ORIGINAL ARTICLE

Enzymatic synthesis of vancomycin derivatives using galactosyltransferase and sialyltransferase

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Analogues of vancomycin and pseudo-vancomycin with new sugar attachments in mono- and di-saccharide form have been enzymatically synthesized by glycosylation with overexpressed glycosyltransferases, β 1,4-galactosyltransferase and α 2,3-sialyltransferases. All four analogues, including galactose-containing derivatives (6 and 8) and galactose- and sialic acid-containing derivatives (7 and 9) were prepared and characterized by HPLC, LC-MS, NMR and MIC test.

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INTRODUCTION

Vancomycin (**1**) belongs to the glycopeptide class of antibiotics, which are effective in the treatment of life-threatening methicillin-resistant Gram-positive bacterial infections, and more importantly, it is useful in the treatment of clinically resistant *Staphylococcus aureus*. As shown in Figure 1, has two hexoses attached as an L-vancosaminyl-1,2-D-glucosyl disaccharide to residue four, 4-hydroxyphenylglycine, of the heptapeptide scaffold.^{1,2} The desvancosamine derivative of vancomycin, pseudo-vancomycin (**2**), which is 2–4 times less active than the parent antibiotic, was obtained as the major product by treatment with trifluoroacetic acid.³ Recent enzymatic and chemoenzymatic alterations have revealed that vancomycin's disaccharide attachment may be one alteration route for new bioactive compounds.^{4,5} Walsh group and coworkers showed regio-specific enzymatic reconstitution of the antibiotic chloroeremomycin from the heptapeptide aglycone by three glycosyltransferases, GtfA, GtfB and GtfC,⁶ and a systematic utility of chemoenzymatic approaches to diversify complex natural product architectures by using glycopeptides glycosyltransferases, GtfD and GtfE, which are the most versatile enzyme for the synthesis of vancomycin analogues.^{7,8}

Glycosyltransferases have proven to be of great importance in oligosaccharide biosynthesis. One glycosyltransferase gene, *HP0826*, encodes the β 1,4-galactosyltransferase (β 1,4-GalT, EC 2.4.2.22) involved in the biosynthesis of the lipopolysaccharide O-chain backbone of *Helicobacter pylori*. It was cloned nearly a decade ago and demonstrated that HP0826 has a highly efficient activity for the synthesis of trisaccharides (Gal α 1, 4Gal β 1, 4GlcNAc) with recombinant bacteria.^{9–11} Another glycosyltransferase gene, *Pm0188*, which encodes multifunctional sialyl transferases (SiaTs), was cloned from

Pasteurella multocida genomic strain Pm70 and four functions, such as α 2,3-SiaT, α 2,6-SiaT, sialidase and *trans*-sialidase, have been identified.¹² Especially, α 2,3-SiaT can transfer a sialic acid (Sia) residue from CMP-Sia to galactosides to form α 2,3 sialyl linkages efficiently at a wide range of pH values (pH 7.5–9.0). In addition, a highly active β 1,4-GalT and α 2,3-SiaT with broad substrate specificity will be a powerful tool for broadening the application of enzymatic approaches to synthesizing the structurally modified glycone of **1**.

To explore new vancomycin analogues with a glycan chain, we report the incorporation of galactose and Sia by enzymatic glycosylations onto D-glucose of **1** and **2** using two purified enzymes, β 1,4-GalT and α 2,3-SiaT. In addition, MIC test of these analogues were then examined for antibiotic activity.

RESULTS

Overexpression and purification of β 1,4-GalT and α 2,3-SiaT

The PCR products were cloned into the *Nde*I/*Hind*III sites of the pET32a(+) expression vector under the control of the T7 promoter. These recombinants were transferred and expressed as full-length enzymes in a heterologous host strain, *E. coli* BL21(DE3). Expression with C-terminal His₆-tagged fusion proteins in soluble form was achieved at 20 °C with 1.0 mM isopropyl β -D-thiogalactopyranoside (data not shown). During the purification process with Co²⁺ affinity chromatography, the target proteins were completely eluted at a concentration of 100 mM imidazole. The molecular weight of the proteins observed by 12% SDS-PAGE analysis was in good agreement with those of calculated values as 32-kDa (β 1,4-GalT) and 53-kDa (α 2,3-SiaT) (Figures 2b and c), and their contents after purification were found to be 21.19 nmol ml⁻¹ and 16.28 nmol ml⁻¹, respectively.

