

## A Systematic Investigation of the Synthetic Utility of Glycopeptide Glycosyltransferases

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**Abstract:** Glycosyltransferases involved in the biosynthesis of bacterial secondary metabolites may be useful for the generation of sugar-modified analogues of bioactive natural products. Some glycosyltransferases have relaxed substrate specificity, and it has been assumed that promiscuity is a feature of the class. As part of a program to explore the synthetic utility of these enzymes, we have analyzed the substrate selectivity of glycosyltransferases that attach similar 2-deoxy-L-sugars to glycopeptide aglycons of the vancomycin-type, using purified enzymes and chemically synthesized TDP  $\beta$ -2-deoxy-L-sugar analogues. We show that while some of these glycopeptide glycosyltransferases are promiscuous, others tolerate only minor modifications in the substrates they will handle. For example, the glycosyltransferases GtfC and GtfD, which transfer 4-*epi*-L-vancosamine and L-vancosamine to C-2 of the glucose unit of vancomycin pseudoaglycon and chloroorienticin B, respectively, show moderately relaxed donor substrate specificities for the glycosylation of their natural aglycons. In contrast, GtfA, a transferase attaching 4-*epi*-L-vancosamine to a benzylic position, only utilizes donors that are closely related to its natural TDP sugar substrate. Our data also show that the spectrum of donors utilized by a given enzyme can depend on whether the natural acceptor or an analogue is used, and that GtfD is the most versatile enzyme for the synthesis of vancomycin analogues.

### Introduction

The glycopeptide antibiotic vancomycin (Figure 1) has gained prominence as the antibiotic of last resort for the treatment of life-threatening infections by methicillin-resistant Gram-positive bacteria. With the global emergence of vancomycin-resistant enterococci (VRE)<sup>1,2</sup> and, more recently, vancomycin-resistant *Staphylococcus aureus* (VRSA),<sup>1,3</sup> the development of novel glycopeptides with improved activity against resistant strains has become a major research focus. In 1988, researchers at Eli Lilly showed that glycopeptides containing lipophilic substituents on the carbohydrate moiety have significant activity against vancomycin-resistant strains.<sup>4</sup> As a result of these studies, the semisynthetic glycopeptide oritavancin (Figure 1), a derivative of the glycopeptide chloroeremomycin, was put into human clinical trials.<sup>1,5</sup>

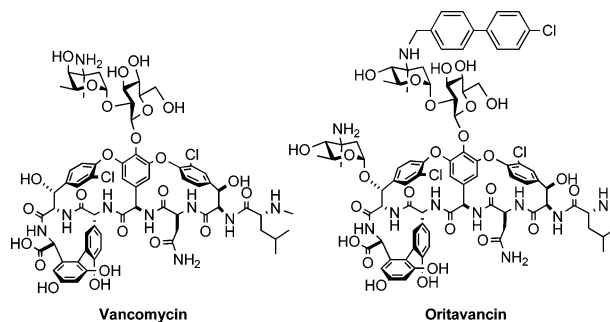


Figure 1. Glycopeptide antibiotics.

Due to the complexity of the glycopeptide class of natural products, early investigations of glycopeptide derivatives involved exploring substituent changes on sites that could be modified easily.<sup>4</sup> Because this precluded studies on glycopeptide derivatives containing unnatural sugars, our laboratory<sup>6</sup> and the Nicolaou group<sup>7</sup> have reported chemical glycosylation strategies to attach unnatural sugars to the vancomycin aglycon. In 1999, we showed that a lipidated vancomycin analogue containing

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(1) Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. T. *Chem. Rev.* **2005**, *105*, 425–448.

(2) (a) Cetinkaya, C.; Falk, P.; Mayhall, C. G. *Clin. Microbiol. Rev.* **2000**, *13*, 686–707. (b) Walsh, C. T.; Fisher, S. L.; Park, I. S.; Prahalad, M.; Wu, Z. *Chem. Biol.* **1996**, *3*, 21–28.

(3) Fridkin, S. K. et al. *N. Engl. J. Med.* **2003**, *348*, 1342–1347.

(4) (a) Nagarajan, R.; Schabel, A. A.; Occolowitz, J. L.; Counter, F. T.; Ott, J. L. *J. Antibiot.* **1988**, *41*, 1430–1438. (b) Nagarajan, R.; Schabel, A. A.; Occolowitz, J. L.; Counter, F. T.; Ott, J. L.; Felty-Duckworth, A. M. *J. Antibiot.* **1989**, *42*, 63–72.

(5) Allen, N. E.; Nicas, T. I. *FEMS Microbiol. Rev.* **2003**, *26*, 511–532.

(6) (a) Ge, M.; Thompson, C.; Kahne, D. *J. Am. Chem. Soc.* **1998**, *120*, 11014–11015. (b) Thompson, C.; Ge, M.; Kahne, D. *J. Am. Chem. Soc.* **1999**, *121*, 1237–1244.

