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N-Terminus alkylation of vancomycin: ligand binding affinity, antimicrobial activity, and site specific nature of quaternary trimethylammonium salt modification

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Abstract

A series of vancomycin derivatives alkylated at the N-terminus amine were synthesized, including those that contain quaternary trimethylammonium salts either directly at the terminal amine site or with an intervening three-carbon spacer. The examination of their properties provide important comparisons with a C-terminus trimethylammonium salt modification that we recently found to improve the antimicrobial potency of vancomycin analogs through an added mechanism of action. The N-terminus modifications disclosed herein were well tolerated, minimally altering model ligand binding affinities (D-Ala-D-Ala) and antimicrobial activity, but did not induce membrane permeabilization that was observed with a similar C-terminus modification. The results indicate that our earlier observations with the C-terminus modification are sensitive to the site as well as structure of the trimethylammonium salt modification, and are not simply the result of non-specific effects derived from introduction of a cationic charge.

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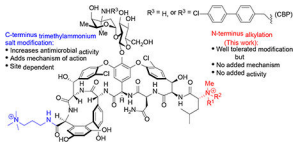

Keywords

vancomycin; glycopeptide antibiotics; site specific mechanism of action; membrane permeabilization; vancomycin peripheral modification

Since the introduction of vancomycin (**1**) into clinical use 60 years ago, it and the related glycopeptide antibiotics have emerged as the antibiotics of last resort.[1–4] They are used to treat Gram-positive bacterial infections, especially those caused by resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA).[5,6] Vancomycin disrupts

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Supporting Information

Full experimental details and ^{1}H NMR spectra of tested compounds.

bacterial cell wall synthesis by binding to the C-terminus D-Ala-D-Ala residues of peptidoglycan precursors including lipid II, a key intermediate in the biosynthesis of peptidoglycan.[6–8] Clinical resistance against vancomycin was first observed in Enterococci (VRE),[9–12] but only after 30 years of clinical use, and was followed by the more recent emergence of vancomycin-intermediate (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA).[13–15] The mechanistic basis of this drug resistance is the late stage structural remodeling of the peptidoglycan precursors, replacing the terminal D-Ala-D-Ala residue with D-Ala-D-Lac and resulting in a decrease (1,000-fold) in the binding affinity between vancomycin and the target ligand.[16,17]

The widespread appearance of VRE and the emergence of VRSA has led to an increased urgency for the development of new antibiotics that overcome this drug resistance.[18,19] Three semi-synthetic glycopeptide antibiotics have been introduced into the clinic.[20–22] Although each exhibits more potent antimicrobial activity than vancomycin, they do not directly address the underlying molecular basis of vancomycin resistance and remain preferentially active against vancomycin-sensitive versus vancomycin-resistant organisms. Complementary to these efforts, we have disclosed synthetic analogs of the glycopeptide antibiotics designed with binding pocket modifications that directly overcome the molecular basis of vancomycin resistance, displaying equipotent activity against vancomycin-sensitive and -resistant organisms.[23–29]

In continued efforts to discover antibiotics that may display more potent and even more durable antimicrobial activity, we have also examined vancomycin analogs that contain peripheral modifications that endow them with additional independent mechanisms of action not found in the parent antibiotics. Thus, subsequent to binding pocket modifications designed to provide dual D-Ala-D-Ala/D-Ala-D-Lac binding to directly overcome the molecular basis of vancomycin resistance, we examined peripheral structural changes in these compounds designed to provide additional synergistic mechanisms of action.[30–33] We reported a C-terminus modification, a quaternary trimethylammonium salt (C1), which was found to provide a binding pocket modified vancomycin analog with a second mechanism of action.[33] This modification induces cell membrane permeabilization without membrane depolarization or disruption in VRE, and is complementary to the parent glycopeptide inhibition of cell wall synthesis. It was further shown that this C-terminus modification may be combined with another reported peripheral change, a (4-chlorobiphenyl)methyl (CBP) addition to the vancomycin disaccharide, which leads to direct inhibition of transglycosylase.[34–36] This provided even more potent compounds whose activity were attributed to three independent and synergistic mechanisms of action, only one of which requires D-Ala-D-Ala/D-Ala-D-Lac binding. It was 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 and their antimicrobial potency follow predictable trends (analog potency and durability improve with 3 > 2 > 1 mechanisms of action).

As a consequence of the results observed with a C-terminus trimethylammonium salt, we have now explored and herein report its introduction at the N- versus C-terminus of a glycopeptide antibiotic. The productive effects of the C-terminus quaternary ammonium salt

incorporation against VRE observed with the pocket modified vancomycin analogs were found to be unique to the trimethylammonium salt (versus other alkylammonium salts) and were shown to correlate directly with its unique impact on cell membrane permeability (versus cell membrane disruption). These and related studies have suggested that the effects are due to a specific mechanism of action as opposed to a non-specific effect of the cation on the bacterial cell membrane integrity. Thus, the impact of a N- versus C-terminus trimethylammonium salt and the combination of two such salts were examined to further distinguish whether the effects are also site-specific. Our interest in the examination of such N-terminus trimethylammonium salts further arose because of its appearance in a naturally occurring glycopeptide antibiotic, M43A (**2a**). M43A and M43D (**2b**), which bear either a N-terminus trimethylammonium salt (**2a**) or a dimethylamine (**2b**) in place of the N-methylamine in vancomycin, have been isolated from a vancomycin-producing strain (*Amycolatopsis orientalis* M43–05865).[37] The characterization of their properties and the potential that **2a** and **2b** benefit from the N-terminus methylation were also addressed in our studies.

Despite extensive semi-synthetic modifications of vancomycin, N-terminus modified vancomycins with enhanced antimicrobial activity are comparatively rare.[38–41] This likely reflects the importance of the N-terminus amino acid residue, N-methyl-D-leucyl, and the role it plays in ligand binding. Both the positively charged protonated secondary amine and the lipophilic iso-butyl group cooperatively contribute to the target ligand binding.[42] The nature of this binding interaction with ligand excludes N-acylation as an amenable modification on the N-terminus.[43,44] However, the impact of N-alkylation on ligand binding and antimicrobial activity has rarely been explored,[45,46] especially against VRE, and the incorporation of a quaternary ammonium salt is even rarer.[47,48]

Two series of N-terminus modifications were examined, one in which the N-methylamine was converted directly to the corresponding trimethylammonium salt or dimethylamine (**2a** and **2b**, M43A and M43D, respectively). In the second series, an intervening three-carbon spacer was placed between the embedded N-terminus methylamine and an added terminal dimethylamine, trimethylammonium salt, or control methyl group (**2c**-**2e**).

The initial series of vancomycin derivatives **2** were synthesized by either nucleophilic substitution[47] (for **2a**, alkylation with MeI) or reductive amination reactions[45] (for **2b**-**e**) under conditions analogous to similar reported reactions. Distinct from reductive amination methods used for modification on the vancosamine moiety conducted under basic conditions, selective reductive amination with $NaBH_3CN$ in the presence of a weak acid led to the exclusive formation of the N-terminus modified vancomycins for all aldehyde substrates.[49] This site of modification was first established by ^{1}H NMR NOESY experiments and further confirmed by trifluoroacetic acid (TFA) mediated cleavage of the disaccharide with clean retention of the amine modification in the aglycon (Supporting Information Figures S1–S15).

Ligand binding studies were performed by differential UV-Vis spectroscopy, following the procedure reported by Perkins[50,51] with the ligand *N,N'*-Ac_2-L-Lys-D-Ala-D-Ala (Ac_2K-D-A-D-A). The antimicrobial activity of **2a**-**e** was established in a standard broth microdilution

assay.[52] The ligand binding constants and antimicrobial minimum inhibitory concentration (MIC) of these derivatives are summarized in Figure 2.

