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Substrate Flexibility of Vicenisaminyltransferase VinC Involved in the Biosynthesis of Vicenistatin

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Abstract: A glycosyltransferase VinC is involved in the biosynthesis of antitumor β-glycoside antibiotic vicenistatin. It catalyzes a glycosyl transfer reaction between dTDP-α-D-vicenisamine and vicenilactam. Previous identification of its broad substrate specificity toward various glycosyl acceptors enabled us to explore the potential of VinC for glycodiversification. In vitro study of the substrate specificity toward several dTDP-sugars with vicenilactam established that VinC displayed activities with α-anomers of several dTDP-2-deoxy-D-sugars such as mycarose, digitoxose, olivose, and 2-deoxyglucose to afford respective β-glycosides. Notably, β-anomers of dTDP-2-deoxy-D-sugars also appeared to be accepted by VinC to form α-glycosides. Furthermore, VinC is capable of catalyzing glycosyl transfer reactions from both the α-anomer and β-anomer of dTDP-L-mycarose, respectively, into β-glycoside and α-glycoside. These results indicate that VinC is a unique glycosyltransferase possessing broad substrate specificity. The mechanism of this axially oriented glycosidic bond formation from the equatorially oriented dTDP-sugar might be explained by conformational change of dTDP-sugar to a boat conformation during the glycosyl transfer reaction. To apply these features of VinC for glycodiversification, 22 sets of structurally diverse glycosides were constructed using unnatural glycosyl donors and acceptors.

Introduction

Glycosyltransferase, which catalyzes a transfer of sugar in the form of nucleoside diphosphosugar (NDP-sugar) to the respective aglycons, is an important enzyme in the biosynthesis of biologically active secondary metabolites because the presence of sugar is, in most cases, necessary for exerting their biological activities.[1] It is therefore a current subject of active research in the field to exploit the usefulness of these glycosyltransferases for structurally diverse glycoside synthesis. In fact, chemoenzymatic glycoside syntheses using glycosyltransferases have been applied to change the sugar fraction of natural glycosides.[2−7] Structural changes of the sugar component in these examples include substitution of the functional group and configurational change. In addition, recent studies have shown examples of change in the aglycon part of natural glycosides using glycosyltransferases.[6−11] Although these examples indicate the importance of a chemoenzymatic approach to create unnatural glycosides, further identification of new glycosyltransferases exhibiting a broad substrate specificity toward both sugar and aglycon components is highly desirable for development of a glycoside library because the success of this approach depends mainly on the substrate specificity of glycosyltransferases themselves.

Vicenistatin (**1**), an antitumor β-glycosidic antibiotic produced by *Streptomyces halstedii* HC 34, comprises amino sugar vicenisamine and 20-membered macrocyclic vicenilactam (Figure 1A).[12] We identified the whole vicenistatin biosynthetic gene cluster (*vin*) and confirmed that VinC is a vicenisaminyltransferase catalyzing the transfer of vicenisamine from dTDP-vicenisamine (**4**) to vicenilactam (**3**) in the last step of vicenistatin biosynthesis (Figure 1B).[13] We recently demonstrated that VinC accepts structurally diverse aglycons to form respective vicenisaminides.[9,11] The broad substrate specificity toward the glycosyl acceptor showed that VinC is an attractive glycosyltransferase for glycodiversification. On the other hand, the substrate specificity of VinC toward a glycosyl donor remains unclear, even though previous isolation of vicenistatin M (**2**) indicated the possibility of D-mycarose transfer to

