 13.8 Hz), 1.07 (d, 3H, J = 6.0 Hz), 0.91 (d, 3H, J = 6.6 Hz), 0.86 (d, 3H, J = 6.6 Hz). Note: additional peaks in the spectrum are buried under a broad singlet due to H₂O. ESI-TOF HRMS m/z 787.2922 ([M + 2H]⁺², C₇₄H₉₂Cl₂N₁₁O₂₃ requires 787.2948).
**Compound 12:** A solution of 1 (4.0 mg, 2.8 μmol) in DMF/DMSO (1/1, 150 μL) was treated with C14 (1 M in DMF/DMSO = 1/1, 13.8 μL, 13.8 μmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 82.8 μL, 82.8 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 55.2 μL, 55.2 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H2O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 20–80% MeCN/H2O–0.07% TFA gradient over 30 min, 3 mL/min, tR = 21.2 min) to afford 12 (2.8 mg, 58%) as a white amorphous solid identical in all respects with authentic material (1H NMR, DMSO-d6).51

Note: This reaction was run on scales of 0.5–10 mg (51–63%) during the optimization of conditions (Table S1). Compounds 13 and 18 were synthesized on 0.7 mg and 0.25 mg scales with respect to their starting material.
Table S1. Optimization of reaction conditions for the coupling of vancomycin (1) and C14.

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<td>5 min</td>
<td>63%</td>
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Experimental for the total synthesis of $4^{3,48}$ has been previously disclosed.

**Compound 13:** A solution of 4 (0.69 mg, 0.48 μmol) in DMF/DMSO (1/1, 30 μL) was treated with C14 (1 M in DMF/DMSO = 1/1, 2.4 μL, 2.4 μmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 14.4 μL, 14.4 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 9.6 μL, 9.6 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H$_2$O (0.2 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 20–80% MeCN/H$_2$O–0.07% TFA gradient over 30 min, 3 mL/min, $t_R = 18.4$ min) to afford 13 (0.53 mg, 64%, typically 61–67%) as a white film: $^1$H NMR (CD$_3$OD, 600 MHz, 298 K) $\delta$ 8.85–8.80 (m, 1H), 8.50–8.45 (m, 1H), 7.98 (s, 1H), 7.82 (d, 1H, $J = 8.7$ Hz), 7.41 (d, 1H, $J = 9.0$ Hz), 7.31 (d, 1H, $J = 2.4$ Hz), 6.93 (d, 1H, $J = 8.4$ Hz), 6.46 (d, 1H, $J = 2.4$ Hz), 6.33 (d, 1H, $J = 2.4$ Hz), 5.54 (d, 1H, $J = 3.0$ Hz), 5.44 (d, 1H, $J = 7.8$ Hz), 5.41 (d, 2H, $J = 2.4$ Hz), 5.37 (d, 1H, $J = 5.4$ Hz), 4.57–4.54 (m, 1H), 4.43–4.31 (m, 2H), 4.29–4.19 (m, 1H), 4.18–3.98 (m, 1H), 3.88–3.70 (m, 3H), 3.69–3.59 (m, 2H), 3.58–3.49 (m, 2H), 3.40–3.32 (m, 4H), 3.29–3.24 (m, 5H), 3.05 (d, 5H, $J = 3.6$ Hz), 3.00 (s, 3H), 2.86 (s, 3H), 2.79 (s, 3H), 2.66 (br s, 9H), 2.64–2.56 (m, 1H), 2.33–2.25 (m, 1H), 2.10–1.92 (m, 5H), 1.83–1.70 (m, 3H), 1.67–1.57 (m, 2H), 1.54 (s, 3H), 1.45–1.25 (m, 23H), 1.20 (d, 3H, $J = 6.6$ Hz), 0.95 (d, 3H, $J = 7.2$ Hz), 0.93–0.87 (m, 6H); ESI-TOF HRMS m/z 857.8965 ([M + 2H]$^{2+}$, C$_{85}$H$_{117}$Cl$_2$N$_{11}$O$_{22}$ requires 857.8948).
Experimental for the synthesis of CBP vancomycin 5\textsuperscript{48} has been previously disclosed.