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Figure 1 The glycopeptide antibiotic vancomycin and pseudo-vancomycin with reduced glycan complexity.

Figure 2 Enzyme reactions with β 1,4-GalT and α 2,3-SiaT from GlcNAc to 3'-sialyllactosamine. **(a)** Schematic representation of the conversion of GlcNAc to 3'-sialyllactosamine by β 1,4-GalT and α 2,3-SiaT. **(b)** SDS-PAGE of purified β 1,4GalT. molecular mass standard (lane 1) and β 1,4GalT (lane 2). **(c)** SDS-PAGE of purified α 2,3-SiaT. α 2,3-SiaT (lane 1) and molecular mass standard (lane 2). **(d)** Mass analysis of 3'-sialyllactosamine from sequential glycosylation of GlcNAc.

Proving the enzymatic activity of β 1,4-GalT and α 2,3-SiaT on *N*-acetylglucosamine

Attempts to overexpress β 1,4-GalT and α 2,3-SiaT were made in the enzymatic synthesis of 4 and 5 from 3 to construct novel glycosylated

vancomycin derivatives that could be directly modified to synthesize glycopeptide analogs (Figure 2a). The galactosylation reaction was carried out on a 30-ml scale with UDP-Gal. After 24 h, 4 was isolated through several purification steps involving the use of DOWEX 1X8-

200 chloride anion-exchange resin and sephadex G10 resin from the reaction mixture. In addition, the sialylation reaction containing CMP-NeuAc and the purified α 2,3-SiaT was performed on the purified disaccharide **4**. The reaction was allowed to proceed for 12 h, and then **5** was isolated through several steps of purification, including activated charcoal chromatography. To obtain a pure sample, the compound was further purified using DOWEX 1X8-200-chloride anion-exchange resin and sephadex G10 resin. As described in the Methods section, MS (Figure 2d) and NMR analyses (data not shown) confirmed the structural identity of **5**. MS analysis of isolated compound **5** showed $m/z=673.2$ $[M-H]^-$, which is identical to the authentic trisaccharide. These results indicated that coupling the trisaccharide **5** production system and overexpressed proteins (β 1,4-GalT from *H. pylori* and α 2,3-SiaT from *P. multocida*) worked very well and suggested that this technique could be applied to the other compounds.

Enzymatic synthesis and analysis of novel glycosylated compounds from pseudo-vancomycin and vancomycin

Compound **2**, which was prepared by trifluoroacetic acid-treated cleavage of the monosaccharide,³ was enzymatically galactosylated with the overexpressed β 1,4-GalT using UDP-Gal as the glycosyl donor (Figure 3a) to generate compound **6**. Then the sialylation of **6** was carried out using overexpressed α 2,3-SiaT and CMP-NeuAc for compound **7**. Glycosylated compounds **6** and **7** were monitored with HPLC analysis (Hypersil ODS) at r.t. for 18.7 and 18.1 min, respectively, and compared with **2** (r.t. 19.8 min). Typically, substrate

conversion yields, 82 and 75% were achieved in **6** and **7** after 14 and 3 h, respectively (Figure 3b). MS analysis of compounds **6** and **7** also showed $m/z = 1469.2$ $[M-H]^-$ and $m/z=1759.8$ $[M-H]^-$, respectively, with high relative abundance at the same r.t (Figure 3c).

Similarly, the glycosylations of **1** were investigated using over-expressed β 1,4-GalT and α 2,3-SiaT, as shown above (Figure 4a). The glycosylated compounds **8** and **9** were also monitored using HPLC analysis (Chromolith performance) at r.t. for 9.2 and 9.6 min, respectively, and compared with **1** (r.t. 10.6 min). Substrate conversion yields, 61 and 50% were observed after 20 h and 90 min, respectively (Figure 4b). MS analysis revealed that the molecular ions MW/2 for **8** and **9** were detected at $m/z = 805.8$ $[M-H]^-$ and $m/z=952.2$ $[M-H]^-$, respectively, at r.t. (Figure 4c).

NMR spectroscopy

The structure elucidations of pseudo-vancomycin (**2**), **6** and **7** were achieved by use of 2D 1H - 1H COSY and 1H - 1H TOCSY spectroscopy. The chemical shifts observed in the proton NMR closely related to vancomycin (**1**) in the published data without sugar residues. The glucose of pseudo-vancomycin was assigned to δ 5.18 (1-CH), δ 3.46 (2-CH), δ 3.39 (3-CH), δ 3.36 (4-CH), δ 3.31 (5-CH) and δ 3.56 and 3.36 (6-CH₂). The anomeric proton (1-CH) of galactose of **6** was assigned to δ 4.31. The specific protons of neuraminic acid [δ 1.94 (CH₃ CO-) and 1.71 and 2.66 (3-CH₂)] were observed in compound **7**. The anomeric protons (1-CH) of glucose and galactose of **6** and **7** were observed with minor shift (Table 1).

Figure 3 Enzymatic incorporations of galactose and sialic acid into pseudo-vancomycin to give compounds **6** and **7**. (a) Overall enzymatic scheme for the compounds **6** and **7** from pseudo-vancomycin. (b) Typical HPLC analysis monitoring the galactosylation and sialylation. (c) Mass analysis of the purified compounds **6** and **7**.

Figure 4 Enzymatic incorporations of galactose and sialic acid into vancomycin to give compounds **8** and **9**. (a) Overall enzymatic scheme for the compounds **8** and **9** from vancomycin. (b) Typical HPLC analysis monitoring the galactosylation and sialylation. (c) Mass analysis of the purified compounds **8** and **9**.

Table 1 NMR data

	Chemical shift		
	Pseudo-vancomycin (2)	6	7
<i>Glucose</i>			
1-CH	5.18	5.23	5.31
2-CH	3.46	3.56	3.68
3-CH	3.39	3.62	3.72
4-CH	3.36	3.51	3.76
5-CH	3.31	3.59	3.60
6-CH ₂	3.56, 3.63	3.62, 3.65	3.78, 3.82
<i>Galactose</i>			
1-CH		4.31	4.51
2-CH		3.38	3.51
3-CH		3.45	4.04
4-CH		3.71	3.88
5-CH		3.55	3.54
6-CH ₂		3.60	3.75, 3.79
<i>Neuraminic acid</i>			
3-CH ₂			1.71, 2.66
4-CH			3.59
5-CH			3.77
5'-(CH ₃ CO)			1.94
6-CH			3.53
7-CH			3.80
8-CH			3.50
9-CH ₂			3.64, 3.68

Biological activity assays of vancomycin analogs

MIC tests were performed by CCARM in accordance with the CLSI guidelines. All enzymatically synthesized vancomycin derivatives purified by preparative HPLC and standard vancomycin were assayed against 20 Gram-positive strains, including MSSA, MRSA, VISA, VREF and VSEF. As shown in Table 2, natural vancomycin was active against several *S. aureus* and *E. faecalis* (CCARM no. 5108) strains, with MIC values in the range of 1–4 $\mu\text{g ml}^{-1}$ for MSSA, MRSA, VISA and 2 $\mu\text{g ml}^{-1}$ for VSEF. In addition, vancomycin was inactive against several VREF strains containing *vanA* or *vanB* genes (MIC > 128 $\mu\text{g ml}^{-1}$). The pseudo-vancomycin derivative (**6**) containing galactose, exhibited poor activity against MSSA, MRSA, VISA and VSEF (MIC = 4–16 $\mu\text{g ml}^{-1}$), but was inactive against VREF, as was **1**. Furthermore, galactosylation of vancomycin antibiotics (**8**) maintained good activity against several MSSA (CCARM no. 3563) (MIC = 1 $\mu\text{g ml}^{-1}$), MSSA (CCARM no. 3561 and 3562) and MRSA (CCARM no. 3699) (MIC = 2 $\mu\text{g ml}^{-1}$) strains. Their activities against the MRSA (CCARM no. 3695, 3696, 3697 and 3698) and VSEF strains were also improved (MIC = 0.5–1 $\mu\text{g ml}^{-1}$). However, the pseudo-vancomycin derivative (**7**) and vancomycin derivative (**9**) containing Sia had very poor activity against MSSA, MRSA and VSEF (MIC = 16–64 $\mu\text{g ml}^{-1}$) or were inactive against all 20 Gram-positive strains. Similar antibacterial activities were demonstrated for Sia of inactive **7** and **9**.

DISCUSSION

In recent years, there has been increasing interest in the development of novel glycopeptide antibiotics owing to the emergence of vancomycin resistance. Especially, sugar modification has been shown to be

Table 2 Comparison of minimal antibacterial activity of vancomycin and their derivatives (MIC, $\mu\text{g ml}^{-1}$)

No.	Strains	CCARM no.	MIC ($\mu\text{g ml}^{-1}$)					Source
			Vancomycin	6	7	8	9	
1	<i>Staphylococcus aureus</i>	27	1	8	>128	2	32	ATCC 29213
2	<i>Staphylococcus aureus</i>	3561	1	4	128	2	32	MSSA, human isolate
3	<i>Staphylococcus aureus</i>	3562	1	4	128	2	32	MSSA, human isolate
4	<i>Staphylococcus aureus</i>	3563	1	8	>128	1	32	MSSA, human isolate
5	<i>Staphylococcus aureus</i>	3695	1	4	128	0.5	32	MSSA, human isolate
6	<i>Staphylococcus aureus</i>	3696	2	8	>128	1	64	MSSA, human isolate
7	<i>Staphylococcus aureus</i>	3697	2	8	>128	1	64	MSSA, human isolate
8	<i>Staphylococcus aureus</i>	3698	1	4	64	0.5	16	MSSA, human isolate
9	<i>Staphylococcus aureus</i>	3699	1	8	128	2	32	MSSA, human isolate
10	<i>Staphylococcus aureus</i>	3502	4	16	>128	8	>128	MSSA, human isolate
11	<i>Enterococcus faecalis</i>	11	4	32	>128	4	64	ATCC 29212
12	<i>Enterococcus faecalis</i>	5012	>128	>128	>128	>128	>128	VREF, vanB, human isolate
13	<i>Enterococcus faecalis</i>	5013	>128	>128	>128	>128	>128	VREF, vanB, human isolate
14	<i>Enterococcus faecalis</i>	5014	>128	>128	>128	>128	>128	VREF, vanB, human isolate
15	<i>Enterococcus faecalis</i>	2015	>128	>128	>128	>128	>128	VREF, vanB, human isolate
16	<i>Enterococcus faecalis</i>	5108	2	8	128	1	32	VSEF, human isolate
17	<i>Enterococcus faecalis</i>	5100	>128	>128	>128	>128	>128	VREF, vanA, human isolate
18	<i>Enterococcus faecalis</i>	5101	>128	>128	>128	>128	>128	VREF, vanA, human isolate
19	<i>Enterococcus faecalis</i>	5102	>128	>128	>128	>128	>128	VREF, vanA, human isolate
20	<i>Enterococcus faecalis</i>	5104	>128	>128	>128	>128	>128	VREF, vanA, human isolate

Abbreviations: MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*; VISA, vancomycin intermediate-resistant *S. aureus*; VREF, vancomycin-resistant *Enterococcus faecalis*; VSEF, vancomycin-susceptible *Enterococcus faecalis*.

a significant factor in the improvement of activity against vancomycin-resistant enterococci.^{13,14} Therefore, the ability to generate additional sugar variants of vancomycin by using dedicated glycosyltransferases would be of particular interest. Diversification of vancomycin through chemoenzymatic strategies to identify new derivatives with novel biological activities has been a practical process; however, these strategies have several limitations, including difficulty synthesizing the sugar donor and substrate specificity.^{8,15} This approach is more convenient than traditional chemical glycosylation of the vancomycin aglycon due to the protection of the aglycon before glycosylation.¹⁶ The diversity of glycones that can be enzymatically incorporated into the vancomycin aglycon also was reduced in other groups through enzymatic glycosylation with their own glycosyltransferases, GtfE, GtfD, GtfC and GtfA.^{5–7,17} Likewise, we have established that the procedure involving enzymatic glycosylation with both the β 1,4-GalT and α 2,3-SiaT cloned from different origins (*H. pylori* and *P. multocida*, respectively) and subsequent purification after heterologous expression in *E. coli* is useful for altering the di- and trisaccharyl chains of vancomycin.

The β 1,4-GalT gene involved in the biosynthesis of lipopolysaccharides in *H. pylori* has been cloned and expressed in *E. coli*, and it was also confirmed that β 1,4-GalT catalyzed the introduction of galactose from UDP-Gal in a β 1,4 linkage to accept residues from **3**.^{9,10} Thus, Wang's group already showed that enzymatic synthesis of the P1 trisaccharide (Gal α 1,4Gal β 1,4GlcNAc), an oligosaccharide analogue, was assessed with *H. pylori* β 1,4-GalT and *Neisseria meningitidis* α 1,4-GalT.¹¹ Those studies provide an efficient approach, including the substrate flexibility of β 1,4-GalT, for the galactosylation of other molecules. In addition, the *Pm0188* gene from *P. multocida* has been cloned, and a truncated protein (tPm0188Ph) was expressed in *E. coli*.¹² They identified its pH-dependent four types of multifunctional activity containing an α 2,3-SiaT (pH 7.5–9.0), an α 2,6-SiaT (pH 4.5–7.0), a sialidase (pH 5.0–5.5), and a *trans*-sialidase

(pH 5.5–6.5). They also showed that α 2,3-SiaT has relaxed acceptor specificity on galactose and β -galactosides. The finding that these kinds of series work strongly supports the possibility of glycosylating galactose and Sia onto D-glucose of **1** and **2** using two purified enzymes, β 1,4-GalT and α 2,3-SiaT.

In this study, we began with enzymatic synthesis of the trisaccharide (NeuAc α 2,3Gal β 1,4GlcNAc, **5**) using two overexpressed glycosyltransferases, β 1,4-GalT and α 2,3-SiaT, to compound **3**, which allowed us to investigate their activities. With the expected substrate specificity of the two glycosyltransferases, continuous galactosylation and sialylation reactions resulted in the incorporation of galactose and Sia to generate derivatives **4** and **5**, respectively. The productions of **4** and **5** were yielded at recovery rates of 91 and 79%, respectively, and this result was also confirmed by MS analyses. The key condition for the enzymatic synthesis of **5** is the use of a buffer at a pH of 8.5 for optimal α 2,3-SiaT activity. Owing to the relaxed substrate specificity described above, we focused second on the actions of β 1,4-GalT and α 2,3-SiaT on compound **1** and **2**, using a slightly modified methodology. Our results showed that both enzymes are promiscuous for the transfer of galactose and Sia to the natural acceptors with high substrate conversion yields (**6**, 82%; **7**, 75%; **8**, 61%; **9**, 50%). All vancomycin derivatives were confirmed by HPLC, MS and NMR analyses. In addition to the difference in production yield between galactosylation and sialylation, the overall turnover rates from **2** were relatively faster than those from **1**.

All enzymatically synthesized derivatives were tested by *in vitro* MIC assays, and they showed antibiotic activity against the MRSA and VSEF strains, with the galactose-containing vancomycin derivative (**8**) having the best MIC values (0.5–1 $\mu\text{g ml}^{-1}$). All other derivatives, including **7** and **9**, showed no activity against the MSSA, MRSA, VISA, VREF and VSEF strains *in vitro*, although the galactose-containing pseudo-vancomycin derivative (**6**) had the least favorable MIC values. Finally, it is interesting to note that galactose-containing derivatives

maintain or improve the antibiotic activity, whereas the galactose/Sia-containing derivatives diminish the activity against resistant strains. Although the exact activity of our derivatives (6–9) remains to be determined, these results are in agreement with earlier results regarding the influence of sugars with regards to their solubility, dimerization constants and conformational flexibility of the aglycon scaffold involved in naturally occurring glycopeptide antibiotics.^{18,19} It is also possible that these vancomycin derivatives containing galactose inhibit transglycosylation by decreasing the affinity to the substrate, and Sia-containing structures will have important roles in cellular recognition and communication because of the anion charge of Sia when they transfer into the cell.^{20,21} Further inhibition studies of our derivatives are required to understand their mechanisms of action in resistant strains.

In summary, we used an enzymatic approach to generate novel vancomycin derivatives using two glycosyltransferases (β 1,4-GalT and α 2,3-SiaT) from other origins and demonstrated the incorporation of galactose and Sia onto the D-glucose glycone of both vancomycin and pseudo-vancomycin. In addition, the MIC test results showed that the antibiotic activities of galactose-containing derivatives against the MRSA and VSEF are the same or better than those of galactose/Sia-containing derivatives. This enzymatic glycosylation strategy utilizing the relaxed substrate specificity of glycosyltransferases, β 1,4-GalT and α 2,3-SiaT, may also be applicable to other glycopeptide antibiotics and other glycosylated natural small molecules, including polyketide and nonribosomal peptide.

METHODS

Materials

Escherichia coli XL1-blue MRF was used as a host cell for the preparation of recombinants plasmids, and *E. coli* BL21(DE3) (Stratagene, La Jolla, CA, USA) was used for the expression of the enzyme. They are typically cultured in Luria-Bertani broth or on Luria-Bertani agar plates at 37 °C.²² Standard vancomycin (GCBA0009, 1) and pseudo-vancomycin (GCBC0016, 2) were purchased from GeneChem (Daejeon, Korea). UDP-galactose (UDP-Gal, U4500), CMP-N-acetylneuraminic acid (CMP-NeuAc, C8271) and N-acetylglucosamine (GlcNAc, A8625, 3) were purchased from Sigma (St Louis, MO, USA). Oligonucleotide primers were synthesized at Geno-Tech (Daejeon, Korea), and all enzymes were purchased from Takara (Kyoto, Japan). The pGEM-T easy vector (Promega, Madison, WI, USA) and pET32a(+) (Novagen, Darmstadt, Germany) were used as vectors for gene cloning and expression, respectively. β 1,4-GalT and α 2,3-SiaT were amplified by polymerase chain reaction (PCR) using the genomic DNA of *H. pylori* (ATCC 43504) and *P. multocida* Pm70 (ATCC 15742), respectively, as a template. PCR was performed on a PCR Thermal Cycler (Takara, Japan), and the PCR products were cloned under the control of the T7 promoter.

Expression and purification of β 1,4-GalT and α 2,3-SiaT

The cloning and expression procedures for β 1,4-GalT and α 2,3-SiaT were performed according to the standard protocol.²² β 1,4-GalT was amplified by PCR with the set of modified primers harboring forward Gal-f (5'-TATCATATGCGTGTGTTTATCATTTTC-3') and reverse Gal-r (5'-GCAAGCTTTACAACTGCCAATATTTC-3'). The primers, Sia-f (5'-TATCATATGAAAACAATCA CGC-3') and Sia-r (5'-GCAAGCTTCAACTGTTTAAACTG-3'), were used to clone α 2,3-SiaT. The restriction sites are indicated by underlined letters. To confirm that there was no mutation, all PCR products (β 1,4-GalT, 822 bp; and α 2,3-SiaT, 1,167 bp) were cloned into the pGEM-T easy vector and sequenced before cloning into the *Nde*I and *Hind*III site of pET32a(+). Plasmids encoding β 1,4-GalT and α 2,3-SiaT were expressed in *E. coli* BL21(DE3) at 20 °C with 1.0 mM isopropyl β -D-thiogalactopyranoside, respectively, and their C-terminal His₆-tagged fusion soluble proteins were purified by Co²⁺ affinity chromatography (TALON Purification Kit, Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. The MW of the proteins were

analyzed and determined by 12% SDS-PAGE. Protein concentrations also determined by the Bradford assay using bovine serum albumin as a standard.²³

Enzymatic effect of β -1,4-GalT and α -2,3 Sia T in NeuAc α 2,3-Gal β -1,4 GlcNAc

Protocols used to enzymatically synthesize Gal β 1,4-GlcNAc (4) and NeuAc α 2,3-Gal β 1,4-GlcNAc (5) using β 1,4-GalT and α 2,3-SiaT were performed as published elsewhere.^{11,12,24} Compound 3 was tested in an enzymatic reaction with β 1,4-GalT in an attempt to prolong the production of 4. The galactosylation reaction was performed with 50 mM HEPES, pH 7.6, 1.0 mM MnCl₂, 40 mM 3, 20 mM UDP-Gal and 100 μ M purified β 1,4-GalT. The reaction mixtures were incubated at 30 °C for 24 h and terminated by boiling for 2 min. The samples were analyzed by thin layer chromatography (TLC) on silica-60 TLC plates (Merck, Darmstadt, Germany) after purification with DOWEX 1X8-200 chloride anion exchange resin and sephadex G10 resin.²⁵ A typical sialylation reaction of α 2,3-SiaT on purified 4 was performed in 100 mM Tris-HCl (pH 8.5) containing 20 mM MgCl₂, 10 mM 4, 10 mM CMP-NeuAc, and 100 μ M purified α 2,3-SiaT. The reactions were allowed to proceed for 12 h at 30 °C, and compound 5 was analyzed by TLC. For the purification of 4 and 5, three kinds of method were used. After adsorbed using SA1500 charcoal (MeadWestvaco, Richmond, VA, USA), those compounds were washed with DW and then eluted by 40% EtOH. Thus, DOWEX 1X8-200 NaCl (0.1 M) anion exchange resin and sephadex G10 resin using FPLC were used to remove salt and small impurity further. Finally, all new compounds, including 4 and 5, were characterized by liquid chromatography-MS (prep. LC-MSD, 1100+G1958, Agilent Technologies, Palo Alto, CA, USA) and NMR analysis.

In vitro galactosylation and sialylation reactions with vancomycin and pseudo-vancomycin

The galactosylation reactions of 1 and 2 were performed respectively in 20 ml of buffer (50 mM HEPES pH 7.6, 5.0 mM MnCl₂, 5.0 mM 1 or 2) with 10 mM UDP-Gal and 100 μ M purified β 1,4-GalT at 30 °C. The reaction mixtures were sampled at various time points. The incubation was terminated by boiling for 5 and 15 min of centrifugation at 13 000 r.p.m. The supernatants, including galactosylated 1 and 2 (8 and 6), were monitored with HPLC analysis using an Adsorbosphere strong anion-exchange analytical columns (Cole-Parmer, Vernon Hills, IL, USA; Hypersil ODS 4.6 $\times$ 250 mm, 5 μ m particle size and Chromolith performance RP-18e, 4.6 $\times$ 100 mm). Furthermore, continuous sialylation reactions of 6 and 8 were performed respectively in 20 ml of buffer (100 mM Tris-HCl pH 8.5, 5.0 mM MgCl₂, 5.0 mM 6 or 8) with 10 mM CMP-NeuAc and 100 μ M purified α 2,3-SiaT at 30 °C. The reaction mixtures were sampled at various time points, and the supernatants, including the sialylated compounds 6 and 8 (7 and 9), were monitored by HPLC as described above.

Purification of vancomycin derivatives (6, 7, 8 and 9) and analytical methods

Two successive preparative HPLC purifications with a mixed solvent system were performed to isolate vancomycin derivatives using SHIMADZU SPD-10Avp (source Q15 resin 200 ml, FineLine Pilot 35 column, Amersham Biosciences, Piscataway, NJ, USA) and K-prep HPLC (ODS-AP 300-S10/20 30 ϕ $\times$ 500 mm column, