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Total Syntheses and Initial Evaluation of [Ψ[C(=S)NH]Tpg^4]vancomycin, [Ψ[C(=NH)NH]Tpg^4]vancomycin, [Ψ[CH$_2$NH]Tpg^4]vancomycin, and Their (4-Chlorobiphenyl)methyl Derivatives: Synergistic Binding Pocket and Peripheral Modifications for the Glycopeptide Antibiotics

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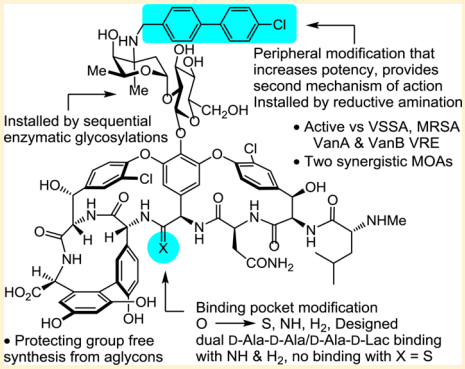
Supporting Information

ABSTRACT: Full details of studies are disclosed on the total syntheses of binding pocket analogues of vancomycin bearing the peripheral L-vancosaminyl-1,2-D-glucosyl disaccharide that contain changes to a key single atom in the residue-4 amide (residue-4 carbonyl O → S, NH, H$_2$) designed to directly address the underlying molecular basis of resistance to vancomycin. Also disclosed are studies piloting the late-stage transformations conducted on the synthetically more accessible C-terminus hydroxymethyl aglycon derivatives and full details of the peripheral chlorobiphenyl functionalization of all of the binding-pocket-modified vancomycin analogues designed for dual D-Ala-D-Ala/D-Ala-D-Lac binding. Their collective assessment indicates that combined binding pocket and chlorobiphenyl peripherally modified analogues exhibit a remarkable spectrum of antimicrobial activity (VSSA, MRSA, and VanA and VanB VRE) and impressive potencies against both vancomycin-sensitive and vancomycin-resistant bacteria (MICs = 0.06−0.005 and 0.5−0.06 μg/mL for the amidine and methylene analogues, respectively) and likely benefit from two independent and synergistic mechanisms of action, only one of which is dependent on D-Ala-D-Ala/D-Ala-D-Lac binding. Such analogues are likely to display especially durable antibiotic activity that is not prone to rapidly acquired clinical resistance.



INTRODUCTION

The glycopeptide antibiotics are among the most important class of drugs used in the treatment of resistant bacterial infections.[1] Vancomycin,[2] teicoplanin,[3] and a set of recently approved semisynthetic derivatives, including oritavancin (August 2014),[4] dalbavancin (May 2014),[5] and telavancin (September 2009),[6] are widely or increasingly used to treat clinically refractory and resistant bacterial infections. The parent member of this group, vancomycin (**1**), was disclosed in 1956[2] and introduced into the clinic in 1958, although its structure was only established 25−30 years later (Figure 1).[7] After more than 50 years of clinical use and even with the additional widespread use of glycopeptide antibiotics for agricultural livestock (avoparcin), worldwide observation of vancomycin-resistant pathogens has only slowly emerged. This was first restricted to vancomycin-resistant enterococci (VRE), initially detected in 1987 after 30 years of clinical use,[8] but recently includes the more feared emergence of vancomycin-resistant *Staphylococcus aureus* (VRSA). which was first detected in 2002.[9] In spite of the increasing prevalence of VRE, such infections presently remain sensitive to other common antibiotic classes, although a time may come when this will no longer be the case. More pressing is the emergence of VRSA, which has already acquired resistance to other common classes of antibiotics. Treatment options in such cases are expected to be limited, and outside the new-generation glycopeptide antibiotics, these presently include antibiotics known to easily evoke resistance (linezolide, daptomycin).[10,11] As a result, these latter antibiotics have been designated or recommended for use as "reserve antibiotics", ones that should be employed sparingly to preserve their effectiveness as drugs of last resort against intractable infections. This has intensified interest in the development of alternative treatments for resistant pathogens that display the remarkable clinical durability of vancomycin.[1,12,13]

The clinical durability can be attributed to several complementary features of vancomycin that result in inhibition of bacterial cell wall biosynthesis and integrity.[14] Foremost of the features responsible for this durability is its primary

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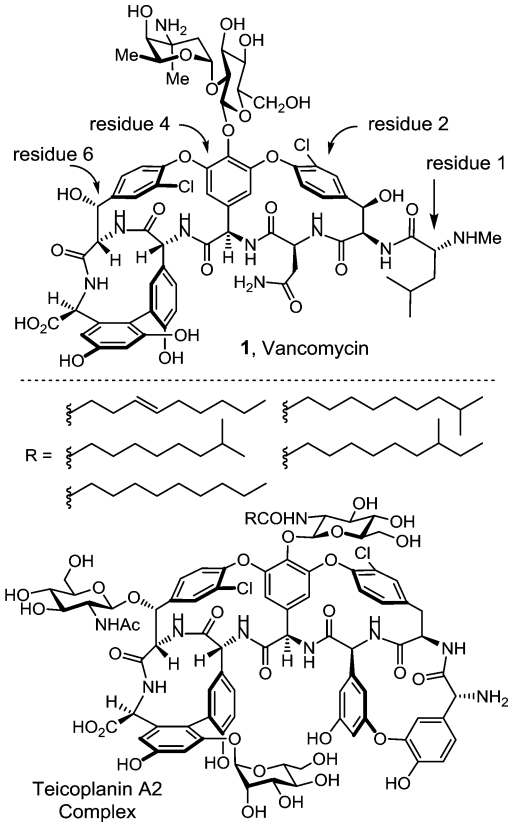

Figure 1. Structures of vancomycin (**1**) and teicoplanin.

biological target (binding to D-Ala-D-Ala). This target is not only unique to bacteria but also a structural component of the bacterial cell wall and a substrate for an enzymatic reaction. It is not a protein or nucleic acid target, and as a consequence, it is not subject to alteration by genetic mutation. Moreover, the ramifications of additional candidate binding sites within the bacterial cell wall (not only D-Ala-D-Ala but also D-Ala-Gly and Gly-Gly) have yet to be defined. Vancomycin's primary mechanism of action involves sequestration of the substrate (D-Ala-D-Ala) for an essential late-stage enzyme (transpeptidase)-catalyzed reaction needed for peptidoglycan cross-linking and bacterial cell wall maturation. However, it is thought also to inhibit transglycosylase-catalyzed incorporation of lipid intermediate II into the repeating polysaccharide backbone of the bacterial cell wall. With this second mechanism of action for vancomycin, it is not yet clear whether this involves direct binding of the appended disaccharide to the enzyme active site or requires binding to cell wall sites (e.g., D-Ala-D-Ala, D-Ala-Gly, or Gly-Gly) that contribute to its localization and subsequent indirect enzyme inhibition. Since there may be two or more mechanisms of action that contribute to the inhibition of bacterial cell wall maturation by vancomycin, full bacterial resistance may require statistically unlikely simultaneous changes to each to overcome all of the contributing mechanisms. Just as importantly, the site of action is at the bacterial cell wall surface and not at an intracellular target. As a result, no bacterial cell wall penetration or import mechanism is needed, and this allows vancomycin to avoid the common resistance mechanisms mediated by efflux pumps, blocked transport, and deactivation by cytosolic metabolic enzymes.[15] Regardless of the origin (and it is likely there are additional features contributing to the durability of vancomycin that are not yet recognized), it is most revealing that the primary mechanism of clinical resistance to the glycopeptide antibiotics (VanA and VanB) was transferred to pathogenic bacteria from nonpathogenic vancomycin-producing organisms that use this inducible mechanism to protect themselves during vancomycin production.[16] This underscores the fact that pathogenic bacteria have not yet independently evolved effective resistance mechanisms to the glycopeptide antibiotics even after more than 50 years of widespread use,[17] suggesting that fundamental solutions to VanA and VanB resistance may provide durable antibiotics with clinical lifetimes lasting 50 more years.

Because of their structural complexity, essentially all analogues of the glycopeptide antibiotics consist of semisynthetic derivatives of the natural products obtained by chemical, enzymatic, or mutasynthesis approaches.[1,12] The most significant of the modifications introduce peripheral hydrophobic groups, and these are found in each of the clinically approved semisynthetic derivatives (Figure 2). For both dalbavancin and telavancin, the long hydrophobic alkyl chains are thought to provide selective membrane anchoring properties and to promote antibiotic dimerization without impacting the binding affinity to the primary biological target D-Ala-D-Ala.[18] It is possible that such semisynthetic changes to the glycopeptide antibiotics also avoid bacterial sensing of the antibiotic challenge, and this may account for their VanB VRE activity first observed with teicoplanin.[19] Additionally, telavancin has been shown to function not only through the traditional mechanism of inhibition of cell wall synthesis by binding of D-Ala-D-Ala but also through disruption of bacterial membrane integrity, a mechanism typically not observed for the glycopeptide antibiotics.[20]

One of the most widely recognized modifications is the 4-chlorobiphenyl (4-CBP) substitution of a peripheral carbohydrate. This has been examined at range of positions in a variety of glycopeptide antibiotics, most notably in oritavancin,[4] the *N*-(4-chlorobiphenyl)methyl derivative of chloroeremomycin, and with vancomycin itself (CBP-vancomycin).[21] In addition to perhaps promoting antibiotic dimerization, membrane anchoring, disruption of bacterial membrane integrity, and potentially avoiding bacterial sensing of the antibiotic challenge, the unique placement of the 4-chlorobiphenyl substituent introduces or potentiates a second mechanism of action. The direct inhibition of transglycosylases mediated by the modified carbohydrate has been identified as a second, now effective, mechanism by which oritavancin exhibits antimicrobial activity.[22,23] Regardless of the origin of the effects, such derivatives often increase the antibiotic potency as much as 100-fold. While increasing bacterial sensitivity to the antibiotics, VanA vancomycin-resistant bacterial strains (minimum inhibitory concentration (MIC) = ca. 10 μg/mL) remain 1000-fold less sensitive than susceptible strains (MIC = ca. 0.01 μg/mL). This suggested to us that combining such peripheral hydrophobic substitutions with vancomycin binding pocket modifications that maintain D-Ala-D-Ala binding and reinstate binding to D-Ala-D-Lac would further increase their antimicrobial activity against not only vancomycin-sensitive but also vancomycin-resistant bacteria to truly remarkable levels.