**Compound 14:** A solution of 5 (2.0 mg, 1.2 \( \mu \)mol) in DMF/DMSO (1/1, 100 \( \mu \)L) was treated with C0 (1 M in DMF/DMSO = 1/1, 6.1 \( \mu \)L, 6.1 \( \mu \)mol), \( N \)-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 36.4 \( \mu \)L, 36.4 \( \mu \)mol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 24.3 \( \mu \)L, 24.3 \( \mu \)mol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H\(_2\)O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 \( \mu \)m, 10 \( \times \) 150 mm, 20–80% MeCN/H\(_2\)O–0.07% TFA gradient over 30 min, 3 mL/min, \( t_R = 13.1 \) min) to afford 14 (1.2 mg, 55%) as a white film: \(^1\)H NMR (CD\(_3\)OD, 600 MHz, 298 K) \( \delta \)9.20–8.99 (m, 1H), 8.73 (s, 1H), 8.48–8.38 (m, 1H), 8.01–7.87 (m, 1H) 7.77–7.41 (m, 13H), 7.32–7.30 (br s, 1H), 7.27 (d, 1H, \( J = 9.0 \) Hz), 7.21–7.16 (m, 1H), 7.10 (br s, 1H), 7.04–6.90 (m, 1H), 6.86–6.79 (br s, 1H), 6.53–6.29 (m, 3H), 5.80 (s, 1H), 5.58–5.29 (m, 5H), 4.60–4.53 (m, 1H), 4.43–3.98 (m, 5H), 3.93–3.36 (m, 9H), 3.21–3.10 (m, 2H), 3.02–2.98 (m, 1H), 2.93–2.87 (m, 6H), 2.86 (br s, 1H), 2.82 (br s, 1H), 2.78 (s, 3H), 2.74 (br s, 1H), 2.66 (s, 2H), 2.21–2.13 (m, 1H), 2.10–1.93 (m, 3H), 1.90–1.80 (m, 1H), 1.78–1.69 (m, 2H), 1.66 (s, 3H), 1.40–1.22 (m, 6H), 1.00 (d, 3H, \( J = 7.2 \) Hz), 0.96 (d, 3H, \( J = 7.2 \) Hz); ESI-TOF HRMS \( m/z \) 866.7940 ([M + 2H]\textsuperscript{2+}, \( C_{74}H_{96}Cl_{13}N_{11}O_{23} \) requires 866.7952).
**Compound 15**: A solution of 5 (1.4 mg, 0.85 μmol) in DMF/DMSO (1/1, 100 μL) was treated with C1 (1 M in DMF/DMSO = 1/1, 4.3 μL, 4.3 μmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 25.5 μL, 25.5 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 17.0 μL, 17.0 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H2O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 20–80% MeCN/H2O–0.07% TFA gradient over 30 min, 3 mL/min, τR = 13.7 min) to afford 15 (0.91 mg, 61%) as a white film: ¹H NMR (CD₃OD, 600 MHz, 298 K) δ 9.15–8.95 (m, 1H), 8.80–8.70 (m, 1H), 8.45–8.35 (m, 1H), 7.78–7.40 m, 13H), 7.38–7.06 (m, 3H), 7.05–6.95 (m, 1H), 6.82 (d, 1H, J = 8.4 Hz), 6.46 (d, 1H, J = 2.4 Hz), 6.38 (s, 2H), 5.85–5.75 (m, 1H), 5.57–5.30 (m, 5H), 4.62–4.51 (m, 1H), 4.40–4.23 (m, 2H), 4.23–4.03 (m, 4H), 3.93–3.80 (m, 1H), 3.78–3.70 (m, 1H), 3.68–3.40 (m, 5H), 3.22–3.02 (m, 12H), 2.99 (s, 1H), 2.86 (s, 1H), 2.83–2.73 (m, 4H), 2.66 (s, 3H), 2.23–2.14 (m, 1H), 2.10–2.00 (m, 3H), 1.87–1.60 (m, 6H), 1.33–1.25 (m, 4H), 1.05–0.93 (m, 6H); ESI-TOF HRMS m/z 873.8042 ([M + 2H]⁺², C₈₅H₉₈Cl₃N₁₁O₂₃ requires 873.8027).
Compound 16: A solution of 5 (1.8 mg, 1.1 µmol) in DMF/DMSO (1/1, 100 µL) was treated with Cyclic C5 (1 M in DMF/DMSO = 1/1, 5.5 µL, 5.5 µmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 21.8 µL, 21.8 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 32.7 µL, 32.7 µmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H2O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10 × 150 mm, 20–80% MeCN/H2O–0.07% TFA gradient over 30 min, 3 mL/min, tR = 12.7 min) to afford 16 (1.2 mg, 61%) as a white film: 1H NMR (CD3OD, 600 MHz, 298 K, rotamers (4:1)) δ 9.04 (s, 0.25H), 8.74 (s, 1H), 8.35 (s, 0.25H), 8.00 (s, 1H), 7.76–7.56 (m, 18H), 7.50–7.48 (m, 4H), 7.30 (d, 1H, J = 9.0 Hz), 7.30 (d, 1H, J = 9.0 Hz), 7.13–7.09 (m, 1.25H), 7.11 (s, 1H), 6.83 (d, 1H, J = 9.0 Hz), 6.48–6.46 (m, 1.25H), 6.40–6.39 (m, 1.25H), 5.84 (br s, 1H), 5.54 (d, 1H, J = 7.8 Hz), 5.49 (d, 1H, J = 4.8 Hz), 5.42 (s, 1H), 5.37–5.34 (m, 2.5H), 4.59 (dd, 0.25H, J = 6.0, 6.0 Hz), 4.37–4.31 (m, 1H), 4.27–4.17 (m, 5H), 4.15–4.04 (m, 4.25H), 3.93–3.86 (m, 1.75H), 3.82–3.75 (m, 1.25H), 3.72 (s, 1H), 3.71–3.64 (m, 3H), 3.59–3.47 (m, 9H), 3.45–3.42 (m, 2.5H), 3.40–3.35 (m, 2H), 3.25–3.21 (m, 1H), 3.18 (d, 1H, J = 2.4 Hz), 3.16–3.12 (m, 1H), 3.09–3.06 (m, 5.5H), 3.04–3.02 (m, 4.25H), 2.97–2.93 (m, 3.5H), 2.89 (s, 3H), 2.82–2.77 (m, 5H), 2.68 (s, 6H), 2.30–2.18 (m, 8.5H), 2.12–2.04 (m, 4.25H), 1.91–1.85 (m, 1.5H), 1.83–1.76 (m, 2H), 1.74–1.65 (m, 3H), 1.42–1.27 (m, 3H), 1.09–0.95 (m, 6H); ESI-TOF HRMS m/z 886.8121 ([M + 2H]2+, C87H101Cl3N11O23 requires 886.8103).
**Compound 17:** A solution of 5 (2.1 mg, 1.3 µmol) in DMF/DMSO (1/1, 100 µL) was treated with C14 (1 M in DMF/DMSO = 1/1, 6.4 µL, 6.4 µmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 38.3 µL, 38.3 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 25.5 µL, 25.5 µmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 min and quenched with the addition of 50% MeOH in H2O (0.2 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10 × 150 mm, 20–80% MeCN/H2O–0.07% TFA gradient over 30 min, 3 mL/min, *tR* = 18.4 min) to afford 17 (1.9 mg, 76%) as a white film: ¹H NMR (CD₃OD, 600 MHz, 298 K, rotamers (2:1)) δ 9.16 (s, 0.5H), 9.09 (s, 0.5H), 8.74 (s, 1H), 8.40 (s, 1H), 7.98 (s, 0.5H), 7.89 (s, 0.5H), 7.80–7.53 (m, 11H), 7.51 (d, 1H, *J* = 7.8 Hz), 7.48–7.42 (m, 3H), 7.38–7.25 (m, 3H), 7.20 (d, 0.5H, *J* = 8.4 Hz), 7.17–7.12 (m, 2H), 7.04 (d, 0.5H, *J* = 2.4 Hz), 6.91 (s, 0.5H), 6.83 (d, 1H, *J* = 8.4 Hz), 6.51 (s, 0.5H), 6.47 (s, 1H), 6.41 (s, 0.5H), 6.37 (s, 1H), 6.29 (d, 0.5H, *J* = 7.8 Hz), 5.81 (br s, 1H), 5.65 (s, 0.5H), 5.58–5.51 (m, 1H), 5.48–5.38 (m, 2H), 5.35 (s, 1H), 5.22 (d, 0.5H, *J* = 2.4 Hz), 5.01 (s, 0.5H), 4.70 (s, 1H), 4.62 (s, 0.5H), 4.60–4.52 (m, 2H), 4.36 (s, 0.5H), 4.28 (s, 1H), 4.19–4.06 (m, 5H), 3.98 (d, 0.5H, *J* = 10.8 Hz), 3.92–3.80 (m, 1.5H), 3.78–3.71 (m, 2H), 3.63–3.59 (m, 2H), 3.57–3.51 (m, 3H), 3.47 (s, 1H), 3.43–3.38 (m, 1.5H), 3.12–3.06 (m, 11H), 3.02–3.00 (m, 8H), 2.92 (d, 1H, *J* = 2.4 Hz), 2.89 (s, 1H), 2.87 (s, 0.5H), 2.83–2.79 (m, 4H), 2.66 (s, 6H), 2.22–2.18 (m, 2H), 2.16–2.10 (m, 0.5H), 2.07–1.99 (m, 4H), 1.95–1.83 (m, 2H), 1.81–1.62 (m, 12H), 1.44–1.19 (m, 41H), 1.09–0.96 (m, 3.5H), 0.93–0.88 (m, 7H), 0.81 (d, 1H, *J* = 4.2 Hz), 0.71 (d, 1H, *J* = 3.6 Hz); ESI-TOF HRMS *m/z* 1928.7987 (M⁺, C₉₅H₁₂₅Cl₃N₁₁O₂₃ requires 1928.8010).
Experimental for the total synthesis of \( \mathbf{8} \) has been previously disclosed.