(7) (a) Nicolaou, K. C.; Mitchell, H. J.; Jain, N. F.; Winssinger, N.; Hughes, R.; Bando, T. *Angew. Chem., Int. Ed.* **1999**, *38*, 240–244. (b) Nicolaou, K. C.; Cho, S. Y.; Hughes, R.; Winssinger, N.; Smethurst, C.; Labischinski, H.; Endermann, R. *Chem.—Eur. J.* **2001**, *7*, 3798–3823.

daunosamine was more active against some resistant bacterial strains than the corresponding vancosamine derivative.<sup>8</sup> We also suggested that glycopeptide derivatives, such as oritavancin, possess a second mechanism of action that is different from vancomycin,<sup>8</sup> which prevents maturation of the bacterial cell wall by binding to the terminal D-alanyl-D-alanine moiety of peptidoglycan precursors, thereby inhibiting the enzymes involved in the final stages of peptidoglycan synthesis. More recently, we could indeed show that lipidated glycopeptides interfere with peptidoglycan synthesis by direct inhibition of the major transglycosylases of *Escherichia coli*<sup>9</sup> and *S. aureus*.<sup>10</sup> These findings provided the impetus for exploring additional carbohydrate derivatives to examine the influence of structural changes in the lipid-disaccharide portion on antibiotic activity and on the ability to inhibit the transglycosylase enzymes. Since chemical glycosylation strategies do not enable rapid exploration of significant numbers of glycopeptide derivatives because of the number of synthetic steps necessary,<sup>11</sup> we became interested in exploring enzymatic approaches to generate glycopeptides.

Recent investigations have shown that some glycosyltransferases have a relaxed substrate selectivity,<sup>12</sup> suggesting that these enzymes may be useful for the chemoenzymatic synthesis of antibiotic analogues containing unnatural carbohydrates. Some of these studies have explored the glycosylation of unnatural aglycon substrates,<sup>13</sup> whereas other studies focused on variations in the sugar substrate structure.<sup>14,15</sup> For example, investigations of GtfE, which transfers D-glucose to the central 4-hydroxyphenylglycine of vancomycin aglycon, have shown that this enzyme transfers a range of unnatural deoxy and amino sugar derivatives to both the vancomycin and teicoplanin aglycons, making it possible to prepare a number of different

glycopeptide analogues rapidly.<sup>15c,d,16</sup> We wanted to know whether the relaxed substrate selectivity of GtfE was typical of the class because, then, the use of these glycosyltransferases in combination with either chemically<sup>17</sup> or enzymatically<sup>18,19</sup> synthesized nucleotide diphosphate (NDP) sugar donors would allow the generation of a large number of sugar-modified analogues in a straightforward way.<sup>20</sup> Accordingly, we decided to compare the donor substrate selectivity of three structurally related glycopeptide glycosyltransferases that attach similar 2-deoxy-L-sugars to glucosylated vancomycin aglycons. In the biosynthesis of chloroeremomycin, GtfA transfers 4-*epi*-L-vancosamine to the benzylic hydroxyl of amino acid 7 of the vancomycin pseudoaglycon **1** to produce chloroorienticin B (**2**).<sup>21</sup> GtfC then transfers 4-*epi*-L-vancosamine to the glucose C-2 hydroxyl of compound **2**.<sup>21</sup> GtfD, on the other hand, is part of the vancomycin biosynthetic cluster and transfers L-vancosamine to the glucose C-2 hydroxyl of pseudoaglycon **1** (Figure 2).<sup>15e</sup>

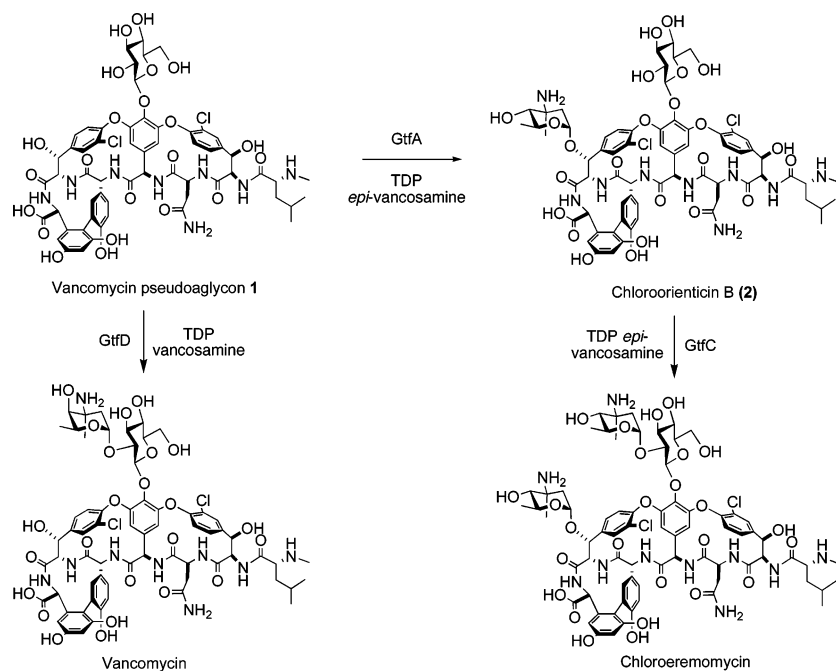
We report here the first comprehensive in vitro study of purified glycosyltransferases involved in the biosynthesis of bacterial secondary metabolites that transfer 2-deoxy-L-sugars. The glycopeptide glycosyltransferases GtfC and GtfD show a moderately relaxed substrate selectivity for the glycosylation of their natural aglycons, whereas GtfA and, in addition, GtfC with vancomycin pseudoaglycon **1** as acceptor only utilize donors that are closely related to their natural NDP sugar substrate. This difference in promiscuity indicates that in order to fully exploit the synthetic utility of glycosyltransferases involved in the biosynthesis of other bioactive natural products, a detailed analysis of the specific enzymes will be necessary.

## Results

**Synthesis of TDP Sugar Donors.** In preliminary studies, we were able to show that GtfD can transfer 4-*epi*-L-vancosamine instead of its natural substrate, L-vancosamine, to vancomycin pseudoaglycon **1**, and that the glucosylated aglycon of the glycopeptide teicoplanin can also serve as a substrate.<sup>15e</sup> A detailed study of the substrate specificity of GtfD, as well as of GtfA and C, was hampered by the synthetically challenging access to the required  $\beta$ -2-deoxy glycosyl thymidine diphosphate (TDP) donors. We have recently developed a chemical route to synthesize  $\beta$ -2-deoxy glycosyl phosphates from 2-deoxy glycosyl chlorides using the phosphate donor tetrabutylammonium dihydrogenphosphate.<sup>22</sup> The glycosyl phosphates are then converted to the desired TDP sugar donors with thymidine 5'-monophosphomorpholidate (TMP morpholidate)<sup>17a</sup> and then

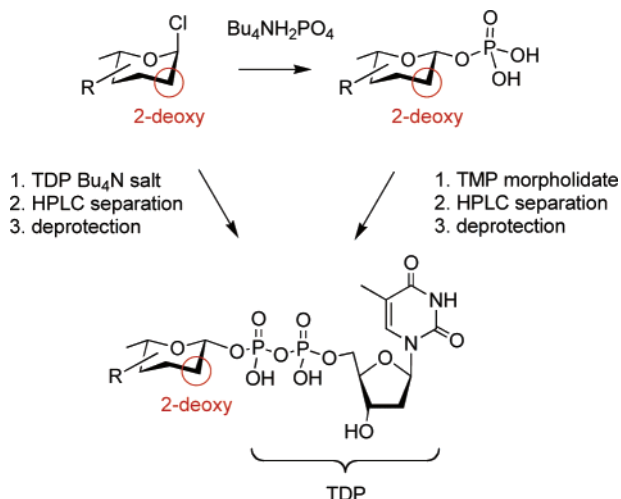
- (8) Ge, M.; Chen, Z.; Onishi, H. R.; Kohler, J.; Silver, L. L.; Kerns, R.; Fukuzawa, S.; Thompson, C.; Kahne, D. *Science* **1999**, *284*, 507–511.
- (9) Chen, L.; Walker, D.; Sun, B.; Hu, Y.; Walker, S.; Kahne, D. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 5658–5663.
- (10) Leimkuhler, C.; Chen, L.; Barrett, D.; Panzone, G.; Sun, B.; Falcone, B.; Oberthür, M.; Donadio, S.; Walker, S.; Kahne, D. *J. Am. Chem. Soc.* **2005**, *127*, 3250–3251.
- (11) Leimkuhler, C.; Chen, Z.; Kruger, R. G.; Oberthür, M.; Lu, W.; Walsh, C. T.; Kahne, D. *Tetrahedron: Asymmetry* **2005**, *16*, 599–603.
- (12) For recent reviews, see: (a) Walsh, C. T. *ChemBioChem* **2002**, *3*, 124–134. (b) Mendez, C.; Salas, J. A. *Trends Biotech.* **2001**, *19*, 449–456.
- (13) (a) Minami, A.; Uchida, R.; Eguchi, T.; Kakinuma, K. *J. Am. Chem. Soc.* **2005**, *127*, 6148–6149. (b) Li, S.-m.; Heide, L. *Curr. Med. Chem.* **2005**, *12*, 419–427. (c) Freil Meyers, C. L.; Oberthür, M.; Heide, L.; Kahne, D.; Walsh, C. T. *Biochemistry* **2004**, *43*, 15022–15036. (d) Eustáquio, A. E.; Gust, B.; Li, S.-m.; Pelzer, S.; Wollleben, W.; Chater, K. F.; Heide, L. *Chem. Biol.* **2004**, *11*, 1561–1572. (e) Rohr, J. et al. *Chem. Biol.* **2004**, *11*, 547–555. (f) Freil Meyers, C. L.; Oberthür, M.; Anderson, J. W.; Kahne, D.; Walsh, C. T. *Biochemistry* **2003**, *42*, 4179–4189. (g) Tang, L.; McDaniel, R. *Chem. Biol.* **2001**, *8*, 547–555.
- (14) For recent examples of syntheses of carbohydrate analogues of bacterial secondary metabolites using an in vivo approach, see: (a) Pérez, M.; Lombó, F.; Zhu, L.; Gibson, M.; Braña, A. F.; Rohr, J.; Salas, J. A.; Méndez, C. *Chem. Commun.* **2005**, 1604–1606. (b) Melançon, C. E., III; Takahashi, H.; Liu, H.-w. *J. Am. Chem. Soc.* **2004**, *126*, 16726–16727. (c) Lombó, F.; Gibson, M.; Greenwell, L.; Braña, A. F.; Rohr, J.; Salas, J. A.; Méndez, C. *Chem. Biol.* **2004**, *11*, 1709–1718. (d) Hoffmeister, D.; Dräger, G.; Ichinose, K.; Rohr, J.; Bechtold, A. *J. Am. Chem. Soc.* **2003**, *125*, 4678–4679. (e) Rodríguez, L.; Aguirrezabalaga, I.; Allende, N.; Braña, A. F.; Méndez, C.; Salas, J. A. *Chem. Biol.* **2002**, *9*, 721–729. (f) Borisova, S. A.; Zhao, L.; Sherman, D. H.; Liu, H.-w. *Org. Lett.* **1999**, *1*, 133–136.
- (15) Examples for the chemoenzymatic synthesis of analogues using purified glycosyltransferases: (a) Lu, W.; Leimkuhler, C.; Oberthür, M.; Kahne, D.; Walsh, C. T. *Biochemistry* **2004**, *43*, 4548–4558. (b) Albermann, C.; Soriano, A.; Jiang, J.; Vollmer, H.; Biggins, J. B.; Barton, W. A.; Lesniak, J.; Nikolov, D. B.; Thorson, J. S. *Org. Lett.* **2003**, *5*, 933–936. (c) Fu, X.; Albermann, C.; Jiang, J.; Liao, J.; Zhang, C.; Thorson, J. S. *Nat. Biotechnol.* **2003**, *21*, 1467–1469. (d) Losey, H. C.; Jiang, J.; Biggins, J. B.; Oberthür, M.; Ye, X.-Y.; Dong, S. D.; Kahne, D.; Thorson, J. S.; Walsh, C. T. *Chem. Biol.* **2002**, *9*, 1305–1314. (e) Losey, H. C.; Peczu, M. W.; Chen, Z.; Eggert, U. S.; Dong, S. D.; Pelzer, I.; Kahne, D.; Walsh, C. T. *Biochemistry* **2001**, *40*, 4745–4755.