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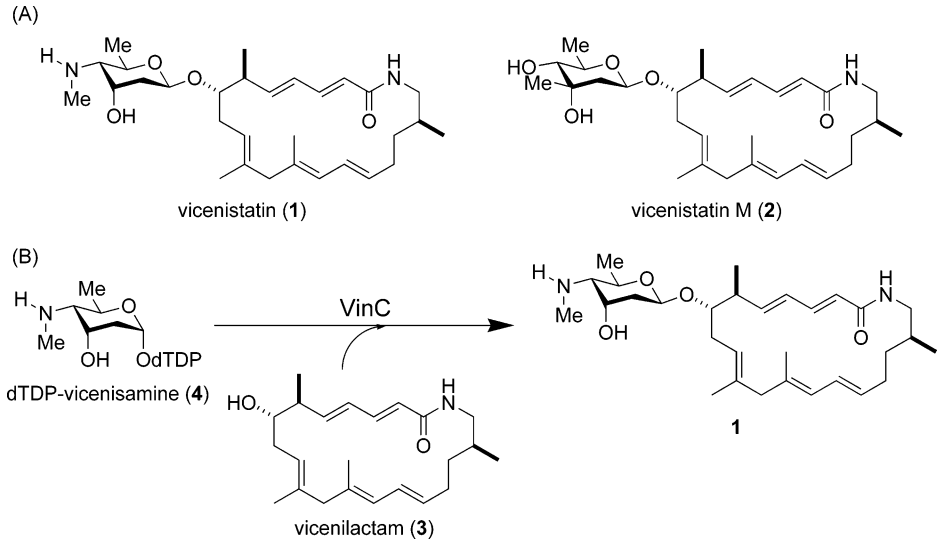

Figure 1. (A) Structure of vicenistatin (**1**) and vicenistatin M (**2**). (B) Glycosylation between dTDP-α-D-vicenisamine (**4**) and vicenilactam (**3**) catalyzed by VinC.

vicenilactam (Figure 1A).[14] For further exploitation of VinC for glycodiversification, more detailed information about the substrate specificity toward the glycosyl donor, especially in the hexose moiety, is indispensable.

Herein, we report the biochemical characterization and in vitro study of the substrate specificity for glycosyl donor in VinC reaction using structurally varied dTDP-sugars including both anomers of D-sugars and L-sugars.

Results and Discussion

Biochemical Properties of VinC. Heterologous expression of *vin*C in *Escherichia coli* was described in our previous paper.[13] The expressed VinC was purified using ammonium sulfate precipitation and DEAE Sepharose fast flow column chromatography. VinC was obtained as an electrophoretically homogeneous state, and the molecular mass was estimated as 46 kDa using SDS-PAGE analysis (Figure S1, see Supporting Information). The molecular weight was confirmed as 46 064 Da (Figure S2) from LC-ESI-MS analysis, which is consistent with the estimated molecular weight (46 066.5 Da) from the amino acid sequence. In addition, native-PAGE analysis results suggest the apparent molecular mass as 88 kDa (data not shown), which strongly indicates that the overexpressed VinC exists in a homodimer.

The highest VinC activity with dTDP-vicenisamine and vicenilactam was observed with 50 mM Tris-HCl buffer, pH 8.0, at ca. 25 °C. The effect of divalent metal cations to VinC activity was eliminated because VinC activity was not changed in the presence of various divalent metal cations (1 mM of Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, or Ca^{2+}) or EDTA (1 mM). No metal requirement for catalytic activity is apparent in other glycosyltransferases such as GtfB and GtfE, which are involved, respectively, in the biosynthesis of chloroeremomycin and vancomycin.[15]

Specificity toward Nucleoside Diphosphate Moiety of NDP-Vicenisamine. To gain additional information about the substrate specificity of VinC toward the NDP moiety, α-anomers and β-anomers of dTDP-, UDP-, and ADP-vicenisamine (**4**−**9**) were prepared using chemical synthesis followed by separation of anomers by preparative HPLC with a ODS-AQ column. These glycosyl donors were subjected separately to VinC reaction with vicenilactam. Subsequent HPLC analysis showed clearly that these α-anomers were accepted by VinC to form vicenistatin. Comparison of their relative activities, which were estimated from the initial formation rate of **1** adjusted by the concentration of VinC, indicated that dTDP-vicenisamine was the best among those tested and that UDP-vicenisamine was also an active substrate, as shown in Table 1. In contrast, ADP-vicenisamine represented low activity among those three glycosyl donors. On the other hand, β-anomers (**5**, **7**, **9**) were not accepted at all, even in the presence of high concentration of VinC (0.2 mM). It was apparent, therefore, that VinC originally catalyzes a stereospecific glycosyl

Table 1. Results of the VinC Reaction with NDP-Sugars (**4**−**21**)[a]

	substrate	product	yield (%)	relative activity
4	dTDP-α-D-vicenisamine	β-glycoside	85	1
5	dTDP-β-D-vicenisamine		no reaction	
6	UDP-α-D-vicenisamine	β-glycoside	61	1.8×10^{-3}
7	UDP-β-D-vicenisamine		no reaction	
8	ADP-α-D-vicenisamine	β-glycoside	49	2.2×10^{-5}
9	ADP-β-D-vicenisamine		no reaction	
10	dTDP-α-D-mycarose	β-glycoside	55	5.7×10^{-2}
11	dTDP-β-D-mycarose	α-glycoside	12	6.1×10^{-8}
12	dTDP-α-D-digitoxose	β-glycoside	54	3.3×10^{-3}
13	dTDP-β-D-digitoxose	ND[b]	18	3.4×10^{-7}
14	dTDP-α-D-olivose	β-glycoside	75	1.1×10^{-4}
15	dTDP-β-D-olivose	α-glycoside	61	6.4×10^{-7}
16	dTDP-α-D-2-deoxyglucose	β-glycoside	69	5.6×10^{-6}
17	dTDP-β-D-2-deoxyglucose	α-glycoside	41	6.2×10^{-7}
18	dTDP-α-L-mycarose	β-glycoside	12	5.2×10^{-5}
19	dTDP-β-L-mycarose	α-glycoside	40	5.6×10^{-5}
20	dTDP-α-D-glucose		no reaction	
21	dTDP-α-D-glucosamine		no reaction	

[a] Yields of this table were calculated using the equation, % yield = $[A_p/(A_p + A_r)]$, where A_p represents the integration of the product peak, and A_r represents the integration of the unreacted aglycon peak. Relative activity is the difference of the initial formation rate of glycoside, which was adjusted by the concentration of VinC, between dTDP-sugar and dTDP-α-D-vicenisamine. [b] ND = Stereochemistry is not determined.

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transfer reaction between dTDP-α-D-vicenisamine (**4**) and vicenilactam (**3**) to form vicenistatin (**1**) in the inversion mechanism (Figure 1B).

The recognition mechanism toward the nucleoside part has been well-demonstrated from crystal structures such as GtfA and GtfD, which are, respectively, homologous to VinC with 32 and 23% homogeneity at the amino acid level.[16,17] Regarding the recognition mechanism toward the thymine or uracil moiety, the carbonyl oxygen and ring nitrogen atoms of the pyrimidine base make contacts through hydrogen-bonding interaction with the main-chain amide and the carbonyl group of the highly conserved valine residue (294V in GtfD, 278V in GtfA) within glycosyltransferases recognizing pyrimidinyl diphosphosugar. This valine residue is also conserved in VinC (305V); therefore, this valine residue might play an important role in dTDP recognition of VinC by these hydrogen-bonding interactions.

Specificity toward the Hexose Moiety of dTDP-Sugar. Chemoenzymatic glycoside synthesis, such as *glycorandomization*, is a powerful method for glycoside synthesis, especially in the sugar component alteration of biologically active glycosides. Previous identification of vicenistatin M from the same strain indicated the possibility of VinC to accept dTDP-mycarose. Therefore, glycosylation between dTDP-D-mycarose and vicenilactam was first investigated.

The α-anomer of dTDP-D-mycarose (**10**), prepared by chemical synthesis from methyl α-D-mannopyranoside followed by HPLC separation, was subjected to VinC reaction. The product was analyzed using HPLC equipped with a photodiode array detector. Compared to the authentic sample,[14] the naturally occurring β-anomer of vicenistatin M was identified (Figure 2A). To our surprise, the β-anomer of dTDP-D-mycarose (**11**) was also accepted, and the α-anomer of vicenistatin M was produced (Figure 2B).

This stereospecific glycosylation from both the α-anomer and β-anomer of dTDP-mycarose to form respective glycosides in the inversion mechanism is unique. Although substrate specificities of several glycosyltransferases toward a glycosyl donor have been investigated,[3−8,10,15,18] to our knowledge, no report has described that glycosyltransferase stereospecifically catalyzes the glycosyl transfer reaction from both anomers of the glycosyl donor. Therefore, we set out to investigate this unique phenomenon of VinC reaction in detail.