Whereas similar ligand binding constants were observed for vancomycin and N-methylated vancomycin **2b**, the N-terminus trimethylammonium salt modification led to a slight decrease (< 2-fold) in the ligand binding affinity (**2a**). A small (2 to 3-fold) decrease was found in the ligand binding constants of N-alkyl substituted vancomycins with three carbon spacer (**2c**-**e**) independent of the terminus substitution pattern (-Me, -NMe_2, or -NMe_3^+). Thus, compared to N-acylation[41] or removal of the amine,[42] alkylation on the N-terminus is a well-tolerated modification. In accordance with the trends in ligand binding affinity, the modified vancomycin derivatives **2a**-**e** displayed antimicrobial activity similar to vancomycin against vancomycin-sensitive *S. aureus* strains, including MRSA, with an approximately 2-fold reduction in activity consistent with the ligand (Ac_2K-D-A-D-A) binding affinities. Similar MIC values against VRE strains were also found for vancomycin and the derivatives **2a**-**e.** Since it is not possible to measure accurately the weak binding (10^2 M^{-1}) of the derivatives to the relevant model ligand present in VRE (Ac_2K-D-Ala-D-Lac), it is unclear whether the small improvements occasionally observed with this series against VRE represent the accuracy of our antimicrobial assays or small improvements in affinity for D-Ala-D-Lac. Most significant of the observations, the conversion of the terminal amine in vancomycin to a trimethylammonium salt (the natural product M43A, **2a**) did not increase or significantly alter ligand binding affinity, nor did it significantly change or improve the antimicrobial activity of vancomycin.

The impact of a C-terminus quaternary trimethylammonium salt modification (C1) on vancomycin was carefully examined in our previous work.[33] An increase in the bacterial cell membrane permeability was observed and this was proved to be an added mechanism of action for these derivatives (Figure 3). The well-known CBP modification on the vancosamine subunit, which increases the antimicrobial activity by independently inhibiting transglycosylase, was found to act synergistically with this C1 modification against VanA VRE. To determine whether a similar synergistic effect would be observed with the N-terminus modified vancomycins, analogous derivatives bearing both the N-terminus and the CBP modifications were examined. These were prepared by reductive amination on the vancosamine moiety of **2a**-**2e** with 4-(4’-chlorophenyl)benzaldehyde, and their antimicrobial activity was established (Figure 4).

In contrast to the nearly identical antimicrobial activity of vancomycin and the N-terminus alkylated vancomycins against *S. aureus* strains (Figure 2), the N-terminus alkylated CBP-vancomycins (**6a**-**e**) showed a decreased activity (2 to 16-fold) against *S. aureus* and VanB VRE strains compared with CBP-vancomycin (**3**) (Figure 4). The loss in activity of **6b**-**e** was less pronounced against VanA VRE (2 to 4-fold) and **6a** displayed a subtle increase (2-fold) in the activity. Notably, those that contain the three-carbon spacer (**6c**-**e**) were significantly less active against all strains tested. Thus, unlike the introduction of the C-terminus trimethylammonium salt (**4** and **5**), its introduction on the N-terminus (**2a**/**2e** and **6a**/**6e**) did not significantly improve the antimicrobial activity of either vancomycin or CBP-vancomycin.

Finally, the question of whether the introduction of two trimethylammonium salts might further enhance the activity of the derivatives was explored. Compounds **2a** and **6a** were further modified by introduction of C1 on the C-terminus to yield the vancomycin derivatives **7** and **8**, which contain trimethylammonium salts located at both the C- and N-terminus. In essence, these two compounds may be viewed as the N-terminus trimethylammonium salts of C1-vancomycin (**4**) and C1-CBP-vancomycin (**5**) detailed in our earlier studies, which displayed productive increases in antimicrobial activity derived from an additional mechanism of action. The MIC values of **7** and **8** against VanA VRE strains were determined and are listed in Figure 5 together with key compounds in the same series.