- (16) Dong, S. D.; Oberthür, M.; Losey, H. C.; Anderson, J. W.; Eggert, U. S.; Peczu, M. W.; Walsh, C. T.; Kahne, D. *J. Am. Chem. Soc.* **2002**, *124*, 9064–9065.
- (17) (a) Wittmann, V.; Wong, C.-H. *J. Org. Chem.* **1997**, *62*, 2144–2147. (b) Fang, X.; Gibbs, B. S.; Coward, J. K. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2701–2706. (c) Arlt, M.; Hinds, G. O. *J. Org. Chem.* **1995**, *60*, 14–15.
- (18) Syntheses of NDP sugars using multienzyme systems: (a) Chen, H.; Thomas, M. G.; Hubbard, B. K.; Losey, H. C.; Walsh, C. T.; Burkart, M. D. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11942–11947. (b) Amann, S.; Dräger, G.; Rupprath, C.; Kirschning, A.; Elling, L. *Carbohydr. Res.* **2001**, *335*, 23–32.
- (19) Enzymatic synthesis of NDP sugars using nucleotidyltransferases: (a) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 521–546. (b) Jiang, J.; Albermann, C.; Thorson, J. S. *ChemBioChem* **2003**, *4*, 443–446. (c) Mizanur, R. M.; Zea, C. J.; Pohl, N. L. *J. Am. Chem. Soc.* **2004**, *126*, 15993–15998.
- (20) Yang, J.; Hoffmeister, D.; Liu, L.; Fu, X.; Thorson, J. S. *Bioorg. Med. Chem.* **2004**, *12*, 1577–1584.
- (21) Lu, W.; Oberthür, M.; Leimkuhler, C.; Tao, J.; Kahne, D.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 4390–4395.
- (22) Oberthür, M.; Leimkuhler, C.; Kahne, D. *Org. Lett.* **2004**, *6*, 2873–2876.



**Figure 2.** Action of the glycosyltransferases GtfA, GtfC, and GtfD involved in the biosynthesis of glycopeptide antibiotics.

**Scheme 1.** Synthetic Strategy toward TDP  $\beta$ -2-Deoxy Sugars



deprotected (Scheme 1).<sup>23</sup> In a shorter approach, the 2-deoxy glycosyl chlorides can be coupled directly with the tetrabutylammonium salt of TDP.<sup>17c</sup> Although the stereoselectivities obtained are lower for the shorter route, it is still possible to obtain sufficient amounts of the desired  $\beta$ -isomer after HPLC separation to characterize the enzymes.

To test the substrate specificity of GtfA, C, and D, we prepared a range of potential TDP 2-deoxy sugar substrates (Figure 3), following the approaches outlined above (see ref 22 and Supporting Information). The TDP sugars were stable under all reaction conditions and could be stored at  $-20\text{ }^{\circ}\text{C}$  without decomposition for a prolonged period of time (weeks to months). The set of TDP 2-deoxy sugars included the natural substrates (TDP L-vancosamine (**3**) and TDP 4-*epi*-L-vancosamine (**4**)) and the corresponding 3-desmethyl derivatives (TDP L-daunosamine (**5**) and TDP L-acosamine (**6**)). In addition,

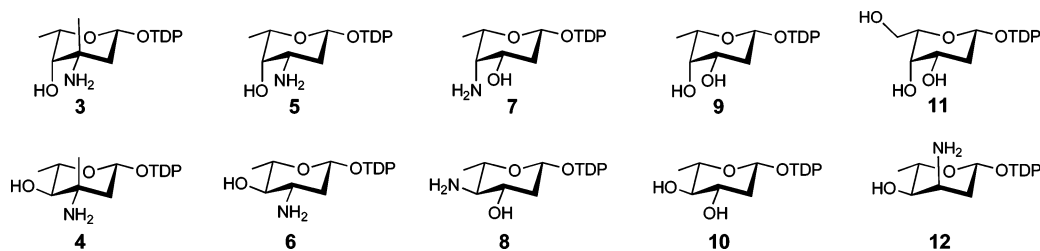
we changed the location of the amino group to position C-4 (TDP 4-amino-2-deoxy-L-fucose (**7**) and TDP 4-amino-2-deoxy-L-rhamnose (**8**)), exchanged the amino for a hydroxy group (TDP 2-deoxy L-fucose (**9**) and TDP 2-deoxy L-rhamnose (**10**)), added a 6-hydroxy group (TDP 2-deoxy L-glucose (**11**)), or changed the stereochemistry of the C-3 amino group (TDP L-ristosamine (**12**)).