In an extension of work first directed at the total syntheses of the naturally occurring glycopeptide antibiotics,[24−27] we recently described studies of the binding pocket redesign of vancomycin[14] that were the first to directly address the underlying molecular basis of clinical resistance to vancomy-

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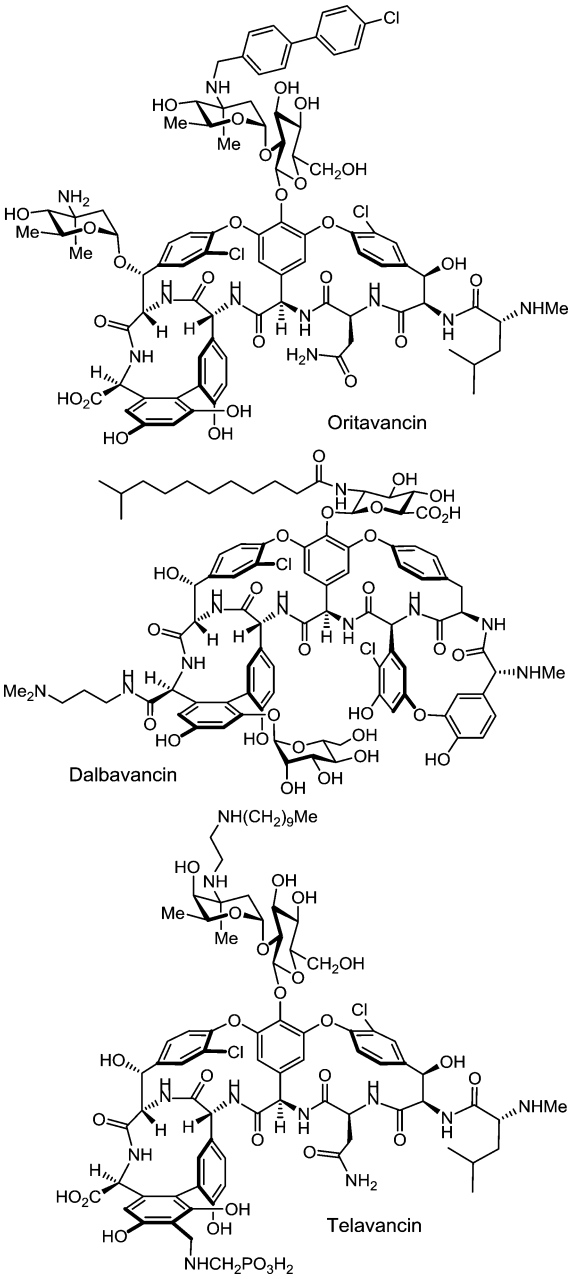
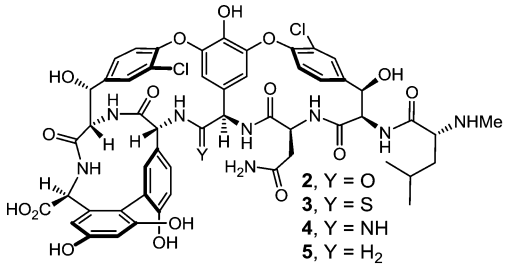


Figure 2. Clinically approved semisynthetic glycopeptide antibiotics.

cin.[28,29] In clinically resistant organisms (VanA and VanB), synthesis of the bacterial cell wall precursors lipid intermediate I and II continue complete with installation of their pendant N-terminus D-Ala-D-Ala, but resistant bacteria sense the antibiotic challenge.[19] Through the use of a two-component cell surface receptor sensing and subsequent intracellular signaling system,[19] they initiate a late-stage remodeling of their peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-Lac[28] to avoid the action of the antibiotic. The vancomycin binding affinity for this altered ligand is reduced 1000-fold,[28] resulting in a corresponding 1000-fold loss in antimicrobial activity. The destabilized binding to D-Ala-D-Lac is due to a combination of the loss of a hydrogen bond central to binding of the ligand to the antibiotic (10-fold) and an even more significant destabilizing lone-pair repulsion between the vancomycin residue-4 carbonyl and D-Ala-D-Lac ester oxygens (100-fold).[30] The elucidation of this inducible mechanism of resistance (VanA and VanB) acquired from nonpathogenic vancomycin-producing organisms[16] also highlighted that such modifications of the vancomycin binding pocket must target compounds that not only establish binding to D-Ala-D-Lac but also maintain D-Ala-D-Ala binding. This not only ensures that they would display antimicrobial activity against vancomycin-resistant bacteria (VanA and VanB) but additionally assures that they would remain active against vancomycin-sensitive bacteria.

Our initial studies provided [Ψ[CH_2NH]Tpg^4]vancomycin aglycon (**5**),[31] which displayed such dual binding properties by virtue of removal of the lone-pair repulsion between the vancomycin residue-4 carbonyl and D-Ala-D-Lac ester oxygens. This change reinstated commensurate activity against VanA VRE, validated the opportunities of the approach, and entailed removal of a single atom from the vancomycin binding pocket (Figure 3). These efforts were followed by the total synthesis of



compound	ligand, K_a (M^{-1}) **6**, X = NH	**7**, X = O	K_a(**6**/**7**)	VanA[a] MIC, μg/mL
2, Y = O	1.7 x 10^5	1.2 x 10^2	1400	640
3, Y = S	1.7 x 10^2	1.1 x 10^1	–	> 640
4, Y = NH	7.3 x 10^4	6.9 x 10^4	1.05	0.5
5, Y = H_2	4.8 x 10^3	5.2 x 10^3	0.9	31

[a]Minimum inhibitory conc., *E. faecalis* (BM4166, VanA VRE).

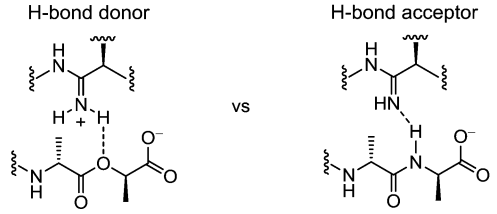


Figure 3. Vancomycin aglycon residue-4 modifications and dual binding behavior of amidine **4**.

[Ψ[C(=NH)NH]Tpg^4]vancomycin aglycon (**4**),[32] providing a modified antibiotic that not only maintains vancomycin's ability to bind the unaltered peptidoglycan D-Ala-D-Ala but also binds the altered ligand D-Ala-D-Lac just as effectively by virtue of its ability to serve as either a hydrogen-bond donor (for D-Ala-D-Lac) or a hydrogen-bond acceptor (for D-Ala-D-Ala). Whereas the former entails binding of the protonated amidine with D-Ala-D-Lac and replaces the destabilizing carbonyl lone-pair interaction with the ester oxygen lone pair with a stabilizing electrostatic interaction and perhaps a reversed hydrogen bond, the latter entails binding of D-Ala-D-Ala with the unprotonated amidine serving as a hydrogen-bond acceptor (Figure 3).[33] Not only did amidine **4** display balanced binding affinities for both target ligands within 2-fold of that exhibited

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by vancomycin aglycon with D-Ala-D-Ala, but it also exhibited effective antimicrobial activity against VanA VRE, being equipotent to the activity that vancomycin displays against sensitive bacterial strains. These latter studies represented the replacement of a single atom in the binding pocket of the antibiotic aglycon (O → NH) to counter a complementary exchange in the cell wall precursors of resistant bacteria (NH → O). Just as remarkable, we established that [Ψ[C(=S)NH]-Tpg^4]vancomycin aglycon (**3**), which served as the penultimate precursor to **4**, fails to bind D-Ala-D-Ala or D-Ala-D-Lac to any appreciable extent and is inactive against both vancomycin-sensitive and vancomycin-resistant bacteria (Figure 3).[32] The expectedly benign conversion of the residue-4 amide to a thioamide with the exchange of a single atom in the binding pocket (O → S) proved sufficient to completely disrupt ligand binding. We attributed this loss in affinity largely to the increased thiocarbonyl bond length and size of the sulfur atom, which are sufficient to sterically displace the ligand out of the binding pocket and completely disrupt the intricate binding of D-Ala-D-Ala. Significantly, the comparison of **3** with **4** highlighted just how remarkable the behavior of amidine **4** is.

Herein we report full details of the recently disclosed[34] total syntheses of [Ψ[C(=NH)NH]Tpg^4]vancomycin, [Ψ[C(=S)NH]Tpg^4]vancomycin, and their (4-chlorobiphenyl)methyl derivatives and previously unreported studies first developed with their corresponding synthetic C-terminus hydroxymethyl precursors. We also report new efforts on the extension of these studies to the total syntheses of [Ψ[CH_2NH]Tpg^4]vancomycin and its corresponding (4-chlorobiphenyl)methyl derivative. The latter new and previously undisclosed studies complete an initial series of totally synthetic vancomycin analogues bearing the peripheral L-vancosaminyl-1,2-D-glucosyl disaccharide as well as their (4-chlorobiphenyl)methyl derivatives. Collectively the compounds represent a key set of analogues of vancomycin and its (4-chlorobiphenyl)methyl derivative containing changes to a single atom in the binding pocket. Their assessment indicates that combined pocket and chlorobiphenyl peripherally modified analogues exhibit a remarkable spectrum of antimicrobial activity (vancomycin-sensitive *S. aureus* (VSSA), methicillin-resistant *S. aureus* (MRSA), and VanA and VanB VRE) and impressive potencies against both vancomycin-sensitive and vancomycin-resistant bacteria and likely benefit from two independent and synergistic mechanisms of action. Like vancomycin, such analogues are likely to display especially durable antibiotic activity not prone to rapidly acquired clinical resistance.

RESULTS AND DISCUSSION

Resynthesis of [Ψ[C(=S)NH]Tpg^4]vancomycin Aglycon and [Ψ[CH_2NH]Tpg^4]vancomycin Aglycon. The total syntheses of the aglycons **3** and **5** were conducted following the approaches previously disclosed,[31,32] presenting the opportunity to further improve on the routes. The first of the reactions that was improved was the E-ring chloride introduction by Sandmeyer substitution of an intermediate diazonium salt (Figure 4). For **8**, it was found that excess reagents (1.1 vs 1.0 equiv of HBF_4/*t*-BuONO) can lead to subsequent reaction of the thioamide, resulting in generation of minor amounts of the corresponding amide. Additionally, it was found that if the Sandmeyer substitution reaction of the intermediate diazonium salt was conducted in 1:1 (vs 2−1.4:1) CH_3CN/H_2O, the small amount of reduced (H vs Cl) byproduct could be eliminated. With the use of stoichiometric amounts of reagents and this

