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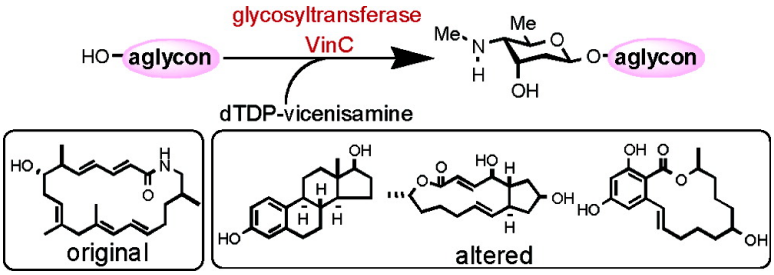
J. Am. Chem. Soc., **2005**, 127 (17), 6148-6149• DOI: 10.1021/ja042848j • Publication Date (Web): 07 April 2005



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Published on Web 04/07/2005

Enzymatic Approach to Unnatural Glycosides with Diverse Aglycon Scaffolds Using Glycosyltransferase VinC

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Glycosides consist of a major class of biologically important metabolites, including various types of antibiotics for clinical use.[1] Among the current interests in drug discovery is to develop glycoside libraries with structural and biological diversities.[2,3] Generally, since both aglycon and the sugar part have to be properly installed in a glycoside to exert its significant biological activity, formation of glycosidic linkage turns out to be a key process for the development of such libraries. Enzymatic synthesis appears to be a method of choice to this end, and it is absolutely necessary to explore potent glycosyltransferases. A key element along this line is the degree of promiscuity of glycosyltransferase toward both glycosyl donor and acceptor.

In the biosynthesis of bioactive natural glycosides, glycosyltransferases are involved mostly in the last-step modification of aglycon, leading to the corresponding ultimate bioactive molecules. Concerning the development of diversity in the sugar part of glycosides, the "glycorandomization approach" developed by Thorson et al. appeared to be a promising solution consisting of in vitro glycosylation through recruitment and gene manipulation of biosynthetic enzymes and acquiring broad promiscuity to the glycosyl donor.[3−5] In contrast, as to the promiscuity for glycosyl acceptors, structural variations to be achieved by currently applicable enzymatic methods are rather limited. Chemical structure that closely resembles the original natural substrate seems to be required as the glycosyl acceptor for the success of utilizing glycosyltransferase in diversification of glycosides.[6] Therefore, exploitation of new glycosyltransferases with significant promiscuity is highly desired. An exception can be found in an example reported recently by Walsh et al., who showed acceptance of varying aglycons having a phenol moiety by NovM functioning in novobiocin biosynthesis.[5] Those viable aglycons included certain simplified coumarin scaffolds and *p*-nitrophenol, which were significantly apart chemically from the natural substrate, novobiocic acid. It appears therefore that certain glycosyltransferases may have significant potential to accept unnatural aglycons having completely different scaffold than those of natural substrate to derive new molecules.

Vicenistatin (**1**), an antitumor polyketide glycoside produced by *Streptomyces halstedii* HC-34, consists of an aminosugar vicenisamine and a 20-membered macrolactam aglycon vicenilactam (**3**).[7] Recently, we have identified the whole gene cluster (*vin*) for the vicenistatin biosynthesis, and VinC has been characterized as the first glycosyltransferase of this sort catalyzing trans-glycosylation from dTDP-vicenisamine (**2**) to the macrocyclic polyketide vicenilactam **3** in the final step of the vicenistatin biosynthesis (Figure 1).[8] Since vicenilactam **3** contains only a hydroxy and an amide functional groups, in addition to C−C double bonds, in such a highly hydrophobic macrocycle, we anticipated that VinC may accept rather hydrophobic aglycons having a scaffold that is different than that of **3**.

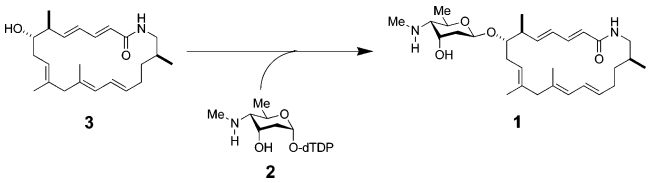

Figure 1. Glycosyltransfer reaction catalyzed by VinC in the last step of vicenistatin biosynthesis.