For further investigation of this unique stereospecific glycosylation of VinC, several dTDP-sugars (**12**−**21**) were tested for VinC reaction (Figure 3). Results indicated that α-anomers and β-anomers of dTDP-D-digitoxose (**12**, **13**), dTDP-D-olivose (**14**, **15**), dTDP-2-deoxy-D-glucose (**16**, **17**), and dTDP-L-mycarose (**18**, **19**) were accepted by VinC to yield respective vicenistatin analogues (Table 1, Figure S3). Generated glycosides, except for **13**, were isolated from large-scale VinC reactions, and the stereochemistries of the anomeric position were determined using ^{1}H NMR, which showed clearly that β-glycosides were produced from α-glycosyl donors and that α-glycosides were generated from β-glycosyl donors. Therefore, it appears that VinC was able to catalyze the stereospecific glycosylation with

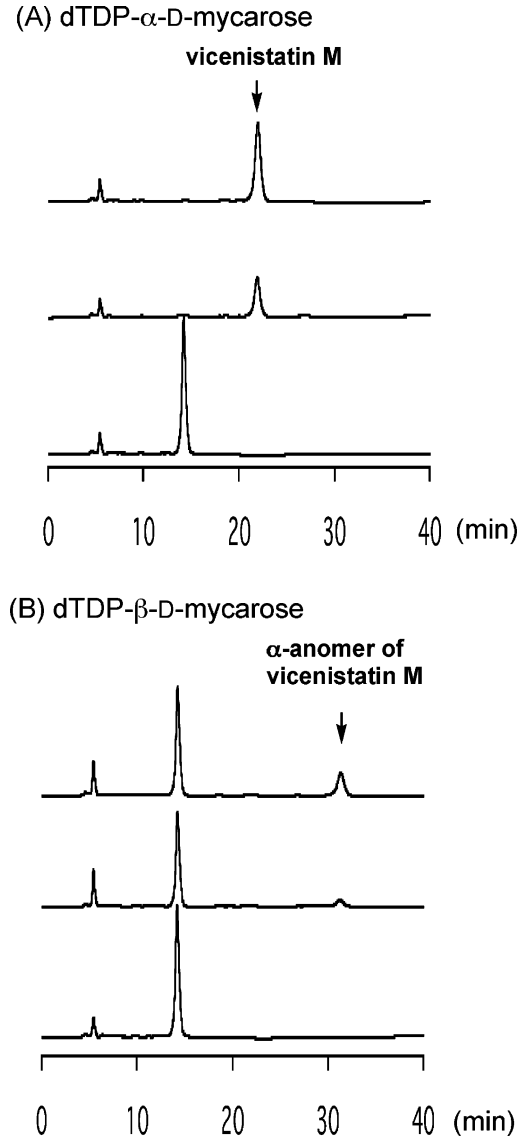

Figure 2. HPLC profiles of the VinC reaction products with (A) α-anomer (**10**), (B) β-anomer (**11**). Top line, co-injection with authentic sample; middle line, enzyme reaction products; bottom line, control experiment without dTDP-mycarose.

structurally diverse dTDP-2-deoxysugar to afford both α-glycosides and β-glycosides, respectively, from β-glycosyl and α-glycosyl donors in the inversion mechanism.

Comparison of the relative activities between these accepted dTDP-D-sugars showed that the α-anomer of dTDP-vicenisamine (**4**) was the best substrate for VinC. The reactivity decreased in order of mycarose (**10**), digitoxose (**12**), olivose (**14**), and 2-deoxyglucose (**16**). In contrast, glucose (**20**) and glucosamine (**21**) were not accepted at all. The fact that dTDP-2-deoxyglucose was accepted and that neither dTDP-glucose nor dTDP-glucosamine was accepted indicates that the steric bulkiness or the hydrophobic interaction at the C2 position of the sugar component is probably important for VinC recognition. In contrast, VinC tolerates the modification at C3, C4, and C6 functional groups within D-series sugars. Furthermore, VinC recognized L-mycarose (**18**, **19**) as well as D-mycarose to afford their respective glycosides, but the marked difference of reactivity between these two glycosyl donors indicates that VinC prefers the D-series sugars as a glycosyl donor. In contrast,