**Compound 18:** A solution of \( \mathbf{8} \) (0.24 mg, 0.15 \( \mu \text{mol} \)) in DMF/DMSO (1/1, 20 \( \mu \text{L} \)) was treated with \( \mathbf{C1} \) (0.1 M in DMF/DMSO = 1/1, 7.4 \( \mu \text{L} \), 0.74 \( \mu \text{mol} \)), \( N \)-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 4.5 \( \mu \text{L} \), 4.5 \( \mu \text{mol} \)), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 3.0 \( \mu \text{L} \), 3.0 \( \mu \text{mol} \)) at 25°C. The reaction mixture was stirred at 25°C for 5 min and quenched with the addition of 50% MeOH in \( \text{H}_2\text{O} \) (0.5 mL) at 25°C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 \( \mu \text{m} \), 10 × 150 mm, 20–80% MeCN/H\( \text{H}_2\text{O} \)–0.07% TFA gradient over 30 min, 3 mL/min, \( t_R \) = 12.9 min) to afford \( \mathbf{18} \) (0.14 mg, 53%) as a white film: \( ^1\text{H} \) NMR (CD\( _3 \)OD, 600 MHz, 298 K) \( \delta \) 9.20–9.05 (m, 1H), 9.01 (s, 1H), 8.64–8.56 (m, 1H), 7.98 (s, 1H), 7.80–7.55 (m, 10H), 7.47 (d, 2H, \( J = 10.2 \) Hz), 7.37–7.31 (m, 1H), 7.29 (d, 1H, \( J = 9.0 \) Hz), 7.25 (s, 1H), 7.09 (d, 1H, \( J = 9.6 \) Hz), 6.86 (d, 1H, \( J = 9.0 \) Hz), 6.84 (s, 1H), 6.73 (d, 1H, \( J = 2.4 \) Hz), 6.47 (s, 1H), 6.39–6.35 (m, 1H), 5.85–5.75 (m, 2H), 5.55–5.40 (m, 3H), 5.35–5.25 (m, 1H), 4.26 (br s, 2H), 4.20–4.05 (m, 4H), 3.95–3.80 (m, 2H), 3.79–3.70 (m, 2H), 3.68–3.55 (m, 3H), 3.54–3.40 (m, 7H), 3.19 (s, 1H), 3.11 (s, 1H), 3.05 (s, 3H), 3.03–2.95 (m, 7H), 3.00 (s, 3H), 2.92 (s, 1H), 2.87 (s, 2H), 2.76 (s, 3H), 2.66 (s, 4H), 2.30–2.15 (m, 6H), 2.14–2.01 (m, 4H), 1.87–1.73 (m, 2H), 1.68 (s, 3H), 1.26 (d, 3H, \( J = 7.2 \) Hz), 1.03 (s, 3H, \( J = 7.2 \) Hz), 0.99 (s, 3H, \( J = 7.2 \) Hz); ESI-TOF HRMS \( m/z \) 866.8129 ([M + 2H]\( ^2 \), \( \text{C}_{85}\text{H}_{102}\text{Cl}_3\text{N}_{11}\text{O}_{22} \) requires 866.8130).
**In vitro antimicrobial assays**

One day before experiments were run, fresh cultures of vancomycin-sensitive *Staphylococcus aureus* (VSSA strain ATCC 25923), methicillin and oxacillin-resistant *Staphylococcus aureus subsp. aureus* (MRSA strain ATCC 43300), vancomycin-resistant *Enterococcus faecalis* (VanA VRE, BM4166), *Enterococcus faecium* (VanA VRE, ATCC BAA-2317), vancomycin-resistant *Enterococcus faecalis* (VanB VRE, strain ATCC 51299), *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC BAA-1710), *Pseudomonas aeruginosa* (ATCC 15442), *Klebsiella pneumoniae* (ATCC 700603) were inoculated and grown in an orbital shaker at 37 °C in 100% Mueller-Hinton broth (VSSA, MRSA and VanB VRE), 100% brain-heart infusion broth (VanA VRE, *A. baumannii* and *K. pneumoniae*) or 100% Luria broth (*E. coli* and *P. aeruginosa*). After 24 h, the bacterial stock solutions were serial diluted with the culture medium (10% Mueller-Hinton broth for VSSA, MRSA and VanB VRE or 10% brain-heart infusion broth for VanA VRE *A. baumannii* and *K. pneumoniae* or 10% Luria Broth for *E. coli* and *P. aeruginosa*) to achieve a turbidity equivalent to a 1:100 dilution of a 0.5 M McFarland solution. This diluted bacterial stock solution was then inoculated in a 96-well V-shaped glass coated microtiter plate, supplemented with serial diluted aliquots of the antibiotic solution in DMSO (4 µL), to achieve a total assay volume of 0.1 mL. The plate was then incubated at 37 °C for 18 h, after which minimal inhibitory concentrations (MICs) were determined by monitoring the cell growth (observed as a pellet) in the wells. The lowest concentration of antibiotic (in µg/mL) capable of eliminating cell growth in the wells is the reported MIC value. The reported MIC values for the vancomycin analogues were determined against vancomycin as a standard in the first well.