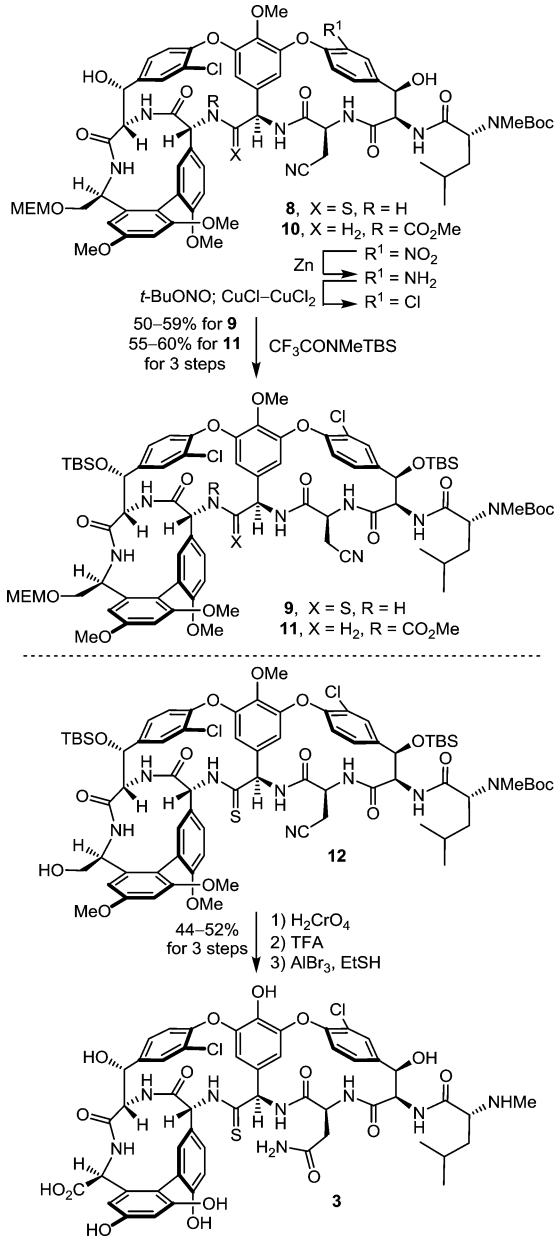

Figure 4. Notable improvements in the total syntheses of **3** and **5**.

solvent adjustment, these minor side reactions were avoided and, following protection of the secondary alcohols, cleanly provided thioamide **9** free of amide contaminant or reduced byproduct. These improvements were subsequently applied in the conversion of **10** to **11** (1.1 vs 1.2 equiv of HBF_4/*t*-BuONO), which was conducted at lower reaction temperatures (−15 °C for diazonium salt formation and −35 to 25 °C for chloride substitution) and with the aryl nitro reduction mediated by zinc nanoparticles (40 equiv, saturated aq. NH_4Cl-acetone, 25 °C, 30 min, quant.) versus the reported H_2/Pd−C to provide **11** with improved conversions. Finally, the final stage of the synthesis of the fully deprotected thioamide aglycon **3** was also improved. Reduction in the amount of reagent used in the Jones oxidation[35] (3 vs 4 equiv of CrO_3, aq. H_2SO_4/acetone, 24 h) under controlled reaction conditions directly oxidized the primary alcohol to the corresponding carboxylic acid (67−75%) in a single step with little competitive oxidative conversion of the thioamide to the

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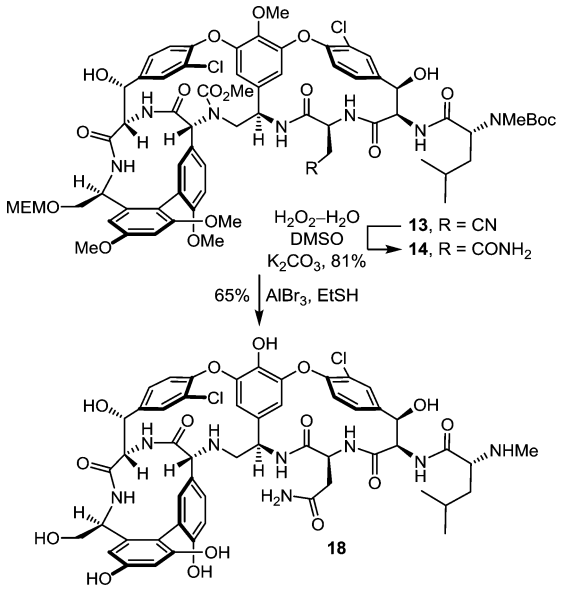
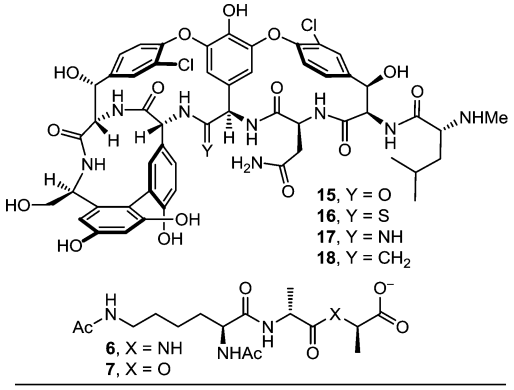
amide (>10:1). As detailed earlier, more popular oxidizing reagents and two-step procedures typically deployed on such occasions, including Dess–Martin periodinane, Swern oxidation, SO_3–Pyr, Bu_4NRuO_4 (TPAP), pyridinium dichromate (PDC),[36] pyridinium chlorochromate (PCC),[37] 2-iodoxybenzoic acid (IBX), and 2,2,6,6-tetramethypiperidine-*N*-oxyl–(bis(acetoxy)iodo)benzene (TEMPO–BAIB), provided little or no desired reaction or complex mixtures of products, even when employed in the presence of thiourea as a diversion additive, and on occasion provided the isolable thioamide *S*-oxide. After trifluoroacetic acid (TFA)-mediated conversion of the residue-3 nitrile to a primary carboxamide,[38] which was accompanied by *N*-Boc removal, global deprotection with the use of increased amounts of reagent, a longer reaction time, and better control of the reaction concentration (0.1 M, $AlBr_3$, EtSH, 25 °C, 72 h) cleanly effected the removal of the four aryl methyl ethers as well as the slower silyl ether deprotections, providing [Ψ[C(=S)NH]Tpg^4]vancomycin aglycon (**3**). Notably, when this final global deprotection was conducted at a more dilute reaction concentration (0.01 vs 0.1 M) under otherwise identical conditions, the major product still contained the residue-2 *tert*-butyldimethylsilyl (TBS) ether. These modified conditions were also adopted for the global deprotection leading to **5** (0.1 M, 100 equiv of $AlBr_3$, EtSH, 25 °C, 78 h), effecting the removal of four aryl methyl ethers, two TBS ethers, the *N*-Boc group, and the methoxycarbonyl carbamate.[39]

Initial Studies Conducted with Synthetic Aglycon C-Terminus Hydroxymethyl Derivatives. Because of their greater accessibility, studies examining and optimizing the sequential enzymatic glycosylation of the unprotected aglycons and the subsequent (4-chlorobiphenyl)methyl introduction were first conducted with the C-terminus hydroxymethyl derivatives. Alongside the residue-4 amide, the studies were first conducted with the residue-4 thioamide and amidine derivatives, both of which were prepared as previously disclosed.[32] Subsequently, the most recent studies were conducted with the residue-4 methylene derivative **18**, which was prepared from **13**[31] as shown in Scheme 1. Without optimization, conversion of the residue-3 nitrile **13** to the corresponding primary carboxamide **14** (60 equiv of H_2O_2, 40 equiv of K_2CO_3, DMSO/H_2O, 25 °C, 2 h, 81%) followed by global deprotection enlisting $AlBr_3$ (100 equiv, EtSH, 0.1 M, 25 °C, 8 h, 65%) provided **18** with removal of the four aryl methyl ethers, the *N*-Boc group, the C-terminus (2-methoxyethoxy)-methyl (MEM) ether, and the methoxycarbonyl carbamate. Its preparation not only provided a sample of **18** for a pilot examination of the initial glycosylation reaction and assured that conditions for the final steps in the preparation of **5** were further optimized but also presented the opportunity to complete the comparison of a key series of aglycon residue-4 analogues, each bearing the C-terminus hydroxymethyl group.

Consistent with expectations and in line with the behavior of **5**, the methylene derivative **18** exhibited antimicrobial activity against VanA VRE (BM4166), reinstating the activity by virtue of its projected dual D-Ala-D-Ala and D-Ala-D-Lac binding (see Figure 3), albeit at a reduced level relative to the corresponding amidine (Figure 5). Thus, like **5**, **18** proved to be less active

Scheme 1





compound	ligand, K_a (M^{-1}) **6**, X = NH	**7**, X = O	K_a(**6**/**7**)	VanA[a] MIC, μg/mL
15, Y = O	1.4 x 10^5	1.3 x 10^2	1100	> 320[b]
16, Y = S	6.2 x 10^2	[c]3.1 x 10^1	–	> 320[b]
17, Y = NH	5.7 x 10^4	6.3 x 10^4	0.9	1[d]
18, Y = H_2	nd	nd	nd	62[d]

[a]Minimum inhibitory conc., *E. faecalis* (BM4166, VanA VRE). [b]Highest conc. tested. [c]Estimate from ill-defined binding curve. [d]Data obtained herein, otherwise data taken from ref. 32.

Figure 5. Aglycon residue-4 analogues bearing a C-terminus hydroxymethyl group.

than the amidine **17**, but it displayed activity precisely in line with expectations based on the ligand binding affinity measured with **5**.

For vancomycin, the carbohydrate introduction has been approached by using either chemical[40–42] or enzymatic[43–46] glycosylations for sequential introduction of the glucose and vancosamine sugars located on the central residue of the aglycon or pseudoaglycon, respectively. Of these and as noted elsewhere,[40,43,44] the enzymatic glycosylations avoid the protection and corresponding deprotection of aglycon precursors required of chemical procedures, providing the fully glycosylated products in two steps from the fully deprotected aglycons. As a consequence, the sequential glycosylations of the modified aglycon derivatives were examined alongside the vancomycin aglycon and its C-terminus hydroxymethyl derivative using the enzymatic approach.[47] The recombinant glycosyltranferases GtfE and GtfD from a

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vancomycin-producing strain of *Amycolatopsis orientalis* (ATCC19795) were expressed in *Escherichia coli* from the corresponding constructs[43a] and were purified to homogeneity (His_6 tag). Notably, although the endogenous glycosyl donors for both enzymes are the TDP-sugars,[43] UDP-sugars have been shown to be equally effective cosubstrates for both enzymes. Since the requisite NDP-sugar precursor UMP morpholidate is commercially available[48] and the corresponding activated TMP is not, we prepared, explored, and adopted UDP-vancosamine for use with GtfD.[47] The UDP-vancosamine possessing the required β-anomer stereochemistry was prepared using a procedure described by Kahne[49] to access TDP-vancosamine with modifications to the synthetic route that incorporate uridine versus thymidine.[47] With the use of the purified enzymes and the synthetic glycosyl donors UDP-glucose[50] (for GtfE) and UDP-vancosamine[47] (for GtfD), conditions were optimized for the two sequential glycosylations of vancomycin aglycon (**2**) as well as its C-terminus hydroxymethyl derivative **15**.[47] Of the two glycosylation reactions, the initial GtfE-catalyzed incorporation of glucose using UDP-glucose exhibited the greatest aglycon substrate sensitivity, and those bearing a C-terminus hydroxymethyl group were established to be much less effective than the corresponding carboxylic acids. Our previously reported optimization efforts focused on this glycosylation reaction and examined simultaneously both **2** and **15** (37 °C).[47] In the case of hydroxymethyl substrate **15**, whose reaction proceeded at a slow rate (Figure 6), preparative