**Characterization of the Substrate Specificity of GtfA, GtfC, and GtfD.** All three enzymes were tested for their ability to transfer the various TDP sugars to the vancomycin pseudoaglycon **1** (Scheme 2) in order to evaluate their possible use as tools for the chemoenzymatic synthesis of novel vancomycin derivatives carrying unnatural 2-deoxy sugars. The results obtained for these transfers are summarized in Table 1. These data show that both *epi*-vancosaminyl transferases GtfA and C were able to transfer L-acosamine in addition to their natural substrate, that is, they tolerated the removal of the 3-C-methyl group (entry 4). Additional modifications, for example, a change of stereochemistry at C-4, led to a dramatic decrease of transfer efficiency (entries 1 and 3). Of all other substrates used, only 2-deoxy L-glucose (GtfA and C) and L-vancosamine, L-daunosamine, and 2-deoxy L-rhamnose (GtfC) were transferred, albeit in trace quantities.

For the glycosylation of vancomycin pseudoaglycon **1**, GtfD showed the most relaxed substrate specificity. Besides its natural substrate, L-vancosamine, it also transferred 4-*epi*-L-vancosamine, L-daunosamine, and L-acosamine with good turnover, that is, both the removal of the C-3-methyl group and the inversion of stereochemistry at C-4 were tolerated (entries 2–4). In addition, 2-deoxy L-glucose, 2-deoxy L-rhamnose, and L-ristosamine were transferred in trace amounts, whereas for substrates containing an amino group at C-4, no product was observed.

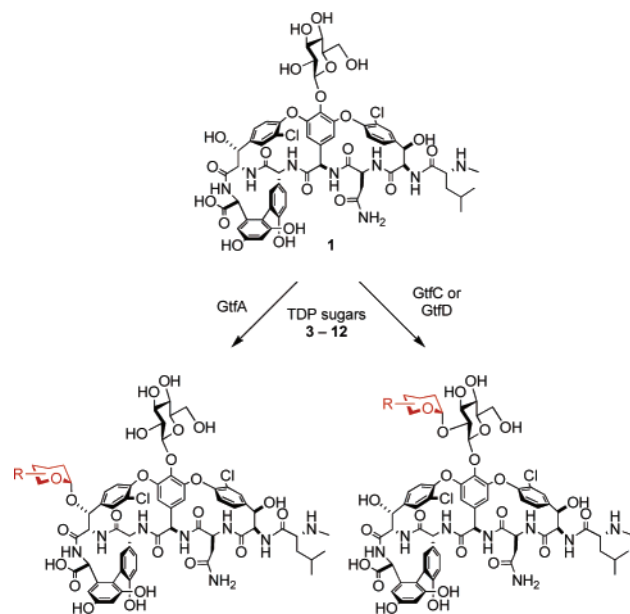
The natural aglycon substrate for GtfC is chloroorienticin B (**2**) (see Figure 2),<sup>21</sup> which contains the *epi*-vancosamine sugar already attached to the benzylic position. To determine if GtfC shows a broader donor substrate selectivity when its

(23) So far, there have been no reports regarding nucleotidyltransferases that accept  $\beta$ -configured 2-deoxy-L-glycosyl phosphates as substrates.



**Figure 3.** TDP sugar substrates used to study the specificity of GtfA, C, and D.

**Scheme 2.** Glycosylation of Vancomycin Pseudoaglycon **1** with GtfA, C, and D



**Table 1.** Results of the Transfers Using TDP Sugars **3–12**<sup>a</sup>

entry	TDP sugar	Enzyme			
		GtfA (aglycon 1)	GtfC (aglycon 1) (aglycon 2)		GtfD (aglycon 1)
1	<b>3</b>	–	trace	+	+
2	<b>4</b>	+	+	+	+
3	<b>5</b>	–	trace	+	+
4	<b>6</b>	+	+	+	+
5	<b>7</b>	–	–	–	–
6	<b>8</b>	–	–	–	–
7	<b>9</b>	–	–	–	–
8	<b>10</b>	–	trace	+	trace
9	<b>11</b>	trace	trace	+	trace
10	<b>12</b>	–	–	+	trace

<sup>a</sup> (+): transfer observed by HPLC; (trace): transfer observed by HPLC, but with  $k_{\text{cat}} < 0.01 \text{ min}^{-1}$ ; (–): no transfer observed by HPLC.

natural aglycon is used, we prepared quantities of chloroorienticin B (**2**) by a large-scale transfer of 4-*epi*-L-vancosamine to aglycon **1** using GtfA. The transfers to chloroorienticin B (**2**) revealed that, in this case, GtfC shows a substrate specificity similar to that of GtfD (Table 1). The enzyme accepted a range of structural changes to its natural substrate, 4-*epi*-L-vancosamine, for example, removal of the C-3-Me, the presence of an axial 3-amino group, and inversion at C-4 and hydroxyl groups at C-3 and C-6.