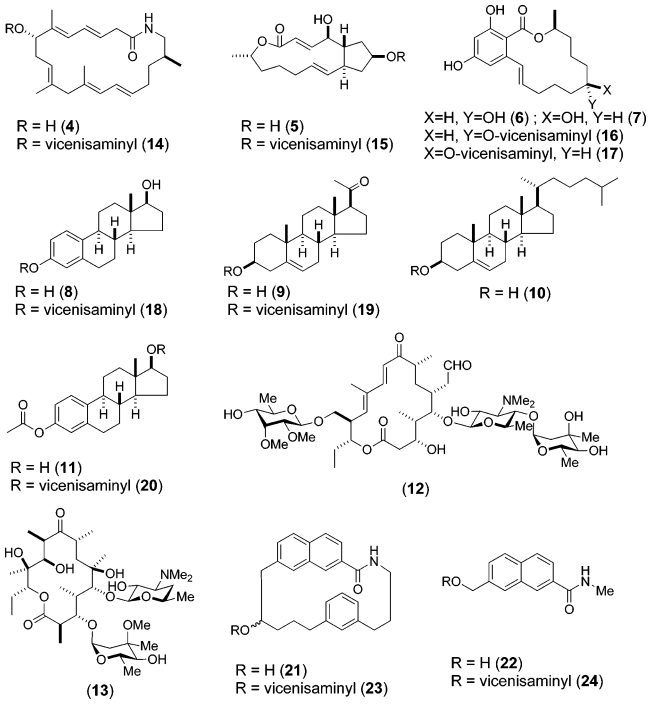

Figure 2. Structures of the substrates and the enzyme reaction products.

Glycosyl transfer reaction by VinC was thus attempted using **2** as a glycosyl donor, and the acceptors tested are shown in Figure 2, that is, a structurally related neovicenilactam (**4**) prepared by alkaline treatment of **3**, brefeldin A (**5**), α- and *β*-zearalenol (**6** and **7**) having similar hydrophobicity to **3** with the least functional groups, *β*-estradiol (**8**), pregnenolone (**9**), cholesterol (**10**), and 3-*O*-acetyl-*β*-estradiol (**11**) as conformationally fixed hydrophobic molecules. In addition, tylosin (**12**) and erythromycin A (**13**) were tested as well.

The enzyme reactions were carried out using heterologously overexpressed and purified VinC, and the reaction products were analyzed mainly by LC−ESIMS.[9] The results clearly indicated that VinC was able to accept **4** (100% conversion by LC−MS analysis), **5** (75%), **6** (51%), **7** (61%), **8** (73%), **9** (58%), and **11** (71%) as a

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10.1021/ja042848j CCC: $30.25

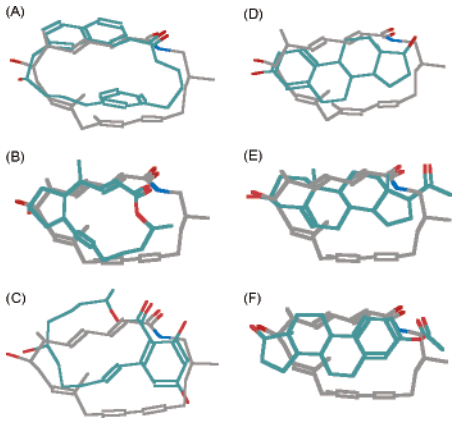

Figure 3. Superimposed view of vicenilactam **3** with (A) mimic **21**, (B) brefeldin A (**5**), (C) α-zearalenol (**6**), (D) *β*-estradiol (**8**), (E) pregnenolone (**9**), and (F) 3-*O*-acetyl-*β*-estradiol (**11**).

glycosyl acceptor. Transglycosylation to cholesterol **10** turned out to be extremely uneffective, and no glycoside formation was observed at all in the reactions with **12** and **13**.

All the vicenisaminide products (**14**−**20**) were isolated by large-scale enzyme reactions and characterized chemically on the basis of spectroscopic analysis. The glycoside bonds were all in *β*-form as determined by ^{1}H NMR spin−spin coupling constants of anomeric positions (**14**, δ_H 4.63, J = 9.6 and 2.0 Hz; **15**; δ_H 4.76, J = 9.6 and 2.0 Hz; **16**, δ_H 4.87, J = 9.6 and 2.0 Hz; **17**, δ_H 4.82, J = 9.6 and 2.0 Hz; **18**, δ_H 5.42, J = 9.2 and 2.0 Hz; **19**, δ_H 4.84, J = 9.6 and 2.4 Hz; **20**, δ_H 4.97, J = 9.2 and 1.6 Hz; vicenistatin, δ_H 5.29, J = 9.5, 3.0 Hz). The site of glycosylation of each vicenisaminide was determined as follows, based on the observation of chemical shift changes in ^{1}H NMR (**15**, C-7, $\Delta\delta_{H-7}$ 0.07 ppm, and $\Delta\delta_{H-4}$ 0 ppm; **16**, C-6′, $\Delta\delta_{H-6'}$ 0.13 ppm; **17**, C-6′, $\Delta\delta_{H-6'}$ 0.05 ppm). The site of glycosylation in **18** was also determined to be at the C-3 position because of apparent chemical shift changes among the A-ring protons: $\Delta\delta_{H-1}$ 0.12 ppm, $\Delta\delta_{H-2}$ 0.22 ppm, $\Delta\delta_{H-4}$ 0.21 ppm, and $\Delta\delta_{H-17}$ 0.01 ppm. Glycosylation to the C-17 position of *β*-estradiol was also successfully achieved using 3-*O*-acetyl-*β*-estradiol (**11**) as a glycosyl acceptor.

