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Enzymatic Glycosylation of Vancomycin Aglycon: Completion of a Total Synthesis of Vancomycin and N- and C-Terminus Substituent Effects of the Aglycon Substrate

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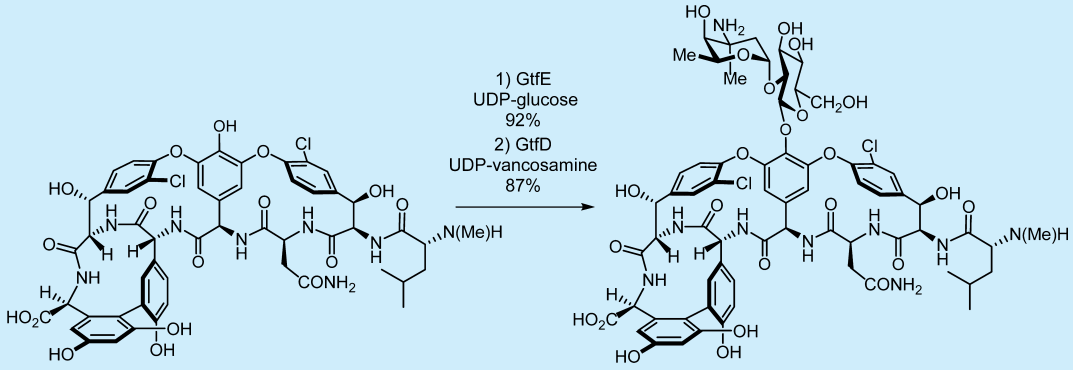
Supporting Information



ABSTRACT: Studies on the further development of the sequential glycosylations of the vancomycin aglycon catalyzed by the glycosyltransferases GtfE and GtfD and the observation of unusual, perhaps unexpected, aglycon substrate substituent effects on the rate and efficiency of the initial glycosylation reaction are reported.

The glycopeptide antibiotics are the most important class of drugs used for the treatment of resistant bacterial infections that include those caused by methicillin-resistant *Staphylococcus aureus* (MRSA).[1,2] After more than 50 years of clinical use, the emergence of vancomycin-resistant Gram-positive pathogens, including not only vancomycin-resistant Enterococci (VRE)[3] but also the more recent vancomycin-resistant *S. aureus* (VRSA),[4] presents an imminent challenge to public health at a time few new antibiotics are being developed. This has led to renewed interest in the search for additional effective treatments for such resistant organisms that possess the long-term durability of the two clinically used glycopeptide antibiotics, vancomycin[5] (Figure 1) and teicoplanin.[1,2,6]

In recent efforts, we reported the total syntheses of two vancomycin aglycon analogues[7,8] designed to address the underlying molecular basis of resistance to the glycopeptide antibiotics.[9] Rational single atom changes to the vancomycin binding pocket designed to achieve dual binding to D-Ala-D-Ala and D-Ala-D-Lac were introduced to reinstate antimicrobial activity against vancomycin-resistant bacteria while maintaining activity against vancomycin-sensitive bacteria.[10,11] The success of the efforts set the stage for not only ongoing continued studies that might further improve their activity and potency[12] but also the subsequent efforts needed to complete the peripheral tailoring of the aglycons with introduction of the L-vancosaminyl-1,2-D-glucosyl disaccharide. Although the attached carbohydrate does not impact in vitro antimicrobial activity or significantly alter target D-Ala-D-Ala or D-Ala-D-Lac binding affinities responsible for inhibition of transpeptidase-

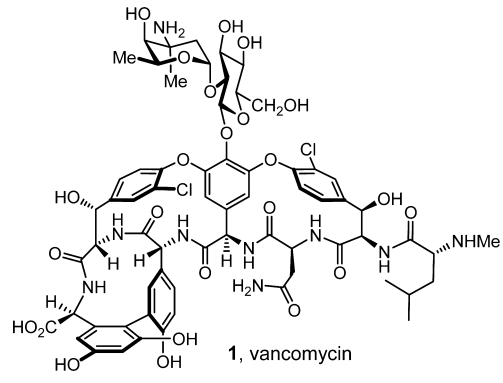


Figure 1. Structure of vancomycin.

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mediated bacterial cell wall cross-linking, the nature of the appended carbohydrate affects in vivo activity, increasing its water solubility and influencing its PK and distribution properties. It has also been suggested that the vancomycin carbohydrate may mediate a second, though less potent, mechanism of action through direct inhibition of transglycosylase enzymes.[13]

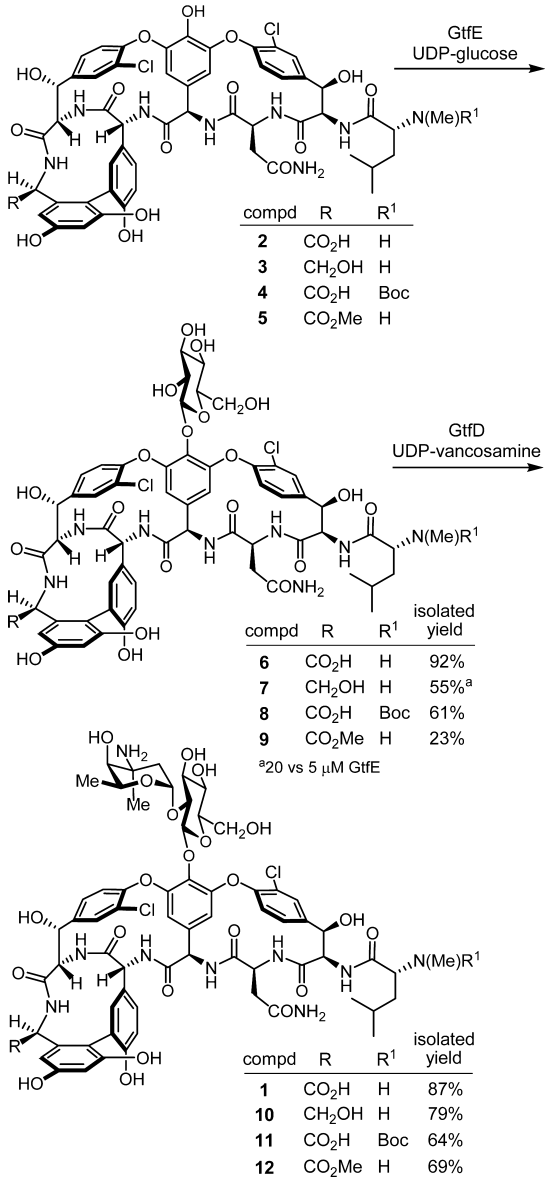
For vancomycin, the carbohydrate introduction has been approached by using either chemical[14−16] or enzymatic[17−20] glycosylations for sequential introduction of the glucose and vancosamine sugars located on the central residue of the aglycon or pseudoaglycon, respectively. Herein, we report studies on the further development of the sequential glycosylations of the vancomycin aglycon catalyzed by the glycosyltransferases GtfE and GtfD and the observation of unusual, perhaps unexpected, substrate substituent effects on the rate and efficiency of the initial glycosylation reaction that were conducted as a prelude to their use with our pocket modified vancomycin aglycon analogues.

Although each enzymatic step has been examined analytically (GtfE with UDP-glucose[17a] and GtfD with TDP-vancosamine[17b]) and alternative monosaccharides have been incorporated at each site,[17−19] and even spacer linked carbohydrate attachments[21] have been examined, a preparative synthesis of vancomycin from the aglycon using the enzymatic approach has not been detailed. This can be attributed to the challenges at the time the enzymes were characterized of converting vancosamine, a 2-deoxy sugar, to the thermodynamically less stable but requisite β-anomer of either thymidine or uridine diphosphoryl-β-2-vancosamine (TDP- or UDP-vancosamine) required of the GtfD cosubstrate.[17] As such and in combination with our earlier efforts that provided a total synthesis of the vancomycin aglycon (**2**),[22] the efforts herein also constitute our completion of a total synthesis of vancomycin itself.

The recombinant glycosyltranferases GtfE and GtfD from the vancomycin producing strain of *A. orientalis* (ATCC19795) were expressed in *E. coli* from the corresponding constructs[17a] and were purified to homogeneity (His_6 tag). Synthetic UDP-vancosamine, possessing the requisite β-anomer stereochemistry, was prepared adapting a procedure recently described by Kahne[23] and used to access TDP-vancosamine, albeit with modifications to the synthetic route that incorporate uridine versus thymidine (Supporting Information). With use of the purified enzymes and the synthetic glycosyl donors UDP-glucose[24] (for GtfE) and UDP-vancosamine (for GtfD), conditions were optimized for the two sequential glycosylations of vancomycin aglycon (**2**) as well as the synthetic derivatives **3**−**5**,[25] bearing modifications to the C- and N-termini of the aglycon (Scheme 1).

Of the two glycosylation reactions, the initial GtfE-catalyzed incorporation of glucose using UDP-glucose exhibited the greatest aglycon substrate sensitivity and the least effective substrate was **3**, bearing a C-terminus hydroxymethyl group. Consequently, optimization efforts focused on this glycosylation reaction, examining simultaneously both the vancomycin aglycon (**2**) and substrate **3** (37 °C). As originally reported,[17a] Tricine (pH 9.0, 75 mM) served as a most satisfactory buffer although Tris (pH 9.0, 75 mM) was essentially equally effective, whereas reactions run in phosphate buffer (pH 8.0, 75 mM) were found to proceed at roughly half the rate and efficiency as Tricine. Although GtfE was originally reported to exhibit a stringent requirement for use at pH 9−10,[26] Tricine (pH 8.5) also supported the reaction effectively. Added detergents

Scheme 1



compd	R	R1	isolated yield
2	CO_2H	H	
3	CH_2OH	H	
4	CO_2H	Boc	
5	CO_2Me	H	
6	CO_2H	H	92%
7	CH_2OH	H	55%[a]
8	CO_2H	Boc	61%
9	CO_2Me	H	23%
1	CO_2H	H	87%
10	CH_2OH	H	79%
11	CO_2H	Boc	64%
12	CO_2Me	H	69%

[a]20 vs 5 μM GtfE

(Triton X-100, CHAPS, Tween 20) were counterproductive, and bovine serum albumin (BSA)[17,18] did not improve or alter the rate of the reactions examined with **2** and **3** during the optimization; thus, neither was employed in the optimized conditions. Increased amounts of glycosyl donor (4 mM UDP-glucose, typically used at 2 mM) did not improve the conversion with **3** which remained slow even with the optimized conditions. Additionally, it was found that the reaction tolerates added DMSO[14c] (≤10% v/v), and this can be used to dissolve less soluble substrates. The added reductant tris(2-carboxyethyl)phosphine (TCEP, 2 mM) was crucial, and the glycosylations failed to proceed in its absence.[17,18] The use of a polyol additive (glycerol, 5% v/v) productively served as an enzyme stabilizer, prolonging enzymatic activity.[18] Finally, although recent studies that we have conducted indicate the $MgCl_2$[18] is not needed for the reaction,[17] our optimization studies were conducted with its presence (1 mM). The net result is that our final conditions for the initial GtfE-catalyzed reaction represent a blend of the original conditions detailed by Walsh[17] and those later described by Wong.[18]

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A comparison of the relative efficiency of the initial glycosylation reaction is shown in Figure 2 when enlisting the

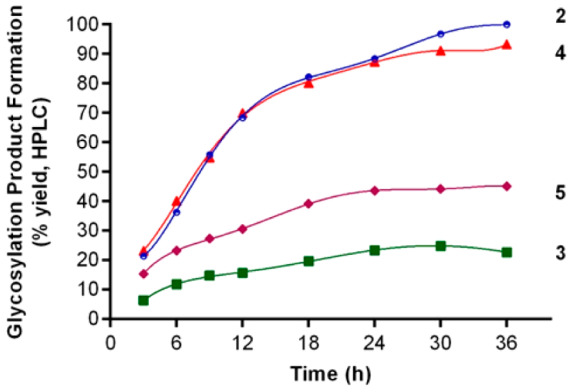

Figure 2. Comparison of the relative rates and efficiency of the GtfE-catalyzed reaction of **2**–**5** (0.5 mM) under the optimized conditions: Tricine (pH 9, 75 mM), TCEP (2 mM), UDP-glucose (2 mM for **2**, **4**, and **5**; 4 mM for **3**), GtfE (5 μM), $MgCl_2$ (1 mM), glycerol (5% v/v), 37 °C.