The comparison of the antimicrobial activity of the vancomycins/CBP-vancomycins bearing a N- or C-terminus trimethylammonium salt is intriguing. Whereas the incorporation of a trimethylammonium salt at either the C-terminus (**4**) or N-terminus (**2a**) of vancomycin provided a subtle increase in antimicrobial potency against VanA VRE strains (**4** > **2a**), their combination in **7** increased the activity by 30 to 60-fold. For the CBP-vancomycin series, C-terminus C1 addition (**5**) provided a 5 to 10-fold increase in activity as reported earlier, whereas the analogous N-terminus C1 addition (**6a**) provided a minor 2-fold increase in activity. Finally, their combination in **8** did not improve the activity compared with **5**.

We also investigated the mechanistic impact of the N- versus C-terminus trimethylammonium salt modifications. The compounds were examined for their ability to induce cell membrane permeability[53,54] (propidium iodide (PI) influx) (Supporting Information Figures S21 and S22). They were also assessed in an assay that measures the induced release of a membrane embedded fluorescent probe (DiSC3, dipropylthiadicarbocyanine iodide) that is indicative of greater membrane disruption, including membrane depolarization (Supporting Information Figure S25). Although activity in both assays has been observed and correlated with the enhanced activity of telavancin and oritavacin,[55,56] our prior studies of the C-terminus trimethylammonium salt indicated that its impact on antibacterial activity correlated only with the former (induced permeability) and not the latter (membrane disruption, depolarization).[33] For example in these studies, the pocket modified residue 4 aminomethylene analog of vancomycin bearing both a peripheral 4-chlorobiphenyl group and the C-terminus trimethylammonium group was found to display potent activity in the permeability assay (PI), but was found to be inactive in the depolarization assay (DiSC3) (data published but provided again in the Supporting Information herein, Figures S23, S24, and S26). Unlike the C-terminus modification that effectively induced membrane permeability (**5**), none of the N-terminus derivatives (**2a**, **2e**, **6a** or **6e**) exhibited this activity, behaving like both vancomycin (**1**) and CBP-vancomycin (**3**) in the assay. However, compound **8** that contains both the C- and N-terminus trimethylammonium salts maintained membrane permeabilization activity found with C1-CBP-vancomycin (**5**), wherein the added N-terminus salt did not significantly alter this activity (the initial rate slowed but final magnitude of the effect not changed).

The activity in the membrane depolarization assay (DiSC3) observed with telavancin and oritavancin (which contains the peripheral 4-chlorobiphenyl modification) is also observed with CBP-vancomycin (**3**). This was found to be enhanced with the C-terminus C1

modification (C1-CBP-vancomycin, **5**) and this enhanced activity was maintained but not improved with C1-CBP-vancomycin-C1 (**8**). Notably and unlike the C-terminus C1 modification, the addition of the N-terminus C1 to CBP-vancomycin (**6a** vs **3**) nearly abolished the depolarization activity seen with **3** and that was improved with **5**.

Thus, regardless of the assay or the mechanistic interpretation of the results, the addition of a N-terminus C1 quaternary ammonium salt did not promote or further improve either membrane permeability or membrane disruption (depolarization) and, in selected instances (**6a**), seems to diminish such activity.

Finally, the unexpected behavior of **7** in both assays, like its potent VRE antimicrobial activity, remain difficult to explain. Although the combined C- and N-terminus C1 modifications enhanced VRE antimicrobial activities to interesting levels (Figure 5), **7** proved inactive in both the membrane permeability (PI, 10 or 100 μM) and membrane disruption (DiSC3, depolarization, 10 μM) assays. As a result, the investigation of the source of its surprisingly effective VRE antimicrobial activity will continue.