For VanA *E. faecalis* (VanA VRE, BM 4166): resistant to erythromycin, gentamicin, chloramphenicol, and ciprofloxacin as well as vancomycin and teicoplanin; sensitive to
daptomycin.
For VanA *E. faecium* (VanA VRE, ATCC BAA-2317): resistant to ampicillin, benzylpenicillin, ciprofloxacin, erythromycin, levofloxacin, nitrofurantoin, and tetracycline as well as vancomycin and teicoplanin, insensitive to linezolid; sensitive to tigecycline and dalfopristine.

**Cell wall permeability assay** S2,S3

One day before experiments were run, cultures of vancomycin-resistant *Enterococcus faecalis* (VanA VRE, BM4166) and *Enterococcus faecium* (VanA VRE, ATCC BAA-2317), were inoculated and grown in an orbital shaker at 37 °C in 100% brain-heart infusion broth for 12 h. The above bacterial solution was subjected to a subculture to obtain fresh mid log phase bacterial cells (total volume of bacterial suspension = 7 mL, incubation time = 6 h, OD<sub>600</sub> = 0.6). After the cultured bacteria was harvested (3000 rpm, 4 °C, 20 min), the white bacterial precipitate was washed and resuspended in 5 mM glucose and 5 mM HEPES buffer (1:1, 500 µL, pH = 7.2). This bacterial suspension (130 µL) was charged in a 96-well black plate with a clear bottom (Corning 3650). The propidium iodide dye (10 µL, 150 µM DMSO solution) was added to the above suspension and the fluorescence was monitored at 25 °C for 5 min at 30 second intervals using a microplate reader (Molecular Devices<sup>®</sup>, Max Gemini EX) at an excitation wavelength of 535 nm and an emission wavelength of 617 nm. The test compound (150 µM, 10 µL) was added to the cell suspension and the fluorescence was monitored at 25 °C for an additional 15 min.