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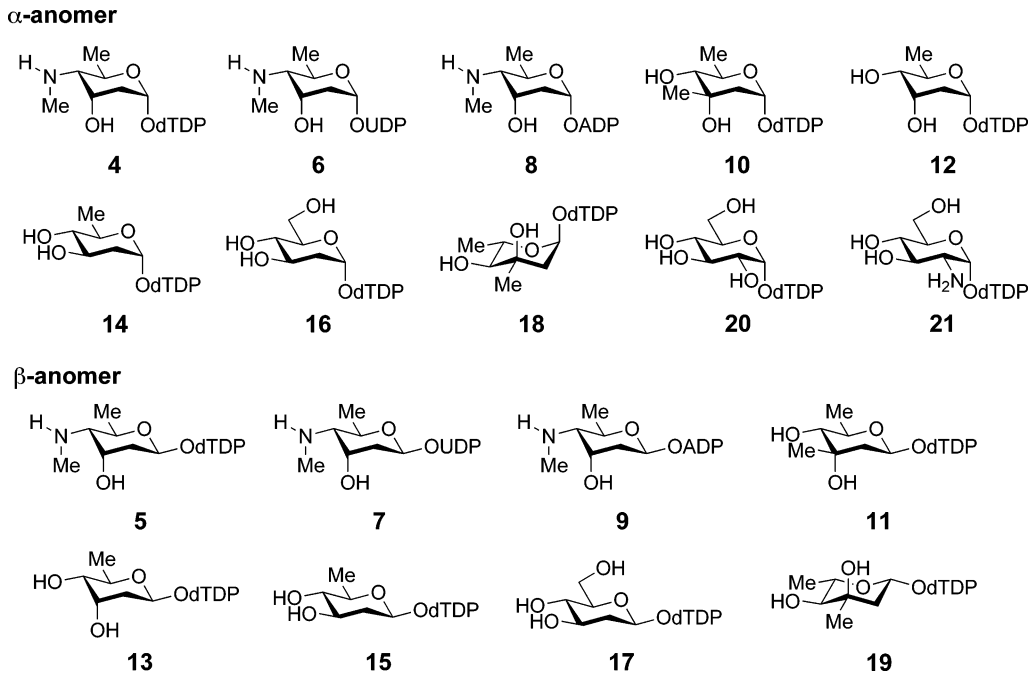

Figure 3. Tested dTDP-sugars for VinC reaction.