It appears therefore that VinC can accommodate various molecules as glycosyl acceptors, including structurally related compounds, such as **4**, but structurally different molecules, such as **5**−**9** and **11**, as well, while **10** in a lesser extent. In other words, even structurally unrelated alcohols to the natural aglycon can be transglycosylated by certain glycosyltransferase as exemplified by VinC, and diverse glycosides of structural and biological interest may well be developed through the present approach.

To figure out the determinative elements of aglycon recognition by VinC, in addition to apparent hydrophobicity, three-dimensional structures of the accepted aglycons were estimated and compared with naturally derived vicenilactam **3**. The structures of all accepted aglycons, except for **5**, were deduced from the molecular mechanics calculation,[10] and the structure of **5** was adopted from its crystal structure (Figure 3).[12] Interesting structural features have been borne out. First of all, gross molecular size may be important since the sizes of all accepted aglycons appeared to be almost the same as **3**. Second, the spatial arrangement of a few polar groups may also be significant. When the glycosyl accepting hydroxy groups of these molecules were superimposed in their 3D structures, the additional oxygen functionality turned out to occupy almost the identical position as that of the amide bond of **3** on the hydrophobic scaffold. Thus, such a hydrogen-bonding interaction appears to be significant in the substrate recognition by VinC. This notion was supported by the rather poor reactivity of **10**, probably due to the lack of hydrogen bonding by the substitution at C-17 with a hydrophobic alkyl side chain. These results appear to imply that a variety of glycosyl acceptors can be designed to fulfill these structural demands. This turned out to be the case since a synthesized racemic mimic (**21**) and its further simplified alcohol (**22**)[13] were in fact reactive in the VinC reaction to form their corresponding vicenisaminides **23** ($[M + H]^+$ = 517, 95% conversion by LC−MS analysis) and **24** ($[M + H]^+$ = 359), respectively, although conversion of **14** was not sufficiently high enough (less than 5% conversion).

In summary, the present studies have successfully demonstrated for the first time significant potential of an enzymatic approach to unnatural glycosides having diverse aglycon structures apart from those found in nature. Particularly, VinC was shown to be useful for the preparation of hydrophobic glycosides with different aglycon scaffolds.

Acknowledgment. This work was supported in part by Grants-in-Aid for Scientific Research from JSPS and a 21st Century COE program from MEXT.

Supporting Information Available: Experimental procedures and characterization data for new compounds (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) In a typical assay, a reaction mixture containing dTDP-vicenisamine (0.9 mM), a glycosyl acceptor in DMSO (0.2 mM for final concentration), and VinC (0.6 mM) was incubated on a shaker at 28 °C for 1.5 hr. The reaction was terminated by the addition of ethyl acetate. The organic layer was separated, concentrated, and redissolved in 30 μL of methanol. The sample was analyzed with a LCQ mass spectrometer (Finnigan) coupled to a NANOSPACE HPLC (SHISEIDO) equipped with a RP-18 GP column (KANTO). HPLC conditions were as follows. After injection of a sample, the column was first washed with 10% MeOH and 0.1% TFA in water for 10 min, and was then eluted with 90% MeOH and 0.1% TFA in water, at a flow rate 50 μL/min. Elution was monitored with a NANOSPACE SI-1 UV detector (SHISEIDO). The percent conversion ratio was calculated by using eq 1, where A_P represents the integration of the product peak, and A_T represents the integration of the unreacted aglycon peak; % conversion = $[A_P/(A_P + A_T)] \times 100$ (eq 1).
(10) Conformational search and energy minimizations were performed using the MM2 force field in Macromodel version 6.5.[11]
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JA042848J