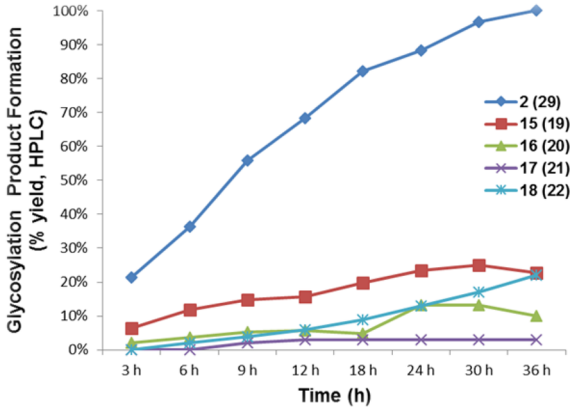
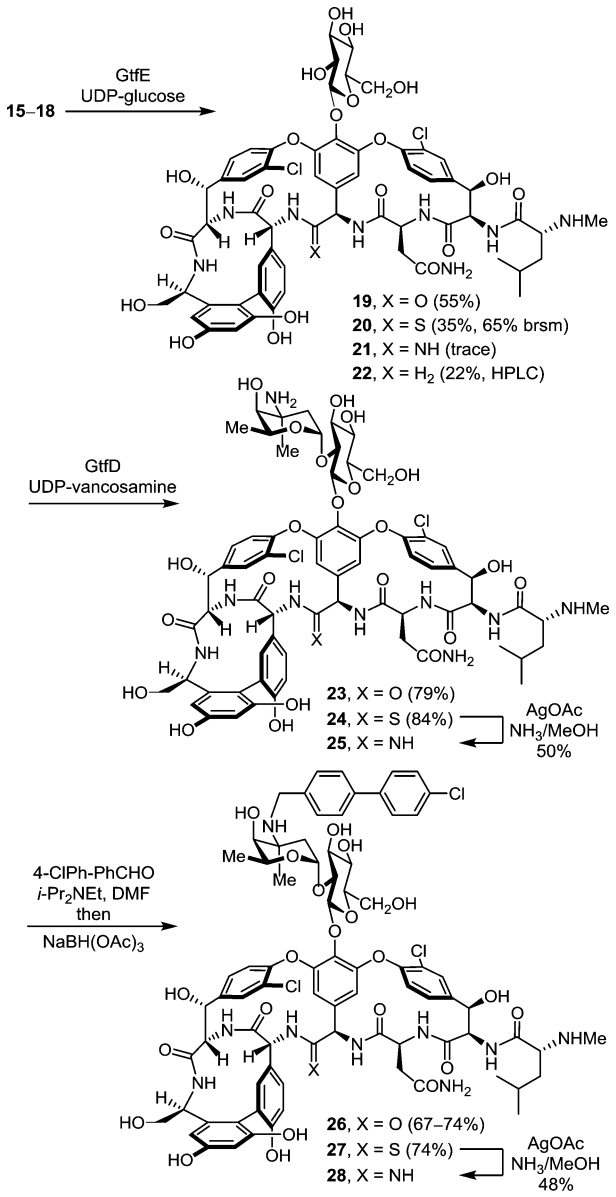


Figure 6. Comparison of the relative rates and efficiencies of the GtfE-catalyzed reactions of **15**–**18** (0.5 mM) vs vancomycin aglycon (**2**) under the optimized conditions: Tricine (pH 9, 75 mM), TCEP (2 mM), UDP-glucose (2 mM for **2**; 4 mM for **15**–**18**), GtfE (5 μM), $MgCl_2$ (1 mM), glycerol (5% v/v), 37 °C.

amounts of product **19** (55%, 48 h)[47] were obtained by increasing the amount of enzyme used (20 vs 5 μM). The residue-4 thioamide and the residue-4 methylene derivative **18** were capable of glycosylation using GtfE and UDP-glucose to provide the pseudoaglycons **20** (35%; 65% based on recovered **16**, 25 μM GtfE) and **22** (HPLC-scale, 22% with 5 μM GtfE), while glycosylation of the residue-4 amidine **17** was not sufficient to provide isolatable amounts of product (Figure 6). Whereas the studies with amide **15** and thioamide **16** were conducted on preparative scales, the studies with amidine **17** and the more recent methylene derivative **18** were conducted only on an analytical scale as a prelude to studies with the corresponding and more effective C-terminus carboxylic acids.

The second glycosylation reaction catalyzed by GtfD using synthetic UDP-vancosamine proceeded to completion rapidly (<3 h) independent of the substrate, displaying no impact of either the C-terminus hydroxymethyl group or the nature of residue 4 (amide, thioamide, or methylene), and the reaction conditions required little optimization (Scheme 2). Aside from

Scheme 2



incorporating glycerol (10% v/v) and reducing the amount of added bovine serum albumin (0.2 vs 1 mg/mL), the conditions used are essentially those originally disclosed[43] for the use of this enzyme and provided both **23** (79%)[47] and **24** (84%) in excellent yields.

Direct conversion of thioamide **24** to the corresponding amidine (10 equiv of AgOAc,[51] sat. NH_3/MeOH, 25 °C, 6 h, 50%, unoptimized)[33] provided **25**, the C-terminus hydroxymethyl analogue of vancomycin containing the residue-4 amidine modification. Importantly, this latter reaction was implemented without competitive deglycosylation, and the entire three-step sequence (conversion of **16** to **25**) could be conducted without protecting groups. Most significantly, the

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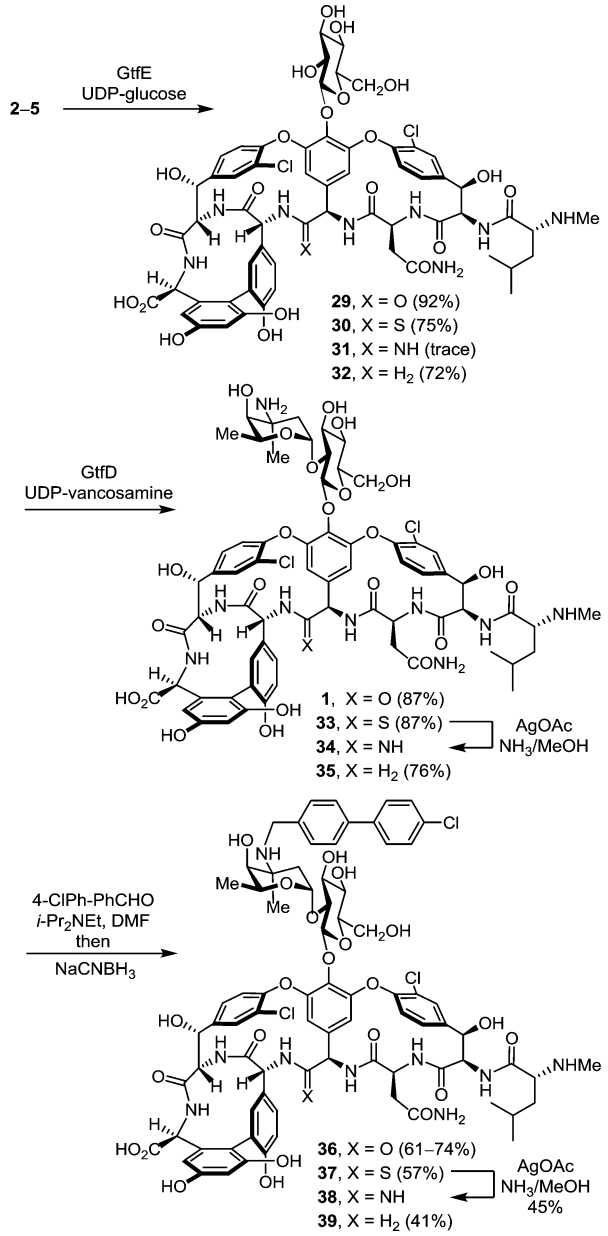
approach defined an effective route to the key residue-4 amidine analogues despite their inability to directly participate effectively in the initial enzymatic glycosylation reaction.

Subsequent introduction of the chlorobiphenyl group into **23** and **24** by selective reductive amination was conducted best with preformation of the imine (1.3−1.5 equiv of 4-(4-chlorophenyl)benzaldehyde, 5 equiv of *i*-Pr_2NEt, DMF, 30 °C, 9−12 h) followed by subsequent imine reduction (100 equiv of $NaBH(OAc)_3$, 30 °C, 2 h) and provided **26** (67−74%) and **27** (74%) using conditions modified ($NaBH(OAc)_3$ vs $NaCNBH_3$) from those disclosed by Kahne for (4-chlorobiphenyl)methyl vancomycin itself (Scheme 2).[52] Of most significance, the reaction of the latter compound occurs without observation of competitive reactions of either the residue-4 thioamide (reduction) or the N-terminal free amine (reductive amination). A final AgOAc-promoted (10 equiv, sat. NH_3/MeOH, 25 °C, 6 h) conversion of thioamide **27** to the amidine provided **28** (48%, unoptimized),[53] the 4-chlorobiphenyl derivative of **25**. By design, the final reaction introducing the amidine as well as the sequential glycosylation reactions and the reductive amination could be conducted effectively on fully functionalized substrates lacking protecting groups and incorporating the vancomycin disaccharide.

Total Syntheses of Vancomycin, [Ψ[C(═S)NH]Tpg^4]-vancomycin, [Ψ[C(═NH)NH]Tpg^4]vancomycin, [Ψ-[CH_2NH]Tpg^4]vancomycin, and Their (4-Chlorobiphenyl)methyl Derivatives. The studies piloted with the C-terminus hydroxymethyl derivatives as well as vancomycin aglycon itself defined the approach and provided the experience needed to address the fully functionalized residue-4-modified aglycons. The two sequential glycosylations of vancomycin aglycon **2**,[47] the freshly prepared synthetic thioamide **3**,[34] amidine **4**,[32] and the more recently reprepared methylene analogue **5**[31] were conducted with the recombinant glycosyltransferases[43] and the synthetic glycosyl donors (UDP-glucose[50]/GtfE and UDP-vancosamine[47]/GtfD) to provide the intermediate pseudoaglycons **29** (92%), **30** (75%, HPLC conversion 86−92%), and **32** (72%, HPLC conversion 85−95%) and subsequently vancomycin (**1**) (87%) and the fully functionalized vancomycin analogues bearing single-atom changes in the binding pocket, [Ψ[C(═S)NH]Tpg^4]-vancomycin (**33**) (87%, HPLC conversion >95%) and [Ψ[CH_2NH]Tpg^4]vancomycin (**35**) (76%, HPLC conversion >95%) (Scheme 3). Notably, in combination with our earlier efforts that provided a total synthesis of **2**,[24a] the studies on the conversion of **2** to **1** also served to complete a total synthesis of vancomycin itself.