In conclusion, a series of N-terminus alkylated vancomycin derivatives were synthesized and the effect of this modification on both ligand binding and antimicrobial activity were investigated. Comparable or slightly decreased (2 to 3-fold) ligand binding affinities were observed for the N-terminus alkylated vancomycin derivatives, indicating that such N-terminus modifications are well tolerated in terms of ligand binding. However, the effect of a trimethylammonium salt modification on the N- versus C-terminus of vancomycin is distinguishable in terms of both antimicrobial activity against VanA VRE and their mechanistic behavior. Whereas the modification on the C-terminus leads to compounds with enhanced activity and an added mechanism of action, the similar modifications on the N-terminus detailed herein do not introduce the additional mechanism of action and do not increase antimicrobial activity. These observations highlight the importance of the site as well as the structure of the C-terminus quaternary trimethylammonium salt modification, and suggest its effects may be due to a specific target and mechanism of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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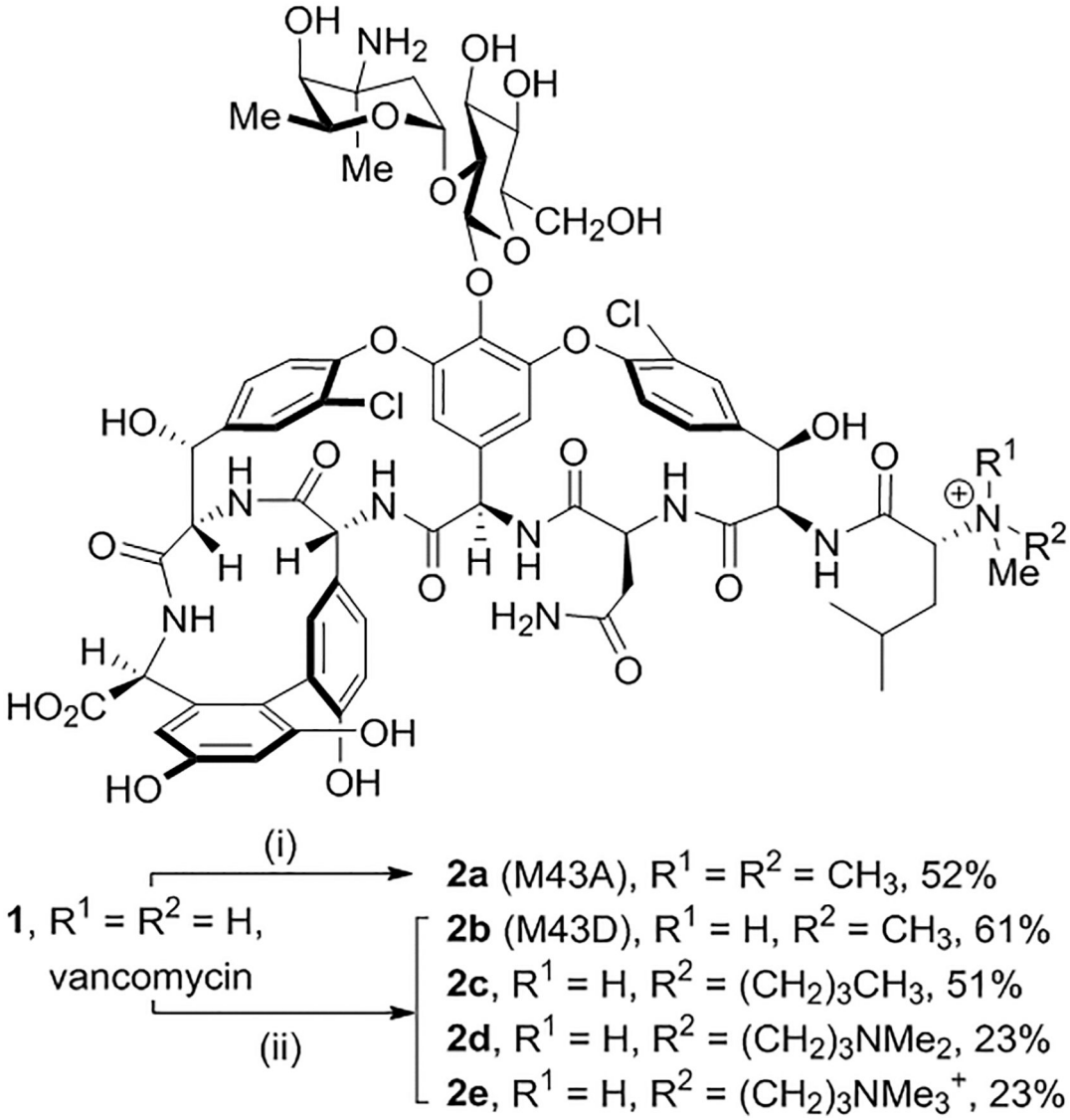