The impact of the structural modifications on cell wall permeability against both VanA VRE examined herein was also examined (Figure S1). Vancomycin (1), C14-vancomycin (12), CBP C1-vancomycin (15), and CBP C1-aminomethylene vancomycin (18) displayed similar induced permeabilities against both vancomycin-resistant *Enterococcus faecalis* (VanA VRE, BM4166) and *Enterococcus faecium* (VanA VRE, ATCC BAA-2317).

Figure S1. Examination of cell wall permeability against VanA *E. faecalis* BM4166 and *E. faecium* ATCC BAA-2317.

Figure S2. Examination of cell wall permeability induced by compounds 1–8 (10 μM added at 5 min) in VanA VRE (*E. faecium* ATCC BAA-2317).
Cell wall depolarization assay

One day before experiments were run, cultures of vancomycin-resistant *Enterococcus faecium* (VanA VRE, ATCC BAA-2317), were inoculated and grown in an orbital shaker at 37 °C in 100% Brain-Heart Infusion for 12 h. The above bacterial solution was subjected to subculture to obtain fresh mid log phase bacterial cells (total volume of bacterial suspension = 7 mL, incubation time = 6 h, OD$_{600}$ = 0.6). After cultured bacteria media was harvested (3000 rpm, 4 °C, 20 min), this bacterial precipitate was washed and resuspended in a mixture of 5 mM glucose, 5 mM HEPES, and 5 mM KCl buffer (1:1:1, pH = 7.2). This bacterial suspension (130 µL) was charged in a 96-well black plate (Corning 3650). The dye (DiSC$_3$:5: 3,3’-Dipropylthiadicarbocyanine iodide, 150 µM DMSO solution, 2.5 µL) was added to the above suspension, preloading the dye in the bacterial cell membrane. The fluorescence was monitored for 10 min at 1 min interval using a microplate reader (Molecular devices, Max Gemini EX) at an excitation wave length of 622 nm and an emission wave length of 670 nm. The test compound (150 µM, 10 µL) was added to the cell suspension and the fluorescence was monitored for a further 30 min.

Resistance development study

The MICs of the vancomycin analogues against vancomycin-resistant *Enterococcus faecalis* (VanA VRE, BM4166) and *Enterococcus faecium* (VanA VRE, ATCC BAA-2317) were determined (see page S13 for protocol). The bacterial suspension (40 μL) in the 96-well plate at sub-MIC concentration (MIC/2) was inoculated with 100% brain-heart infusion broth and the bacteria were grown in an orbital shaker at 37 °C for 6 h until the value of OD$_{600}$ became 0.6. A new MIC assay was performed with the same protocol (see S13). This process was repeated for 50 passages, and the fold increase in MIC was determined at each passage.


Cell wall biosynthesis inhibition assay

Cultures of vancomycin-resistant *Enterococcus faecalis* (VanA VRE, BM4166) and *Enterococcus faecium* (VanA VRE, ATCC BAA-2317) were inoculated and grown in an orbital shaker at 37 °C in 100% brain-heart infusion broth for 12 h. The above bacterial solution was subjected to a subculture to obtain fresh mid log phase bacterial cells (total volume of bacterial suspension = 5 mL, incubation time = 6 h, OD$_{600}$ = 0.6). Tetracycline (5 mg/mL, 130 μL) was added to the above bacterial suspension to ensure complete inhibition
of protein synthesis and incubated at 37 °C for 30 min. Vancomycin analogues were added and the mixture was incubated at 37 °C for a further 30 min. After the bacteria was harvested (3000 rpm, 4 °C, 20 min), this bacterial precipitate was washed and resuspended in 5 mM glucose and 5 mM HEPES buffer (500 μL, 1:1, pH = 7.2). This bacterial suspension was heated at 100 °C for 15 min and centrifuged (13000 rpm, 25 °C, 10 min). The entire volume of supernatant was directly purified by semi-preparative reverse-phase HPLC without further manipulation (Agilent Technologies, Zorbax SB-C18, 5 μm, 9.4 × 150 mm, 1–40% MeCN/H₂O–0.07% TFA gradient over 40 min, 3 mL/min, tᵣ = 11.9 min) to afford UDP Mur N-Ac depsipentapeptide (19) as a white film identical in all respects with authentic material (¹H NMR, D₂O). S7

The amount of UDP Mur N-Ac pentapeptide (19) was quantified by use of calibration curves (Figure S5 for E. faecalis BM4166, Figure S6 for E. faecium ATCC BAA-2317) based on the area under the curve (AUC).