A comparison of the relative efficiencies of the initial glycosylation reactions with **3** and **5** conducted on an analytical scale alongside that of **2** is shown in Figure 7. Unlike the significant impact of the C-terminal hydroxymethyl group but like the well-tolerated N-terminus substitutions,[43d,47] modifications to the vancomycin binding pocket itself had a minimal impact on both the rate and overall efficiency of the initial GtfE-catalyzed reaction. However, like the observations made with amidine **17**, amidine aglycon **4** failed to undergo successful GtfE-catalyzed glycosylation. Although small amounts of product could be detected by HPLC, the aglycon itself underwent competitive conversion to several byproducts under the basic conditions (pH 9) required for the reaction. The second glycosylation reaction catalyzed by GtfD using the cosubstrate UDP-vancosamine proceeded rapidly (<1.5 h) regardless of the aglycon substrate, displaying no significant

Scheme 3



impact of the nature of residue 4 (amide, thioamide, or methylene), and the conditions required no further optimization (Scheme 3). For [Ψ[C(═NH)NH]Tpg^4]vancomycin (**34**), direct conversion of thioamide **33** to the corresponding amidine (10 equiv of AgOAc, sat. NH_3/MeOH, 25 °C, 6 h) provided **34**.[53]

Subsequent introduction of the chlorobiphenyl group with [Ψ[C(═S)NH]Tpg^4]vancomycin (**33**) and [Ψ[CH_2NH]-Tpg^4]vancomycin (**35**) by reductive amination (1.5 equiv of 4-(4-chlorophenyl)benzaldehyde, 5 equiv of *i*-Pr_2NEt, DMF, 50−70 °C, 2 h; 100 equiv of $NaCNBH_3$, 70 °C, 5 h) provided **37** (57%)[53] and **39** (41%), respectively,[53] on the unprotected vancomycin analogues without optimization using conditions piloted with CBP-vancomycin (**36**) itself (61−74%).[52] Direct AgOAc-promoted (10 equiv, sat. NH_3/MeOH, 25 °C, 6 h) conversion of thioamide **37** to the amidine provided **38** (45%),[53] the chlorobiphenyl derivative of **34**. Significantly, the reductive amination was conducted without competing reaction

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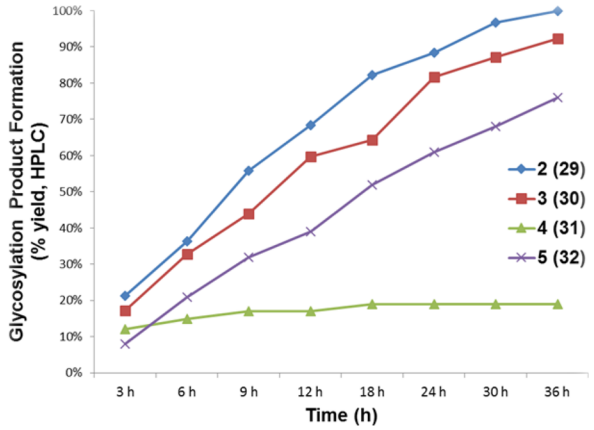
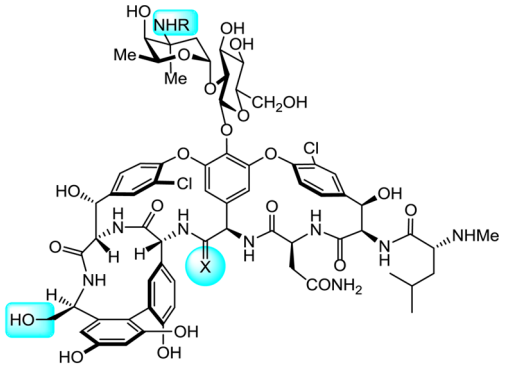


Figure 7. Comparison of the relative rates and efficiencies of the GtfE-catalyzed reactions of **2**–**5** (0.5 mM). Conditions: Tricine (pH 9, 75 mM), TCEP (2 mM), UDP-glucose (2 mM for **2**; 4 mM for **3**–**5**), GtfE (5 μM), $MgCl_2$ (1 mM), glycerol (5% v/v), 37 °C.

of either the thioamide or the N-terminus and residue-4 secondary amines, the entire three or four step sequence could be conducted without protecting groups, and the amidine introduction was implemented without competitive deglycosylation. Finally, it is worth noting that the enzymatic glycosylations were conducted on ca. 1–3 mg of substrate with 1 mol % enzyme and 4 equiv of UDP-glucose or UDP-vancosamine, reflecting our piloted laboratory scale. However, the expression and purification of the enzymes and the chemical synthesis of UDP-vancosamine,[47] along with the commercial availability of UDP-glucose,[50] were conducted on scales that would easily support laboratory preparations on much larger scales (ca. 100-fold) than exemplified herein and could easily be scaled beyond even this level.

Antimicrobial Activity. The pocket-modified vancomycin analogues containing the C-terminus hydroxymethyl group (**23**–**25**) and their chlorobiphenyl derivatives (**26**–**28**), as well as the fully functionalized vancomycin analogues [Ψ[C(=S)NH]Tpg^4]vancomycin, [Ψ[C(=NH)NH]Tpg^4]-vancomycin, and [Ψ[CH_2NH]Tpg^4]vancomycin (**33**–**35**) and their (4-chlorobiphenyl)methyl derivatives (**37**–**39**), were examined alongside the corresponding vancomycin (residue-4 amide) derivatives. As shown in Figure 8, the antimicrobial activities of the compounds were evaluated against a panel of Gram-positive bacteria that included VSSA, MRSA, and both VanA (*Enterococcus faecalis* and *Enterococcus faecium*) and VanB (*E. faecalis*) VRE, of which VanA is the most stringent of the resistant organisms.[54] Notably, one VanA VRE tested (*E. faecium* ATCC BAA-2317) represents an emerging challenging multidrug-resistant VanA VRE that is resistant not only to vancomycin and teicoplanin but also ampicillin, benzylpenicillin, ciprofloxacin, erythromycin, levofloxacin, nitrofurantoin, and tetracycline. It is also insensitive to linezolid but remains sensitive to tigecycline and dalfopristine.[54] The activities of the C-terminus hydroxymethyl derivatives paralleled those observed with the corresponding C-terminus carboxylic acids and will not be discussed separately below. However, they displayed the same trends and near identical absolute MIC values, reinforcing the generality of the observations and significance of the conclusions.

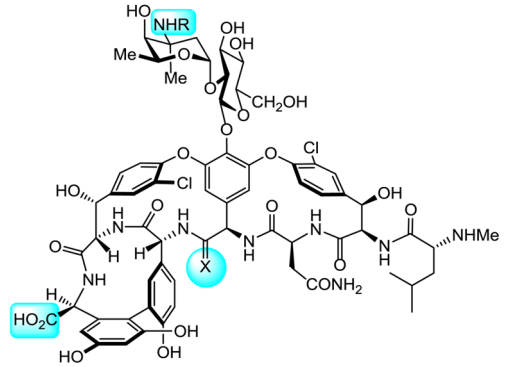
First and as expected, the activities of the pocket-modified vancomycin analogues **33**–**35** matched the in vitro antimicrobial activities of the corresponding aglycon analogues **3**–**5** on



Antimicrobial Activity, MIC[a] (μg/mL)

	sensitive	MRSA	VanA		VanB
	S. aureus[b]	*S. aureus*[c]	*E. faecalis*[d]	*E. faecium*[e]	*E. faecalis*[f]
R = H					
23, X = O	0.5	0.5	250	250	8
24, X = S	>32	>32	>32	>32	>32
25, X = NH	nd[g]	nd[g]	2	2	nd[g]
R = CBP, (4-chlorobiphenyl)methyl					
26, X = O	0.03	0.03	2	4	0.13
27, X = S	4	8	8	4	4
28, X = NH	0.13	0.13	0.02	0.02	0.06

[a]MIC = Minimum inhibitory concentration. [b]ATCC 25923. [c]ATCC 43300. [d]BM 4166. [e]ATCC BAA-2317. [f]ATCC 51299. [g]not determined.



Antimicrobial Activity, MIC[a] (μg/mL)

	sensitive	MRSA	VanA		VanB
	S. aureus[b]	*S. aureus*[c]	*E. faecalis*[d]	*E. faecium*[e]	*E. faecalis*[f]
R = H					
1, X = O	0.5	0.5	250	250	8
33, X = S	>32	>32	>32	>32	>32
34, X = NH	nd[g]	nd[g]	0.5	0.5	nd[g]
35, X = H_2	nd[g]	nd[g]	31	31	nd[g]
R = CBP, (4-chlorobiphenyl)methyl					
36, X = O	0.03	0.03	2.5	2.5	0.03
37, X = S	2	2	4	4	2
38, X = NH	0.03	0.06	0.005	0.005	0.06
39, X = H_2	0.5	0.25	0.13	0.06	0.5

[a]MIC = Minimum inhibitory concentration. [b]ATCC 25923. [c]ATCC 43300. [d]BM 4166. [e]ATCC BAA-2317. [f]ATCC 51299. [g]not determined.

Figure 8. In vitro antimicrobial activity.

which they are based (see Figure 3). Although it is well-established that the attached unmodified carbohydrate does not alter the in vitro antimicrobial activity (potency) or influence the target D-Ala-D-Ala or D-Ala-D-Lac binding, the disaccharide impacts the in vivo activity, increasing the water solubility, influencing the pharmacokinetic and distribution properties,

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and contributing a potential second mechanism of action. An analogous impact on the vancomycin analogues **33**−**35** might be expected since each represents the change of a single atom in the binding pocket (residue-4 carbonyl O → S, NH, H_2), and they would be the preferred compounds (vs **3**−**5**) with which to probe in vivo activity. Within this series, vancomycin displayed potent activity against VSSA and MRSA (MIC = 0.5 μg/mL) but was ineffective against VanA VRE (MIC = 250 μg/mL) and only modestly active against VanB VRE (MIC = 8 μg/mL) under the assay conditions employed. Consistent with its lack of binding to either D-Ala-D-Ala or D-Ala-D-Lac, thioamide **33** proved to be inactive as an antimicrobial agent against both sensitive and resistant bacteria (MICs >32 μg/mL). Both amidine **34**[34] and the methylene analogue **35** reinstated the activity against VanA VRE with MICs of 0.5 and 31 μg/mL, respectively, precisely in line with expectations based on the relative dual D-Ala-D-Ala and D-Ala-D-Lac binding affinities of their aglycons and matching the activities observed with the corresponding aglycons **4** and **5** (see Figure 3). Of most significance, amidine **34** displayed a potency against VanA VRE that matched the activity vancomycin displays against sensitive bacteria (VSSA and MRSA, MICs = 0.5 μg/mL).

Given the distinct origins of their impacts on the antimicrobial activity of vancomycin, we expected that incorporation of the peripheral chlorobiphenyl modification into the structure of the binding-pocket-modified vancomycin analogues would further increase their antimicrobial activity against not only vancomycin-sensitive but also vancomycin-resistant bacteria to truly remarkable levels. Although this conceivably could have been demonstrated by substitution of the synthetic aglycons **2**−**5**, we expected that the most definitive assessment of the dual impact would be a direct comparison of CBP-vancomycin (**36**) with **37**−**39** wherein a series of key changes in a single atom in the binding pocket were introduced, despite the synthetic challenges this posed. This choice of both the site of modification and the use of the chlorobiphenyl modification proved key to understanding the behavior of such analogues and revealed unique insights into the origin of the effects.