**Kinetic Characterization of the Successful Transfers.** To compare the efficiency of the different enzymes to transfer both their natural and non-natural TDP substrates, we undertook a kinetic characterization of the robust transfers.

GtfA transfers L-acosamine (entry 4) with a  $k_{\text{cat}}$  of  $0.18 \text{ min}^{-1}$ , about 10-fold slower than that of the natural substrate, 4-*epi*-

L-vancosamine ( $2.3 \text{ min}^{-1}$ , entry 2). The overall catalytic efficiency, however, decreases only about 2-fold for the unnatural substrate because of substantially lower  $K_{\text{m}}$  for L-acosamine (38 versus  $218 \mu\text{M}$ ). The only other TDP sugar accepted by GtfA, 2-deoxy-L-glucose, was transferred in trace quantities with a  $k_{\text{cat}} < 0.001 \text{ min}^{-1}$  (entry 6).

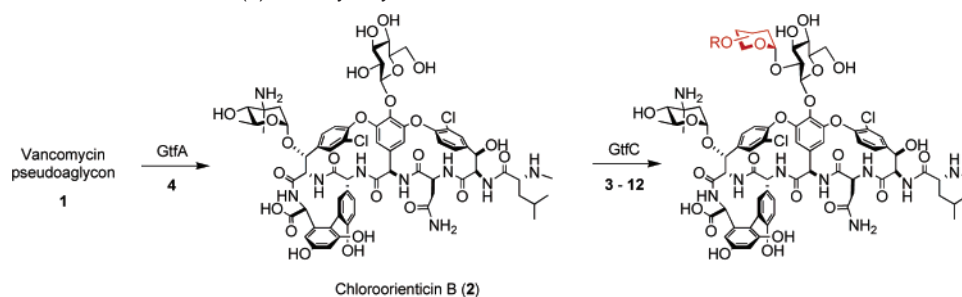
Using the unnatural substrate vancomycin aglycon **1** as the acceptor substrate, GtfC transferred its natural substrate, 4-*epi*-L-vancosamine, with a  $k_{\text{cat}}$  of  $9.7 \text{ min}^{-1}$  (entry 2). Removal of the 3-C-methyl group in the sugar donor (TDP L-acosamine (**6**)) led to a 1000-fold decrease of the  $k_{\text{cat}}$  value ( $< 0.01 \text{ min}^{-1}$ , entry 4), and the  $k_{\text{cat}}$  values for other substrates for which transfer was observed (TDP sugars **3**, **5**, **10**, and **11**) were even lower ( $< 0.001 \text{ min}^{-1}$ , entries 1, 3, 5–7). GtfC shows a dramatically higher catalytic efficiency for its natural aglycon substrate, chloroorienticin B (**2**). This is reflected in the  $K_{\text{m}}$  value ( $4 \mu\text{M}$ ) for this aglycon, which is more than 200-fold lower than that for vancomycin pseudoaglycon **1** ( $K_{\text{m}} = 923 \mu\text{M}$ ). GtfC transfers its natural substrate, 4-*epi*-L-vancosamine, to aglycon **2** with a  $k_{\text{cat}}$  of  $41 \text{ min}^{-1}$  and a  $K_{\text{m}}$  of  $199 \mu\text{M}$  (entry 2). The removal of the 3-C-methyl group in the sugar substrate (TDP L-acosamine (**6**)) is well tolerated and actually leads to an increase of catalytic efficiency because the 3-fold reduction of  $k_{\text{cat}}$  is compensated by the lower  $K_{\text{m}}$  value for this NDP sugar (entry 4).

A change of stereochemistry at C-4 (TDP L-vancosamine (**3**) and TDP L-daunosamine (**5**)), on the other hand, leads to a larger decrease of  $k_{\text{cat}}$  (ca. 100-fold) and overall catalytic efficiency (entries 1 and 3). In addition, other TDP substrates, such as TDP 2-deoxy-L-rhamnose (**10**), TDP 2-deoxy-L-glucose (**11**), and TDP L-ristosamine (**12**), were also transferred with a comparable decrease of the turnover rate (entries 5–7).

In contrast to GtfC, the vancosaminyl transferase GtfD transfers the TDP sugars with differing stereochemistry at C-4, TDP L-vancosamine (**3**) and TDP 4-*epi*-L-vancosamine (**4**), with almost the same  $k_{\text{cat}}$  ( $128$  and  $135 \text{ min}^{-1}$ , respectively, entries 1 and 2) and with a 6-fold increase of  $K_{\text{m}}$  for the unnatural substrate **4** ( $38$  versus  $232 \mu\text{M}$ ). For this enzyme, however, the removal of the C-3 methyl group has a more pronounced effect on  $k_{\text{cat}}$  (500- and 50-fold decrease for L-daunosamine and L-acosamine, respectively, entries 3 and 4), whereas almost the same  $K_{\text{m}}$  values are observed. The transfers that were detected by HPLC for the TDP sugars **10–12** occurred with a  $k_{\text{cat}} < 0.001 \text{ min}^{-1}$  (entries 5–7).