Figure 1.

Synthesis of N-terminus modified vancomycins. Reagents: (i) MeI, $NaHCO_3$, MeOH, (ii) aldehyde, $NaBH_3CN$, AcOH, H_2O, CH_3CN, THF.

	MIC (μg/mL)					K_a (10^6 M^{-1})[f]
	Sensitive	MRSA	VanA	VanA	VanB	
	S. aureus[a]	*S. aureus*[b]	*E.faecalis*[c]	*E. faecium*[d]	*E. faecalis*[e]	
1	0.5	0.5	250	250	8	1.3 ± 0.1
2a	0.5	1	125	63	4	0.87 ± 0.09
2b	1	1	250	250	8	1.5 ± 0.4
2c	1	1	125	250	8	0.46 ± 0.02
2d	1	1	>250	63	8	0.73 ± 0.12
2e	0.5	1	>250	250	8	0.49 ± 0.07

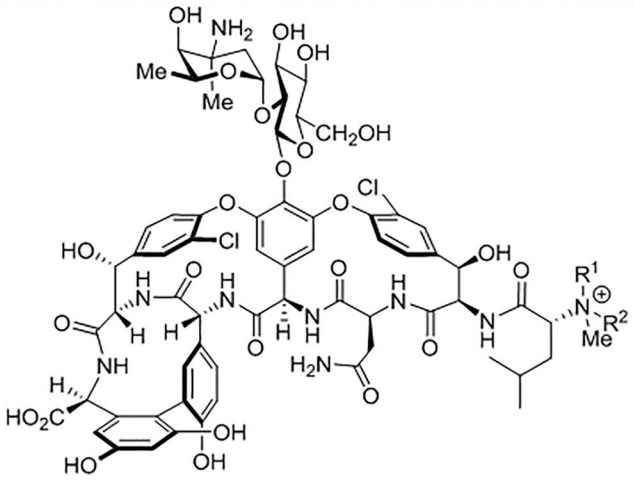


Figure 2.

Antimicrobial activity and ligand binding constant of vancomycin (**1**) and compounds **2a-e**. VanA: vancomycin and teicoplanin resistant. VanB: vancomycin resistant, teicoplanin sensitive. [a]ATCC 25923. [b]ATCC 43300. [c]BM 4166. [d]ATCC BAA-2317. [e]ATCC 51299. [f]Association constant for Ac_2K-D-A-D-A.