In line with reports of its impact, introduction of the (4-chlorobiphenyl)methyl group into vancomycin (**36** vs **1**) resulted in 100-fold improvements in the activity against VanA and VanB VRE (MIC = 2.5 vs 250 μg/mL) and 20-fold improvements against VSSA and MRSA (MIC = 0.03 vs 0.5 μg/mL) in the strains examined. In spite of the increases in potency, it remained 100-fold less effective against VanA VRE than against sensitive bacterial strains. Both amidine **38** and the methylene analogue **39** displayed the same 100-fold increases in activity against VanA VRE, exhibiting remarkable MICs of 0.005 and 0.06−0.13 μg/mL, respectively. Just as significantly, introduction of the chlorobiphenyl group into either the vancomycin amidine (to give **38**) or the vancomycin methylene analogue (to give **39**) resulted in compounds with remarkable spectra of activity at these impressive potencies. Both were equally effective against both vancomycin-sensitive bacteria (VSSA and MRSA) and vancomycin-resistant bacteria (VanA and VanB VRE), of which VanA VRE proved especially sensitive to the analogues. Both analogues exhibited MICs below 1 μg/mL across the bacterial panel, and amidine **38** was found to be on average 15-fold more potent than the methylene analogue **39**, precisely in line with their relative dual ligand binding affinities. Moreover, amidine **38** not only matched the activity that CBP-vancomycin (**36**) displays against vancomycin-sensitive bacteria (VSSA and MRSA) but also exhibited this extraordinary potency against VanA and VanB vancomycin-resistant bacteria. In fact, the activity of **38** against the most stringent of the resistant bacteria, VanA VRE, was nearly 10-fold better than the potency it displayed against the sensitive bacteria, representing a 500-fold increase in activity relative to **36** and a 50000-fold increase in activity relative to vancomycin (**1**) itself. Thus, the introduction of chlorobiphenyl into the pocket-modified vancomycin analogues **38** (MICs = 0.005−0.06 μg/mL) and **39** (MICs = 0.06−0.5 μg/mL) synergistically increased their potency against both vancomycin-sensitive and vancomycin-resistant bacteria. Insights into this behavior came from an examination of **37**, the chlorobiphenyl derivative of vancomycin thioamide (**33**). Introduction of the (4-chlorobiphenyl)methyl group into **33** to give **37** reinstates impressive and equally potent activity (MIC = 2−4 μg/mL) against all vancomycin-sensitive and vancomycin-resistant strains despite its inability to bind the primary cell wall target D-Ala-D-Ala/D-Ala-D-Lac. It is unlikely that such effective activity can be achieved simply by the effects of antibiotic membrane anchoring, antibiotic dimerization, or disruption of bacterial membrane integrity. Rather, it likely reflects potent antimicrobial activity derived from a second mechanism of action impacting cell wall synthesis unrelated to D-Ala-D-Ala/D-Ala-D-Lac binding. In line with observations made with CBP-vancomycin and analogues containing damaged binding pockets, this most likely involves potent transglycosylase inhibition mediated by direct binding to the enyzme.[22,23] Because of the insights derived from the comparative examination of the thioamides **33** and **37**, the behavior of the CBP-vancomycin amidine **38** and CBP-vancomycin methylene analogue **39** likely represents a spectrum of activity and potency derived from inhibition of bacterial cell wall synthesis through two synergistic mechanisms, one involving inhibition of transpeptidase-catalyzed cell wall cross-linking through dual substrate (D-Ala-D-Ala and D-Ala-D-Lac) binding and the second involving direct inhibition of transglycosylase independent of such ligand binding. If this is the case, it suggests that resistance is even more unlikely to emerge against such analogues since it would entail simultaneous bacterial changes to two distinct targets of the antibiotics, one of which is not subject to direct genetic alterations. Therefore, both **38** and **39** are superb candidates for preclinical development. Their preliminary assessments not only indicate that they address the present-day emerging vancomycin resistance and exhibit remarkable spectra of activity and superb antimicrobial potency but also that they are endowed with a unique combination of characteristics that may allow them to display the 50-year clinical durability of vancomycin.

Although at this stage still speculative, the four chlorobiphenyl derivatives **36**−**39** are also uniquely poised to help unravel the subtleties of the mechanisms of action of such modified glycopeptide antibiotics. Because of its inability to bind either D-Ala-D-Ala or D-Ala-D-Lac, CBP-[Ψ[C(═S)NH]-Tpg[4]]vancomycin (thioamide **37**) derives its antimicrobial activity (MIC = 2−4 μg/mL) exclusively through a distinct second mechanism of action that does not involve ligand binding and likely involves direct inhibition of transglycosylase.[22,23] By virtue of its inability to bind D-Ala-D-Lac, CBP-vancomycin (**36**) also likely derives its similarly potent activity against vancomycin-resistant organisms (VanA VRE, MIC = 2.5 μg/mL) by this same mechanism potentially involving only the direct inhibition of transglycosylase, whereas its more potent

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activity against vancomycin-sensitive organisms (VSSA and MRSA, MIC = 0.03 μg/mL) is derived from the equally potent and synergistic inhibition of both transpeptidase (via D-Ala-D-Ala binding and substrate sequestration) and transglycosylase (direct enzyme inhibition). As a result of the binding pocket redesign and ability to exhibit fully effective dual D-Ala-D-Ala and D-Ala-D-Lac binding combined with the peripheral chlorobiphenyl-mediated potential direct inhibition of transglycosylase, CBP-[Ψ[C(=NH)NH]Tpg^4]vancomycin (**38**) picks up the ability to effectively inhibit transpeptidase in vancomycin-resistant bacteria (VanA VRE) via D-Ala-D-Lac binding, maintains the ability to inhibit transpeptidase in vancomycin-sensitive bacteria (VSSA and MRSA) via D-Ala-D-Ala binding, allows the potential indirect transglycosylase inhibition through ligand binding, and benefits potentially from an equally potent and synergistic direct inhibition of transglycosylase independent of D-Ala-D-Ala/D-Ala-D-Lac binding. The net result is an antibiotic that benefits from two equally potent, independent, and synergistic mechanisms of action and that displays remarkable antimicrobial potencies (MIC = 0.06−0.005 μg/mL) against both vancomycin-sensitive and vancomycin-resistant bacteria. In contrast but similarly interestingly, the potency of CBP-[Ψ[CH_2NH]Tpg^4]vancomycin (**39**) (MIC = 0.5−0.06 μg/mL) suggests that the principal mechanism by which it acts is through the potential chlorobiphenyl-mediated direct inhibition of transglycosylase, but now with a second less-potent contribution derived from its balanced, albeit reduced, dual ligand binding affinities for inhibition of transpeptidase in either vancomycin-sensitive and vancomycin-resistant bacteria. It is remarkable that the series appears to display the combined activity of two independent mechanisms, which act synergistically with one another, to provide predictable potency trends derived independently from the binding pocket modifications and the peripheral carbohydrate substitution. Kahne has shown that although the potency of most lipid-linked glycopeptides or their aglycons lose activity against VanA strains when their binding pocket is chemically damaged,[52] indicating that ligand binding is important to their activity, a small subset (including CBP-vancomycin) retain good antimicrobial activity even when their binding pocket is chemically damaged.[22] Moreover, it is such derivatives that were shown by Kahne and Walker to effectively inhibit transglycosylase without substrate or ligand binding, suggesting that they directly bind and inhibit the enzyme. CBP-[Ψ[C(=S)NH]Tpg^4]vancomycin embodies these same characteristics, displays the same VanA VRE potency, and likely will display the same behavior toward transglycosylases. As pointed out by Kahne, is likely that this activity against VanA strains requires specific positioning of the hydrophobic substituent attached to the vancomycin disaccharide. As a consequence, it is especially notable that our studies were conducted with single-atom changes to the binding pocket of vancomycin and CBP-vancomycin and not conducted on simpler, more accessible aglycon derivatives.

CONCLUSIONS

Clinical uses of vancomycin include treatment of patients on dialysis, allergic to β-lactam antibiotics, or undergoing cancer chemotherapy.[1] However, the most widely recognized use of vancomycin is the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections.[1] The prevalence of MRSA in intensive care units (ICUs) (60% of SA infections in the United States are MRSA)[55] and its movement from a hospital-acquired to a community-acquired infection in the last 10 years has increased the number and intensified the need to treat such resistant bacterial infections. In addition, vancomycin-resistant bacterial strains are also on the rise, with U.S. ICU clinical isolates of vancomycin-resistant *Enterococcus faecalis* approaching 30%,[55] albeit in strains presently sensitive to other antibiotics. Most feared is the recent emergence of MRSA strains now resistant or insensitive to vancomycin (VRSA and VISA). This poses a major health problem and has intensified efforts to develop antibiotics that not only combat this resistance but also display the durability of vancomycin.[7]

Herein we have provided full details of the completion of the total syntheses and evaluation of [Ψ[C(=S)NH]Tpg^4]-vancomycin (**33**), [Ψ[C(=NH)NH]Tpg^4]vancomycin (**34**), and [Ψ[CH_2NH]Tpg^4]vancomycin (**35**), fully adorned analogues of vancomycin that contain single-atom changes or exchanges in the binding pocket (residue-4 carbonyl O → S, NH, H_2) designed to probe and directly address the molecular basis of vancomycin resistance; their corresponding C-terminus hydroxymethyl analogues **23**−**25**, on which the chemistry was piloted; and their corresponding chlorobiphenyl derivatives (**26**−**28** and **36**−**39**). The latter derivatives constitute combined pocket and peripherally modified vancomycin analogues anticipated to exhibit remarkable properties.

By design, the sequential enzyme-catalyzed glycosylation reactions of the modified aglycons, the final amidine introductions, and the intermediate reductive aminations used for the chlorobiphenyl substitutions were conducted without the need of protecting groups, establishing the foundation and providing the methodology for potential semisynthetic or biosynthetic preparations of such glycopeptide analogues. As expected, the activities of the pocket-modified vancomycin analogues **33**−**35** matched the in vitro antimicrobial activities of the corresponding aglycon analogues **3**−**5** on which they are based. In line with expectations based on the behavior of the corresponding aglycons and in stark contrast to one another, the vancomycin amidine and, to a lesser extent, the vancomycin methylene analogue reestablish potent antimicrobial activity against VanA VRE, whereas vancomycin thioamide is inactive even against vancomycin-sensitive bacteria; results that parallel the dual D-Ala-D-Ala and D-Ala-D-Lac binding affinities of the corresponding aglycons. The introduction of a peripheral chlorobiphenyl modification converts vancomycin thioamide into an effective antimicrobial agent that is active against vancomycin-sensitive and vancomycin-resistant bacteria (MIC = 2−4 μg/mL), even though it is not capable of D-Ala-D-Ala/D-Ala-D-Lac binding. It also converts the vancomycin amidine and vancomycin methylene analogues into compounds with remarkable spectra of activity (VSSA, MRSA, and VanA and VanB VRE) and truly impressive potencies (MIC = 0.06−0.005 and 0.06−0.5 μg/mL, respectively) that are likely derived from inhibition of cell wall biosynthesis through at least two distinct mechanisms, only one of which is dependent on D-Ala-D-Ala/D-Ala-D-Lac binding. In addition to indicating that the described peripheral and productive pocket modifications are synergistic, such analogues are likely to be even less prone to acquired clinical resistance than vancomycin and to display durable antibiotic activity.