## Discussion

In recent years, there has been an increasing interest in the development of novel glycopeptide antibiotics due to the emergence of resistance to vancomycin. Research showed that changes in the carbohydrate portion of lipid-containing vancomycin derivatives can have a significant effect on activity against both glycopeptide sensitive and resistant bacteria,<sup>4,8</sup> which makes

**Scheme 3.** Synthesis of Chloroorienticin B (2) and Glycosylation with GtfC**Table 2.** Kinetic Data for the Successful Transfers<sup>a</sup>

entry	TDP sugar	GtfA (aglycon 1)	GtfC (aglycon 1)	GtfC (aglycon 2)	GtfD (aglycon 1)
1	<b>3</b>	—	$k_{\text{cat}} < 0.001$	$k_{\text{cat}} = 0.6$ $K_{\text{m}} = 31$ $k_{\text{cat}}/K_{\text{m}} = 0.02$	$k_{\text{cat}} = \mathbf{128}$ $K_{\text{m}} = \mathbf{38}$ $k_{\text{cat}}/K_{\text{m}} = \mathbf{3.4}$
2	<b>4</b>	$k_{\text{cat}} = \mathbf{2.3}$ $K_{\text{m}} = \mathbf{218}$ $k_{\text{cat}}/K_{\text{m}} = \mathbf{0.01}$	$k_{\text{cat}} = 9.7$	$k_{\text{cat}} = \mathbf{41}$ $K_{\text{m}} = \mathbf{199}$ $k_{\text{cat}}/K_{\text{m}} = \mathbf{0.21}$	$k_{\text{cat}} = 135$ $K_{\text{m}} = 232$ $k_{\text{cat}}/K_{\text{m}} = 0.58$
3	<b>5</b>	—	$k_{\text{cat}} < 0.001$	$k_{\text{cat}} = 0.24$ $K_{\text{m}} = 88$ $k_{\text{cat}}/K_{\text{m}} = 3 \times 10^{-3}$	$k_{\text{cat}} = 0.28$ $K_{\text{m}} = 43$ $k_{\text{cat}}/K_{\text{m}} = 0.007$
4	<b>6</b>	$k_{\text{cat}} = 0.18$ $K_{\text{m}} = 38$ $k_{\text{cat}}/K_{\text{m}} = 5 \times 10^{-3}$	$k_{\text{cat}} < 0.01$	$k_{\text{cat}} = 14$ $K_{\text{m}} = 15$ $k_{\text{cat}}/K_{\text{m}} = 0.9$	$k_{\text{cat}} = 2.4$ $K_{\text{m}} = 36$ $k_{\text{cat}}/K_{\text{m}} = 0.07$
5	<b>10</b>	—	$k_{\text{cat}} < 0.001$	$k_{\text{cat}} = 0.5$ $K_{\text{m}} = 34$ $k_{\text{cat}}/K_{\text{m}} = 0.01$	$k_{\text{cat}} < 0.001$
6	<b>11</b>	$k_{\text{cat}} < 0.001$	$k_{\text{cat}} < 0.001$	$k_{\text{cat}} = 0.04$ $K_{\text{m}} = 90$ $k_{\text{cat}}/K_{\text{m}} = 4 \times 10^{-4}$	$k_{\text{cat}} < 0.001$
7	<b>12</b>	—	—	$k_{\text{cat}} = 0.25$ $K_{\text{m}} = 100$ $k_{\text{cat}}/K_{\text{m}} = 2 \times 10^{-3}$	$k_{\text{cat}} < 0.001$

<sup>a</sup> The kinetic data for the transfers of the natural substrate pairs are shown in bold.  $k_{\text{cat}}$  [ $\text{min}^{-1}$ ],  $K_{\text{m}}$  [ $\mu\text{M}$ ],  $k_{\text{cat}}/K_{\text{m}}$  [ $\mu\text{M}^{-1} \text{min}^{-1}$ ]. (—): no transfer (see Table 1).

an efficient synthetic access to such derivatives crucial for the development of analogues with even better activity.

A chemoenzymatic approach could enable the rapid generation of a large number of novel glycopeptides that contain changes in the carbohydrate portion, and the glycosyltransferase GtfE has shown promise in this regard.<sup>15c–e</sup> To determine whether promiscuity is a general quality of glycosyltransferases involved in the biosynthesis of glycopeptide antibiotics, we undertook the first systematic evaluation of the synthetic utility of three glycopeptide glycosyltransferases, GtfA, GtfC, and GtfD, which transfer similar 2-deoxy-L-sugars (4-*epi*-L-vancosamine or L-vancosamine, respectively, see Figure 2).

Our results for the transfers of a variety of TDP substrate analogues<sup>22</sup> to the natural aglycon acceptors show that the three enzymes differ markedly with respect to their promiscuity toward these substrates. Whereas GtfA accepted only one other TDP sugar besides its natural substrate, both GtfC and GtfD are comparably promiscuous and were able to transfer a number of 2-deoxy sugar analogues of the natural substrates L-*epi*-vancosamine and L-vancosamine, respectively.

For GtfA and GtfC, the substrate specificity can change substantially when unnatural acceptor substrates were glycosylated, which reduces the synthetic utility of these enzymes. We have reported earlier that GtfA transfers even its natural sugar substrate 4-*epi*-L-vancosamine to two alternate substrates, the fully deglycosylated vancomycin aglycon and epivancomy-

cin (vancomycin carrying 4-*epi*-L-vancosamine instead of L-vancosamine), with catalytic turnovers ( $k_{\text{cat}} < 0.05 \text{ min}^{-1}$ ) that are too slow to be synthetically useful.<sup>21</sup> Our results from the present study demonstrate that GtfC also shows a significant decrease in its ability to transfer 4-*epi*-L-vancosamine to the unnatural acceptor, vancomycin pseudoaglycon **1**, compared to the natural substrate, aglycon **2**. More importantly, GtfC was also considerably less promiscuous toward unnatural sugar substrates in the transfers to aglycon **1**, which limits the use of GtfC as a tool for the chemoenzymatic synthesis of glycopeptide analogues.