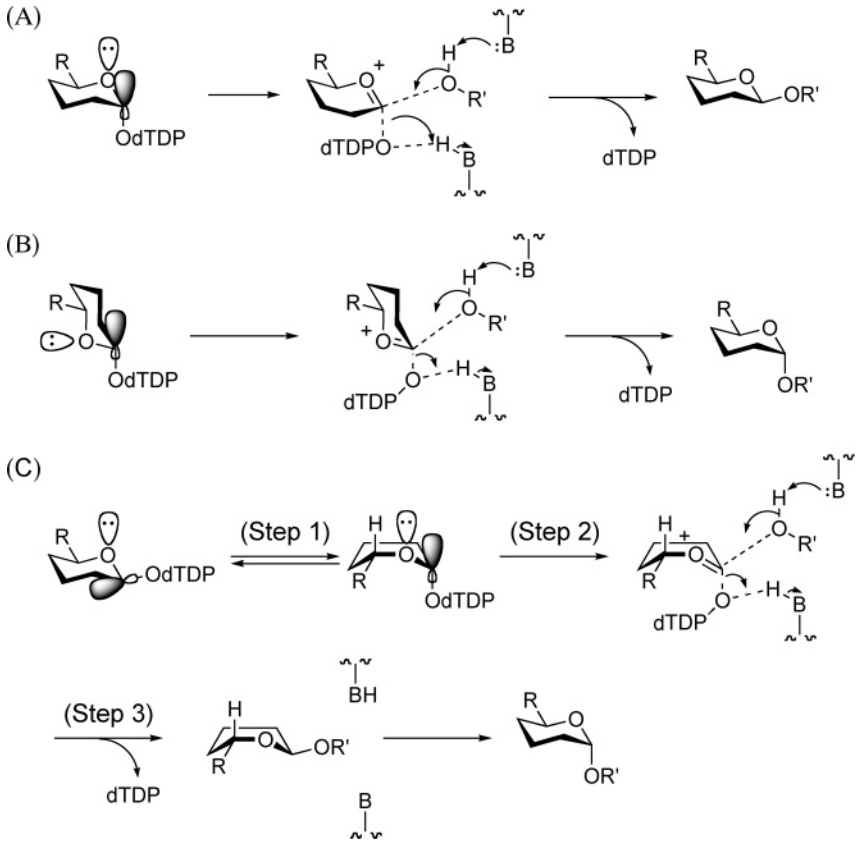

Figure 4. Glycosylation mechanism of (A) β-glycoside formation from the α-glycosyl donor, (B) α-glycoside formation from the β-glycosyl donor in the direct inversion mechanism, and (C) α-glycoside formation from the β-glycosyl donor passing through a conformational change of the hexose part.

β-anomers of dTDP-D-sugar exhibited decreased activity compared to respective α-anomers, which implies that VinC preferentially recognized the α-configuration of dTDP-D-sugar.

Reaction Mechanism for Glycoside Formation. Generally, regioselectivity and stereoselectivity are strictly controlled in the glycosyl transfer reaction by glycosyltransferase. For glycosylation with D-series sugars, the α-anomer of the relevant NDP-sugar is used as a glycosyl donor. The reactions involving simple inversion and double inversion (retention) at the anomeric position give rise, respectively, to β-glycoside and α-glycoside. On the basis of its amino acid sequence, VinC belongs to glycosyltransferase family 1.[19−21] Furthermore, in the VinC reaction with the natural substrate dTDP-vicenisamine, the α-anomer is only accepted to form the β-glycoside, which

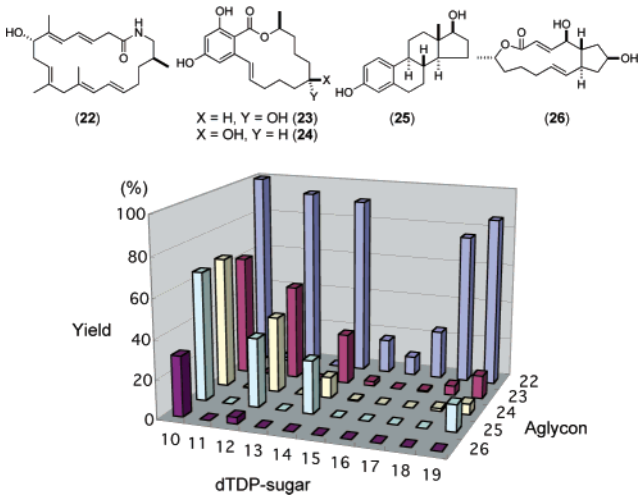

Figure 5. Glycodiversification between glycosyl donors (**10**−**19**) and acceptors (**22**−**26**).

indicates that VinC originally catalyzes the glycosyl transfer reaction in the inversion mechanism, just as other family 1 glycosyltransferases do.

This reaction is considered to proceed via the oxocarbenium ionic intermediate. Formation of the oxocarbenium ionic intermediate is facilitated by the interaction between the unshared electron pair of the endocyclic oxygen and the σ^* orbital of the C1−O1 bond. The reaction is completed by a nucleophilic attack of a hydroxy group to the anomeric carbon of the intermediate (Figure 4A). However, when altered dTDP-sugars were subjected to VinC reaction, the unexpected α-glycoside formations from β-anomers of dTDP-D-sugars were observed. In these cases, such an interaction cannot occur in a stable $^4C_1$ chair conformation having an equatorially oriented dTDP group. The $^4C_1$ conformation for these sugars in solution was confirmed by their ^{1}H NMR data (see Supporting Information), which indicates that the direct inversion mechanism, as shown in Figure 4B from the β-anomer of dTDP-D-sugar, seems implausible.