ASSOCIATED CONTENT

Supporting Information

Full experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) (a) Cooper, G. L.; Given, D. B. In *Vancomycin: A Comprehensive Review of 30 Years of Clinical Experience*; Park Row Publications: Indianapolis, IN, 1986; pp 1−5. (b) *Glycopeptide Antibiotics*; Nagarajan, R., Ed.; Marcel Dekker: New York, 1994. (c) Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. T. *Chem. Rev.* **2005**, *105*, 425.

(2) McCormick, M. H.; Stark, W. M.; Pittenger, G. E.; Pittenger, R. C.; McGuire, J. M. *Antibiot. Annu.* **1955−1956**, 606.

(3) Parenti, F.; Beretta, G.; Berti, M.; Arioli, V. *J. Antibiot.* **1978**, *31*, 276.

(4) (a) Nicas, T. I.; Mullen, D. L.; Flokowitsch, J. E.; Preston, D. A.; Snyder, N. J.; Zweifel, M. J.; Wilkie, S. C.; Rodriguez, M. J.; Thompson, R. C.; Cooper, R. D. *Antimicrob. Agents Chemother.* **1996**, *40*, 2194. (b) Nagarajan, R.; Schabel, A. A.; Occolowitz, J. L.; Counter, F. T.; Ott, J. L.; Felty-Duckworth, A. M. *J. Antibiot.* **1989**, *42*, 63. (c) Markham, A. *Drugs* **2014**, *74*, 1823.

(5) (a) Candiani, G.; Abbondi, M.; Borgonovi, M.; Romano, G.; Parenti, F. *J. Antimicrob. Chemother.* **1999**, *44*, 179. (b) Anderson, V. R.; Keating, G. M. *Drugs* **2008**, *68*, 639.

(6) (a) Judice, J. K.; Pace, J. L. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 4165. (b) Corey, G. R.; Stryjewski, M. E.; Weyenberg, W.; Yasothan, U.; Kirkpatrick, P. *Nat. Rev. Drug Discovery* **2009**, *8*, 929.

(7) (a) Harris, C. M.; Kopecka, H.; Harris, T. M. *J. Am. Chem. Soc.* **1983**, *105*, 6915. (b) Williamson, M. P.; Williams, D. H. *J. Am. Chem. Soc.* **1981**, *103*, 6580.

(8) (a) Leclercq, R.; Derlot, E.; Duval, J.; Courvalin, P. *N. Engl. J. Med.* **1988**, *319*, 157. (b) Courvalin, P. *Clin. Infect. Dis.* **2006**, *42*, S25.

(9) (a) Weigel, L. M.; Clewell, D. B.; Gill, S. R.; Clark, N. C.; McDougal, L. K.; Flannagan, S. E.; Kolonay, J. F.; Shetty, J.; Killgore, G. E.; Tenover, F. C. *Science* **2003**, *302*, 1569. (b) Howden, B. P.; Davies, J. K.; Johnson, P. D.; Stinear, T. P.; Grayson, M. L. *Clin. Microbiol. Rev.* **2010**, *23*, 99. (c) Walsh, T. R.; Howe, R. A. *Annu. Rev. Microbiol.* **2002**, *56*, 657.

(10) (a) Brickner, S. J. *Curr. Pharm. Des.* **1996**, *2*, 175. (b) Scheetz, M. H.; Knechtel, S. A.; Malczynski, M.; Postelnick, M. J.; Qi, C. *Antimicrob. Agents Chemother.* **2008**, *52*, 2256. (c) Brickner, S. J.; Barbachyn, M. R.; Hutchinson, D. K.; Manninen, P. R. *J. Med. Chem.* **2008**, *51*, 1981.

(11) (a) Baltz, R. H.; Miao, V.; Wrigley, S. K. *Nat. Prod. Rep.* **2005**, *22*, 717. (b) Baltz, R. H. *Curr. Opin. Chem. Biol.* **2009**, *13*, 144.

(12) (a) Malabarba, A.; Nicas, T. I.; Thompson, R. C. *Med. Res. Rev.* **1997**, *17*, 69. (b) Najarajan, R. *J. Antibiot.* **1993**, *46*, 1181. (c) Van Bambeke, F. V.; Laethem, Y. V.; Courvalin, P.; Tulkens, P. M. *Drugs* **2004**, *64*, 913. (d) Butler, M. S.; Hansford, K. A.; Blaskovich, M. A.; Halai, R.; Cooper, M. A. *J. Antibiot.* **2014**, *67*, 631. For recent approaches, see: (e) Fowler, B. S.; Laemmerhold, K. M.; Miller, S. J. *J. Am. Chem. Soc.* **2012**, *134*, 9755. (f) Griffith, B. R.; Krepel, C.; Fu, X.; Blanchard, S.; Ahmed, A.; Edmiston, C. E.; Thorson, J. S. *J. Am. Chem. Soc.* **2007**, *129*, 8150. (g) Jain, R. K.; Trias, J.; Ellman, J. A. *J. Am. Chem. Soc.* **2003**, *125*, 8740. (h) Weist, S.; Kittel, C.; Bischoff, D.; Bister, B.; Pfeifer, V.; Nicholson, G. J.; Wohlleben, W.; Süssmuth, R. D. *J. Am. Chem. Soc.* **2004**, *126*, 5942.

(13) (a) Süssmuth, R. D. *ChemBioChem* **2002**, *3*, 295. (b) von Nussbaum, F.; Brands, M.; Hinzen, B.; Weigand, S.; Häbich, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 5072.

(14) James, R. C.; Pierce, J. G.; Okano, A.; Xie, J.; Boger, D. L. *ACS Chem. Biol.* **2012**, *7*, 797.

(15) (a) Wright, G. D. *Chem. Commun.* **2011**, *47*, 4055. (b) Walsh, C. T. *Nature* **2000**, *406*, 775.

(16) Marshall, C. G.; Lessard, I. A.; Park, I.; Wright, G. D. *Antimicrob. Agents Chemother.* **1998**, *42*, 2215.

(17) Identified mechanisms of resistance: VanA and VanB (inducible D-Ala-D-Ala to D-Ala-D-Lac, 1000-fold), VanC (D-Ala-D-Ser, 20-fold), and thickened cell wall (increased number of target sites, 10-fold). See: Courvalin, P. *Clin. Infect. Dis.* **2006**, *42*, S25.

(18) (a) Allen, N. E.; Hobbs, J. N., Jr.; Nicas, T. I. *Antimicrob. Agents Chemother.* **1996**, *40*, 2356. (b) Sharman, G. J.; Try, A. C.; Dancer, R. J.; Cho, Y. R.; Staroske, T.; Bardsley, B.; Maguire, A. J.; Cooper, M. A.; O'Brien, D. P.; Williams, D. H. *J. Am. Chem. Soc.* **1997**, *119*, 12041. (c) Allen, N. E.; Nicas, T. I. *FEMS Microbiol. Rev.* **2003**, *26*, 511.

(19) (a) Hong, H.-J.; Hutchings, M. I.; Buttner, M. J. *Adv. Exp. Med. Biol.* **2008**, *631*, 200. (b) Koteva, K.; Hong, H.-J.; Wang, X. D.; Nazi, I.; Hughes, D.; Naldrett, M. J.; Buttner, M. J.; Wright, G. D. *Nat. Chem. Biol.* **2010**, *6*, 327. (c) Ikeda, S.; Hanaki, H.; Yanagisawa, C.; Ikeda-Dantsuji, Y.; Matsui, H.; Iwatsuki, M.; Shiomi, K.; Nakae, T.; Sunakawa, K.; Omura, S. *J. Antibiot.* **2010**, *63*, 533. (d) Kwun, M. J.; Novotna, G.; Hesketh, A. R.; Hill, L.; Hong, H. *Antimicrob. Agents Chemother.* **2013**, *57*, 4470.

(20) Higgins, D. L.; Chang, R.; Debabov, D. V.; Leung, J.; Wu, T.; Krause, K. M.; Sandvik, E.; Hubbard, J. M.; Kaniga, K.; Schmidt, D. E., Jr.; Gao, Q.; Cass, R. T.; Karr, D. E.; Benton, B. M.; Humphrey, P. P. *Antimicrob. Agents Chemother.* **2005**, *49*, 1127.

(21) Cooper, R. D. G.; Snyder, N. J.; Zweifel, M. J.; Staszak, M. A.; Wilkie, S. C.; Nicas, T. I.; Mullen, D. L.; Butler, T. F.; Rodriguez, M. J.; Huff, B. E.; Thompson, R. C. *J. Antibiot.* **1996**, *49*, 575.

(22) (a) Ge, M.; Chen, Z.; Onishi, H. R.; Kohler, J.; Silver, L. L.; Kerns, R.; Fukuzawa, S.; Thompson, C.; Kahne, D. *Science* **1999**, *284*, 507. (b) Chen, L.; Walker, D.; Sun, B.; Hu, Y.; Walker, S.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5658.

(23) Goldman, R. C.; Baizman, E. R.; Longley, C. B.; Branstrom, A. A. *FEMS Microbiol. Lett.* **2000**, *183*, 209.

(24) (a) Boger, D. L.; Miyazaki, S.; Kim, S. H.; Wu, J. H.; Castle, S. L.; Loiseleur, O.; Jin, Q. *J. Am. Chem. Soc.* **1999**, *121*, 10004. (b) Boger, D. L.; Kim, S. H.; Mori, Y.; Weng, J.-H.; Rogel, O.; Castle, S. L.; McAtee, J. J. *J. Am. Chem. Soc.* **2001**, *123*, 1862. (c) Crowley, B. M.; Mori, Y.; McComas, C. C.; Tang, D.; Boger, D. L. *J. Am. Chem. Soc.* **2004**, *126*, 4310. (d) Shimamura, H.; Breazzano, S. P.; Garfunkle, J.; Kimball, F. S.; Trzupek, J. D.; Boger, D. L. *J. Am. Chem. Soc.* **2010**, *132*, 7776.

(25) (a) Evans, D. A.; Wood, M. R.; Trotter, B. W.; Richardson, T. I.; Barrow, J. C.; Katz, J. L. *Angew. Chem., Int. Ed.* **1998**, *37*, 2700. (b) Evans, D. A.; Dinsmore, C. J.; Watson, P. S.; Wood, M. R.; Richardson, T. I.; Trotter, B. W.; Katz, J. L. *Angew. Chem., Int. Ed.* **1998**, *37*, 2704. (c) Evans, D. A.; Barrow, J. C.; Watson, P. S.; Ratz, A. M.; Dinsmore, C. J.; Evrard, D. A.; DeVries, K. M.; Ellman, J. A.; Rychnovsky, S. D.; Lacour, J. *J. Am. Chem. Soc.* **1997**, *119*, 3419. (d) Evans, D. A.; Dinsmore, C. J.; Ratz, A. M.; Evrard, D. A.; Barrow, J. C. *J. Am. Chem. Soc.* **1997**, *119*, 3417.