GtfD, on the other hand, is more promising in this context because of its ability to transfer 2-deoxy sugars, such as L-vancosamine, 4-*epi*-L-vancosamine, and L-daunosamine, to vancomycin pseudoaglycons with modifications in the glucose portion<sup>15d</sup> and to different teicoplanin aglycons.<sup>15e,16,24</sup> The latter observation is especially significant because the chemical glycosylation approach is so far limited to the vancomycin system.

Finally, it is interesting to note that the differences in substrate specificities described above for the three enzymes correlate with their turnover rates for their natural substrate pairs, that is, the faster enzymes, GtfC and GtfD, also proved to be the most promiscuous ones. This result is in agreement with our

(24) Kruger, R.; Lu, W.; Oberthür, M.; Tao, J.; Kahne, D.; Walsh, C. T. *Chem. Biol.* **2005**, *12*, 121–130.

earlier results regarding the promiscuity of the two glucosyltransferases involved in the biosynthesis of chloroeremomycin and vancomycin, in which case the faster GtfE showed also the more relaxed substrate specificity compared to that of the slower GtfB.<sup>15d,e</sup> If this holds true for other glycosyltransferases involved in the biosynthesis of bacterial secondary metabolites, this result might aid the search for other synthetically useful enzymes because, then, the turnover rate for the natural donor–acceptor pair could serve as an indicator for enzyme promiscuity.

## Conclusion

This first systematic study of a subgroup of glycosyltransferases that transfer 2-deoxy-L-sugars to natural product aglycons shows that these enzymes can differ rather dramatically in terms of their substrate specificity. The rather high promiscuity of enzymes studied earlier, for example, GtfE, does not seem to be a general quality of glycosyltransferases per se. It follows that to further expand the repertoire of synthetically useful glycosyltransferases, a detailed study of other enzymes will be necessary. The set of TDP 2-deoxy-L-sugar analogues obtained through chemical synthesis, however, will facilitate the screening of such glycosyltransferases.

For the generation of vancomycin analogues carrying unnatural sugars, GtfD is the most promising enzyme and can be used to attach 2-deoxy sugars that are structurally related to the natural sugar substrate L-vancosamine, for example, 4-*epi*-L-vancosamine, L-daunosamine, and L-acosamine. We are currently using both GtfD and GtfE as reagents in the synthesis of lipidated glycopeptide analogues to study the influence of modifications in the carbohydrate portion on biological activity.

## Experimental Section

**Synthesis of TDP Sugars 3–12.** TDP  $\beta$ -L-vancosamine (**3**), TDP 4-*epi*- $\beta$ -L-vancosamine (**4**), TDP  $\beta$ -L-daunosamine (**5**), TDP  $\beta$ -L-acosamine (**6**), and TDP 2-deoxy- $\beta$ -L-fucose (**9**) were synthesized as

previously described.<sup>22</sup> The synthesis of TDP sugars **7**, **8**, and **10–12** is described in the Supporting Information.

**Chemoenzymatic Synthesis of Chloroorienticin B (2).** Vancomycin pseudoaglycon **1**, obtained from vancomycin by acid degradation (trifluoroacetic acid/H<sub>2</sub>O 9:1, room temperature, 8 h) and HPLC purification, was enzymatically glycosylated with TDP 4-*epi*-L-vancosamine (**4**) using the chloroeremomycin biosynthetic glycosyltransferase GtfA to yield the doubly glycosylated chloroorienticin B (**2**).<sup>21</sup> The reaction conditions were as follows: 75 mM Tris (pH = 7.0), 8 mM MgCl<sub>2</sub>, 2.5 mM TCEP, 1 mg/mL BSA, 10% (v/v) DMSO, 500  $\mu$ M TDP 4-*epi*-L-vancosamine (**4**), 500  $\mu$ M vancomycin pseudoaglycon **1**, 5  $\mu$ M GtfA. The reaction was monitored by HPLC, and additional aliquots of GtfA were added twice daily until the reaction proceeded to 50% completion (up to 3 days). Glycopeptide **2** was purified by preparative HPLC (Vydac C<sub>18</sub> column, 0–30% acetonitrile, 0.1% TFA in 25 min) and was verified by LCMS.

**Kinetic Analysis of GtfA, GtfC, and GtfD.** The glycosyltransferases GtfA, GtfC, and GtfD were expressed and purified as previously described.<sup>15e,21</sup> The enzymes were tested for activity against TDP sugars **3–12** using a previously reported HPLC-based assay.<sup>15e</sup> In GtfA and GtfD assays, vancomycin pseudoaglycon **1** was used as the glycopeptide acceptor scaffold, while chloroorienticin B (**2**) was used in GtfC assays as we have previously shown that chloroorienticin B is the preferred substrate for GtfC.<sup>21</sup> Kinetic parameters for GtfA using sugar TDP 4-*epi*-L-vancosamine (**4**) have been previously reported,<sup>21</sup> and the same methodology was used to generate kinetic parameters for sugars that demonstrated suitable activity.

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**Supporting Information Available:** Full author list for refs 3a and 13e; experimental procedures and compound characterization for the synthesis TDP sugars **7**, **8**, and **10–12**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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