The mechanism for these α-glycoside formation reactions is explainable according to the mechanism of β-glycosidase, which catalyzes a cleavage of the β-glycosidic bond. In the β-glycosidase reaction, the conformational change of the hexose part in the active site is generally required for inducing the scissile bond to *pseudoaxial* orientation to fulfill the stereoelectronic requirements described above.[22−24] This conformational change permits the oxocarbenium ion formation, followed by a nucleophilic attack of water, thereby leading to the cleavage of the β-glycosidic bond. Therefore, it seems likely that the NDP moiety of dTDP-β-sugar occupies a *pseudoaxial* position, as in the case of β-glycosidase (Figure 4C, step 1). This conformational change of dTDP-β-sugar in the active site causes the interaction between the unshared electron pair of the endocyclic oxygen and the σ^* orbital of C1−O1 bond and facilitates the formation of the oxocarbenium ionic intermediate. A boat conformation or a twisted-boat conformation seems to be suitable for the intermediate structure because this conformation resembles the transition-state conformation. Therefore, when the NDP substituent occupies a *pseudoaxial* position, VinC reaction proceeds smoothly via the oxocarbenium ionic intermediate, followed by a nucleophilic attack to afford α-glycoside, according to the general mechanism of inverting glycosyltransferases (Figure 4C, steps 2 and 3).

Similar examples have already been found for glycosylation catalyzed by DesVII, which originally catalyzes D-desosamine transfer in the inverting mechanism. In addition, dTDP-β-L-rhamnose is reportedly transferred to 12-membered and 14-membered macrolactones to give the respective α-glycosides by DesVII.[25] Although details of the L-rhamnose transfer have not been discussed, similar conformational change might also occur as in the dTDP-L-β-mycarose transfer by VinC. An additional example can be found in a glycosyl transfer reaction by UrdGTII.[26] These examples present the possibility that some glycosyltransferases catalyze stereospecific glycosylation to form both anomers.

Construction of Glycoside Library. A goal of chemoenzymatic glycoside synthesis, such as *glycorandomization*, is construction of a glycoside library with structurally diverse scaffolds. Although glycoside diversification has been partially achieved by changing either the sugar or aglycon part, few examples are known to demonstrate changes of both parts simultaneously.[6,7,10] The VinC has a relaxed specificity toward both parts and catalyzes a unique stereospecific glycosylation. Therefore, we set out to explore the potential of VinC for glycodiversification.

As an effort toward glycodiversification, 50 sets of VinC reaction using dTDP-sugars (**10**−**19**) and aglycons including neovicenilactam (**22**), α-zearalenol (**23**), β-zearalenol (**24**), β-estradiol (**25**), and brefeldinA (**26**) were carried out; the VinC reaction products were analyzed using HPLC-PDA and LC-MS. Figure 5 shows that, when α-anomers of dTDP-mycarose, dTDP-digitoxose, and dTDP-olivose were subjected to VinC reaction, the respective glycosides were observed in 12 of 15 reactions, but the α-anomer of dTDP-2-deoxyglucose was transferred only to neovicenilactam. These results indicate that 2,6-dideoxysugar is a more suitable substrate for glycodiversification with structurally diverse glycosides in this case. In contrast, regarding glycosylation from the β-anomer of dTDP-D-sugar, the expected glycosides were observed only when neovicenilactam **22** was used as a glycosyl donor and the other aglycons were not accepted at all. In addition, the respective glycosides were obtained in six reactions from VinC reaction with dTDP-L-mycarose. In all, formation of 22 glycosides was achieved from 50 sets of VinC reactions. These results indicate that VinC is an attractive glycosyltransferase for glycodiversification.

Conclusion

This in vitro study of substrate specificity toward glycosyl donors in VinC reaction showed that VinC is a promiscuous

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glycosyltransferase to accept various dTDP-sugars as a substrate. In fact, VinC displayed activities with several dTDP-2-deoxy-D-sugars in addition to dTDP-L-mycarose. Moreover, VinC was capable of catalyzing the unexpected β-glycoside and α-glycoside formations, respectively, from α-anomers and β-anomers of dTDP-sugars. The reaction mechanism for axially oriented glycoside formation from the equatorially oriented dTDP-sugar might be explained using the process involving conformational change to a boat or a twisted-boat conformation followed by a nucleophilic attack by a hydroxy group. To apply this feature of VinC, 22 glycosides were constructed using unnatural glycosyl donors and acceptors. These results strongly suggest the potential of VinC for construction of glycoside libraries.

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Supporting Information Available: Experimental procedures and preparation of NDP-sugars and vicenistatin analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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