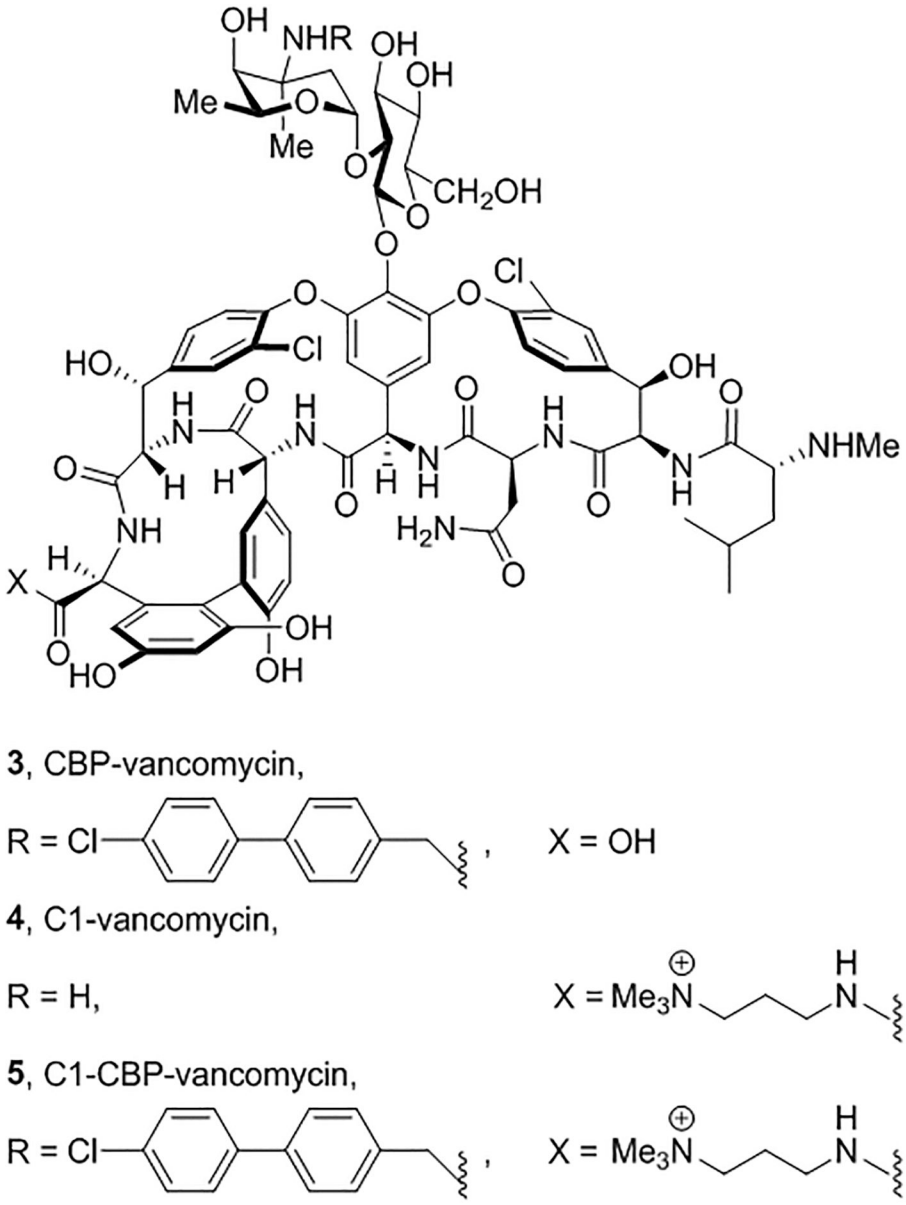
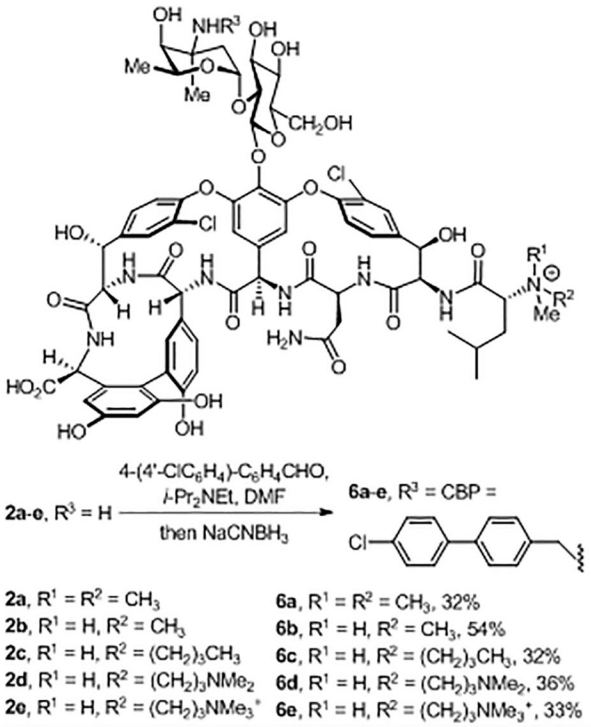