(26) (a) Nicolaou, K. C.; Takayanagi, M.; Jain, N. F.; Natarajan, S.; Koumbis, A. E.; Bando, T.; Ramanjulu, J. M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2717. (b) Nicolaou, K. C.; Natarajan, S.; Li, H.; Jain, N. F.; Hughes, R.; Solomon, M. E.; Ramanjulu, J. M.; Boddy, C. N. C.; Takayanagi, M. *Angew. Chem., Int. Ed.* **1998**, *37*, 2708. (c) Nicolaou, K. C.; Jain, N. F.; Natarajan, S.; Hughes, R.; Solomon, M. E.; Li, H.;

DOI: 10.1021/jacs.5b01008
J. Am. Chem. Soc. 2015, 137, 3693−3704

Ramanjulu, J. M.; Takayanagi, M.; Koumbis, A. E.; Bando, T. *Angew. Chem., Int. Ed.* **1998**, *37*, 2714.
(27) (a) Boger, D. L. *Med. Res. Rev.* **2001**, *21*, 356. (b) Nicolaou, K. C.; Boddy, C. N. C.; Brase, S.; Winssinger, N. *Angew. Chem., Int. Ed.* **1999**, *38*, 2096. (c) Evans, D. A.; DeVries, K. M. *Drugs Pharm. Sci.* **1994**, *63*, 63.
(28) (a) Bugg, T. D. H.; Wright, G. D.; Dutka-Malen, S.; Arthur, M.; Courvalin, P.; Walsh, C. T. *Biochemistry* **1991**, *30*, 10408. For reviews, see: (b) Walsh, C. T. *Science* **1993**, *261*, 308. (c) Walsh, C. T.; Fisher, S. L.; Park, I.-S.; Prahalad, M.; Wu, Z. *Chem. Biol.* **1996**, *3*, 21. (d) Lessard, I. A.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 11028. (e) Healy, V. L.; Lessard, I. A.; Roper, D. I.; Knox, J. R.; Walsh, C. T. *Chem. Biol.* **2000**, *7*, R109.
(29) (a) Perkins, H. R. *Pharmacol. Ther.* **1982**, *16*, 181. (b) Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 1332. (c) Schaefer, M.; Schneider, T. R.; Sheldrick, G. M. *Structure* **1996**, *4*, 1509.
(30) McComas, C. C.; Crowley, B. M.; Boger, D. L. *J. Am. Chem. Soc.* **2003**, *125*, 9314.
(31) Crowley, B. M.; Boger, D. L. *J. Am. Chem. Soc.* **2006**, *128*, 2885.
(32) (a) Xie, J.; Pierce, J. G.; James, R. C.; Okano, A.; Boger, D. L. *J. Am. Chem. Soc.* **2011**, *133*, 13946. (b) Xie, J.; Okano, A.; Pierce, J. G.; James, R. C.; Stamm, S.; Crane, C. M.; Boger, D. L. *J. Am. Chem. Soc.* **2012**, *134*, 1284.
(33) Okano, A.; James, R. C.; Pierce, J. G.; Xie, J.; Boger, D. L. *J. Am. Chem. Soc.* **2012**, *134*, 8790.
(34) Okano, A.; Nakayama, A.; Schammel, A. W.; Boger, D. L. *J. Am. Chem. Soc.* **2014**, *136*, 13522.
(35) (a) Bowden, K.; Heilbron, I. M.; Jones, E. R. H.; Weedon, B. C. L. *J. Chem. Soc.* **1946**, 39. (b) Bowers, A.; Halsall, T. G.; Jones, E. R. H.; Lemin, A. J. *J. Chem. Soc.* **1953**, 2548.
(36) (a) Corey, E. J.; Suggs, J. W. *Tetrahedron Lett.* **1975**, *16*, 2647. (b) Corey, E. J.; Boger, D. L. *Tetrahedron Lett.* **1978**, *19*, 2461.
(37) Corey, E. J.; Schmidt, G. *Tetrahedron Lett.* **1979**, *20*, 399.
(38) Moorthy, J. N.; Singhal, N. *J. Org. Chem.* **2005**, *70*, 1926.
(39) Throughout the studies with the thioamide intermediates, because of low-level contaminants in EtOAc as it is often presently supplied, its wash with 5% aq. Na_2CO_3 was mandatory to prevent oxidation of the thioamide when EtOAc was used as either an extraction or chromatography solvent.
(40) (a) Ge, M.; Thompson, C.; Kahne, D. *J. Am. Chem. Soc.* **1998**, *120*, 11014. (b) Thompson, C.; Ge, M.; Kahne, D. *J. Am. Chem. Soc.* **1999**, *121*, 1237. (c) Leimkuhler, C.; Chen, Z.; Kruger, R. G.; Oberthur, M.; Lu, W.; Walsh, C. T.; Kahne, D. *Tetrahedron: Asymmetry* **2005**, *16*, 599.
(41) (a) Nicolaou, K. C.; Mitchell, H. J.; Jain, N. F.; Winssinger, N.; Hughes, R.; Bando, T. *Angew. Chem., Int. Ed.* **1999**, *38*, 240. (b) Nicolaou, K. C.; Mitchell, H. J.; Jain, N. F.; Bando, T.; Hughes, R.; Winssinger, N.; Natarajan, S.; Koumbis, A. E. *Chem.—Eur. J.* **1999**, *5*, 2648.
(42) Ritter, T. K.; Mong, K.-K. T.; Liu, T.; Nakatani, T.; Wong, C. H. *Angew. Chem., Int. Ed.* **2003**, *42*, 4657.
(43) (a) Losey, H. C.; Peczuh, M. W.; Chen, Z.; Eggert, U. S.; Dong, S. D.; Pelczer, I.; Kahne, D.; Walsh, C. T. *Biochemistry* **2001**, *40*, 4745. (b) Oberthur, M.; Leimkuhler, C.; Kruger, R. G.; Lu, W.; Walsh, C. T.; Kahne, D. *J. Am. Chem. Soc.* **2005**, *127*, 10747. (c) Losey, H. C.; Jiang, J.; Biggins, J. B.; Oberthur, M.; Ye, X.; Dong, S. D.; Kahne, D.; Thorson, J. S.; Walsh, C. T. *Chem. Biol.* **2002**, *9*, 1305. (d) Dong, S. D.; Oberthur, M.; Losey, H. C.; Anderson, J. W.; Eggert, U. S.; Peczuh, M. W.; Walsh, C. T.; Kahne, D. *J. Am. Chem. Soc.* **2002**, *124*, 9064. For enzymatic acyltransferase functionalization of the carbohydrates, see: (e) Kruger, R. G.; Lu, W.; Oberthur, M.; Tao, J.; Kahne, D.; Walsh, C. T. *Chem. Biol.* **2005**, *12*, 131.
(44) (a) Thayer, D. A.; Wong, C. H. *Chem.—Asian J.* **2006**, *1*, 445. (b) Thayer, D. A.; Yu, H. N.; Galan, M. C.; Wong, C. H. *Angew. Chem., Int. Ed.* **2005**, *44*, 4596.
(45) (a) Solenberg, P. J.; Matsushima, P.; Stack, D. R.; Wilkie, S. C.; Thompson, R. C.; Baltz, R. H. *Chem. Biol.* **1997**, *4*, 195. (b) Oh, T.-J.; Kim, D. H.; Kang, S. Y.; Yamaguchi, T.; Sohng, J. K. *J. Antibiot.* **2011**, *64*, 103.
(46) (a) Fu, X.; Albermann, C.; Zhang, C. S.; Thorson, J. S. *Org. Lett.* **2005**, *7*, 1513. (b) Fu, X.; Albermann, C.; Jiang, J. Q.; Liao, J. C.; Zhang, C. S.; Thorson, J. S. *Nat. Biotechnol.* **2003**, *21*, 1467.
(47) (a) Nakayama, A.; Okano, A.; Feng, Y.; Collins, J. C.; Collins, K. C.; Walsh, C. T.; Boger, D. L. *Org. Lett.* **2014**, *16*, 3572.
(48) Presently there are four commercial suppliers of UMP morpholidate. We employed material obtained from Sigma-Aldrich ($176/g).
(49) Oberthur, M.; Leimkuhler, C.; Kahne, D. *Org. Lett.* **2004**, *6*, 2873.
(50) Commercially available (Sigma-Aldrich).
(51) Corey, E. J.; Boger, D. L. *Tetrahedron Lett.* **1978**, *19*, 5.
(52) (a) Chen, Z.; Eggert, U. S.; Dong, S. D.; Shaw, S. J.; Sun, B.; LaTour, J. V.; Kahne, D. *Tetrahedron* **2002**, *58*, 6585. (b) Kerns, R.; Dong, S. D.; Fukuzawa, S.; Carbeck, J.; Kohler, J.; Silver, L.; Kahne, D. *J. Am. Chem. Soc.* **2000**, *122*, 12608.
(53) The sequence was conducted only twice on a preparative scale and consequently is not yet optimized.
(54) Vancomycin-sensitive *S. aureus* (VSSA, ATCC 25923): sensitive to vancomycin, teicoplanin, oritavancin, daptomycin, linezolid, quinupristin−dalfopristin, fussidic acid, azithromycin, telithromycin, gentamycin, penicillin V, nafcillin, ampicillin, oxacillin, ciprofloxacin, levofloxacin, garenoxacin, and moxifloxacin. Methicillin-resistant *S. aureus* (MRSA, ATCC 43300): sensitive to vancomycin, teicoplanin, daptomycin, linezolid, tigecycline, and ciprofloxacin; resistant to methicillin, amoxicillin, amoxicillin with clavulanic acid, cephalexin, enrofloxacin, erythromycin, azithromycin, gentamycin, clindamycin, lincomycin−spectinomycin, neomycin, oxacillin, penicillin G, streptomycin, trimethoprim−sulfamethoxazole, and tetracycline. VanA *E. faecalis* (VanA VRE, BM 4166): resistant to erythromycin, gentamicin, chloramphenicol, and ciprofloxacin as well as vancomycin and teicoplanin; sensitive to daptomycin. 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. VanB *E. faecalis* (VanB VRE, ATCC 51299): resistant to vancomycin, streptomycin, gentamicin; sensitive to teicoplanin, ampicillin, tetracycline, and ciprofloxacin.
(55) (a) Cardo, D.; Horan, T.; Andrus, M.; Dembinski, M.; Edwards, J.; Peavy, G.; Tolson, J.; Wagner, D. *Am. J. Infect. Control* **2004**, *32*, 470. (b) Laxminarayan, R. Antibiotic Resistance: The Unfolding Crisis. In *Extending the Cure: Policy Responses to the Growing Threat of Antibiotic Resistance*; Laxminarayan, R., Malani, A., Howard, D., Smith, D. L., Eds.; Resources for the Future: Washington, DC, 2007; Chapter 1, pp 25−37. (c) Walsh, C. T.; Fischbach, M. A. *Sci. Am.* **2009**, *301* (1), 44.

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J. Am. Chem. Soc. 2015, 137, 3693−3704