optimized conditions on an analytical scale not only with vancomycin aglycon (**2**) and its C-terminus hydroxymethyl derivative **3** but also with the N-terminus Boc derivative **4** and the C-terminus methyl ester **5**. Whereas Boc protection of the N-terminus amine had no effect on the rate and efficiency of the reaction analogous to reports of the use of the corresponding N-Alloc derivative,[17d] the substrate solubility did benefit from added DMSO (10% v/v). By contrast, glycosylation of the methyl ester **5** proceeded significantly more slowly, behaving much more like **3** but only slightly better, and added DMSO (10% v/v) in the reaction solvent system did improve this reaction, providing **9** in approximately 8%–12% higher conversions. However, extended reaction times led to some competitive hydrolysis of the methyl ester under the basic buffer conditions, complicating efforts to effectively monitor this substrate over an extended period (i.e., >24–36 h). Nonetheless, it is clear that modification at the N-terminus amine of vancomycin aglycon is well tolerated, whereas C-terminus modification with removal of the acidic carboxylic acid results in a slower, less effective initial glycosylation. It is unclear if the variation in yield and rate is due to the C-terminus modification or to altered physical properties, but the prospect that it is substrate induced raises interesting questions regarding substrate recognition at the enzyme active site. It will be interesting to further probe the tolerance of more significantly modified vancomycin intermediates in these glycosylation reactions in future studies. In the case of the hydroxymethyl substrate **3**, whose reaction proceeded at the slowest rate, preparative amounts of product **7** (55%, 48 h) were obtained by increasing the amount of enzyme used (20 vs 5 μM).

In contrast, the second glycosylation reaction catalyzed by GtfD using synthetic UDP-vancosamine proceeded to completion rapidly (<3 h) regardless of the vancomycin-derived substrate (**6**–**9**), displaying no impact of either the C- or N-terminus disposition of the substrate, and the conditions required little optimization. Aside from incorporating the use of glycerol (10% v/v) and reducing the amount of added BSA (0.2 vs 1 mg/mL), our reaction conditions [substrate (0.5 mM), Tricine (pH 9.0, 75 mM), TCEP (2 mM), UDP-vancosamine (2 mM), GtfD (5 μM), $MgCl_2$ (2 mM), BSA (0.2 mg/mL), glycerol (10% v/v)] remain essentially the same as originally disclosed for use of this enzyme.[17]

Finally, the preparative use of the enzymatic glycosylations on **2**–**5** were conducted herein on ca. 2 mg of substrate with 1 mol % enzyme and 4 equiv of UDP-glucose or UDP-vancosamine, reflecting the scale on which we anticipated conducting the initial glycosylyations of our pocket modified analogues of vancomycin aglycon. However, the laboratory expression and purification of the enzymes and the chemical synthesis of UDP-vancosamine, along with the commercial availability of UDP-glucose,[24] were conducted on scales that would easily support laboratory preparations much larger (ca. 100-fold) than exemplified. It is worth noting that although the endogenous glycosyl donors for both enzymes are the TDP-sugars,[17] UDP-sugars have been shown to be utilized as effectively as cosubstrates by both enzymes. Moreover, the requisite NDP-sugar synthetic precursor UMP morpholidate is commercially available,[27] whereas the corresponding activated TMP is not, leading to our synthesis, exploration, and adoption of UDP-vancosamine for use with GtfD.

In addition to serving as the completion of our past efforts,[22] providing a total synthesis of vancomycin, the work set the stage for glycosylation of pocket modified vancomycin analogues needed to further explore their properties. In this regard, the observation of unusual aglycon C-terminus effects on the rate of initial GtfE-catalyzed glycosylation and the ability to address them by using additional enzyme under optimized reaction conditions permitted the exploration of binding pocket modifications initially on the more accessible earlier stage synthetic intermediates. These and related studies will be disclosed in due course. Most significantly and as noted elsewhere,[14,17,18] the enzymatic glycosylations avoid protection and the corresponding deprotection of aglycon precursors required of chemical procedures, providing the fully glycosylated products in two steps from the fully deprotected aglycons.

■ ASSOCIATED CONTENT

Supporting Information

Full experimental details for the preparation and characterization of **1**, **6**–**13**, UDP-vancosamine, and copies of their ^{1}H NMR spectra are provided. 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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(27) Presently, there are four commercial suppliers of UMP morpholidate. We employed material obtained from Sigma−Aldrich ($176/g).

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