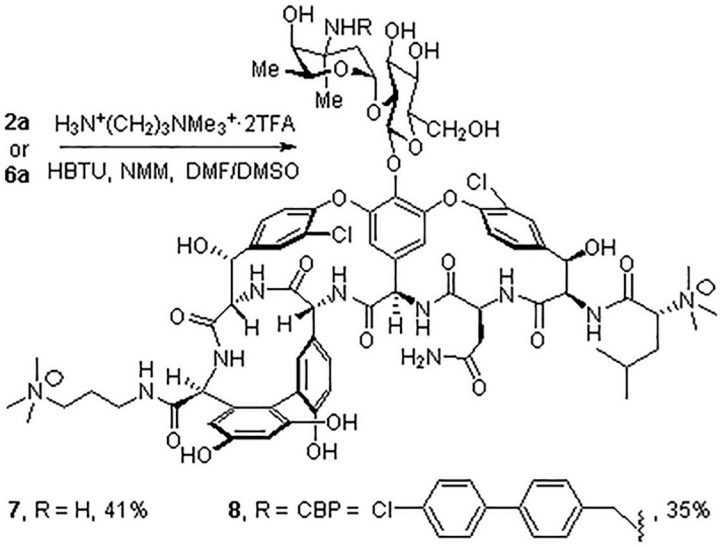
Figure 3.
Reported vancomycin derivatives bearing CBP and C1 modifications discussed in this study.



	MIC (μg/mL)				
	Sensitive	MRSA	VanA	VanA	VanB
	S. aureus[a]	*S. aureus*[b]	*E. faecalis*[c]	*E. faecium*[d]	*E. faecalis*[e]
3	0.08	0.08	2.5	2.5	0.08
6a	0.15	0.3	1.2	1.2	0.15
6b	0.3	0.3	5	5	0.3
6c	1.2	1.2	10	5	1.2
6d	0.6	1.2	10	5	0.6
6e	1.2	0.6	10	5	0.6

Figure 4.

Synthesis and antimicrobial activity of N-terminus modified CBP-vancomycins **6a-e**. VanA: vancomycin and teicoplanin resistant. VanB: vancomycin resistant, teicoplanin sensitive. [a]ATCC 25923. [b]ATCC 43300. [c]BM 4166. [d]ATCC BAA-2317. [e]ATCC 51299.



Compound	MIC (μg/mL)	
	VanA VRE *E. faecalis*	VanA VRE *E. faecium*
vancomycin (**1**)	250	250
vancomycin-C1 (**2a**)	125	63
C1-vancomycin (**4**)	63	31
C1-vancomycin-C1 (**7**)	8	4
CBP-vancomycin (**3**)	2.5	2.5
CBP-vancomycin-C1 (**6a**)	1.2	1.2
C1-CBP-vancomycin (**5**)	0.25	0.5
C1-CBP-vancomycin-C1 (**8**)	0.6	0.3

Figure 5.

Synthesis of double trimethylammonium salt derivatives **7** and **8** and the comparison of antimicrobial activity of the positively charged vancomycin derivatives. VanA: vancomycin and teicoplanin resistant.