Figure S5. Calibration curve (VRE, VanA E. faecalis BM4166).

Figure S6. Calibration curve (VRE, VanA E. faecium ATCC BAA-2317).

**Hemolysis assay**

The blood cells in pig whole blood (2 mL, Pel-Free Biologicals, non-sterile, sodium citrate) were harvested (3000 rpm, 4 °C, 20 min), and the red blood precipitate was washed and resuspended in phosphate buffer saline (pH 7.4). This diluted red blood cell stock solution (384 μL) was incubated with the antibiotic solution in DMSO (16 μL) in a 1 mL microtube to achieve the final concentration of the test compounds. The mixture was then incubated at 37 °C for 1 h. The solution was diluted with phosphate buffer saline (pH 7.4, 200 μL) at 25 °C and centrifuged (3000 rpm, 4 °C, 20 min). The supernatant (200 μL) was transferred to a microtiter plate. A positive control (0.2 % vol% Triton X-100, 100% total hemolysis) and the negative control (no antibiotic, 0% hemolysis) were prepared. \( A_{350} \) was measured using a microplate reader (Molecular Devices®, Max Gemini EX). The % hemolysis was determined by calculating the following equation shown below (eq. 1).

\[
\text{Hemolysis (\%)} = \frac{(A_{\text{test}} - A_{\text{zero}})}{(A_{\text{total}} - A_{\text{zero}})} \times 100 \quad \text{(eq. 1)}
\]

\( A_{\text{test}} \): Absorbance with test compound  
\( A_{\text{total}} \): Absorbance of 100% hemolysis  
\( A_{\text{zero}} \): Absorbance of 0% hemolysis


The key compounds in the series were examined for in vitro toxicity that might result from the combined mechanisms of action, especially the introduction of structural modifications (quaternary ammonium salt) that might impact host as well as bacterial cell wall integrity. The compounds were examined for red blood cell hemolytic activity, resulting from membrane lysis. Although the differences in mammalian and bacterial cell wall composition are extensive, including the more highly anionic composition of the bacterial cell wall responsible for a preferential and differential cation binding, lysis of mammalian cell membranes (red blood cells) are potential off-target consequences of cationic compounds that impact bacterial cell membrane integrity. The standard red blood cell hemolysis assay was conducted and measures the extent of red blood cell lysis after 1 h exposure to candidate compounds (pH 7.4, PBS, 37 °C, 1 h). No compound in the series, including 18, exhibit any
hemolytic activity even at concentrations >1000-fold above their MICs (Figure S7A). Because this set of observations did not distinguish between any of the derivatives (no hemolytic activity with any derivative), we extended the time of the assay out to 24 h (Figure S7B). However, red blood cells deteriorate under the conditions of the assay as time progresses and such extended time assays are not recommended or utilized by any in the field. So the results should not be taken as reflective of potential toxicity. However, it is notable that 18 was the best compound in the series even with an extended exposure, displaying little hemolytic activity and behaving no different than the control linezolid which does not act on the bacterial cell membrane. It was also substantially better than vancomycin itself which was no different than the control tigecycline that also does not act on the bacterial cell wall membrane, and it was much better and readily distinguishable from control daptomycin that acts by permeabilizing (not lysing) the bacterial cell membrane. The only compound in the series examined that performed worse than the control daptomycin was the C-terminus C14 quaternary ammonium salt of vancomycin (12). Importantly this combined set of studies indicate compound 18, as well as 15, have less of an impact on mammalian red blood cell membranes than even vancomycin itself. Finally, the extraordinary potency of the key analog 18 would also be expected to minimize any nonselective toxicity because the amounts required for observation of antimicrobial activity are so low.
Figure S7. Hemolytic assay of red blood cells. (A) % Hemolysis observed versus concentration expressed as fold concentration over measured MIC alongside vancomycin, daptomycin, linezolid and tigecycline controls. (B) % Hemolysis versus time at MIC concentration alongside vancomycin, daptomycin, linezolid and tigecycline controls.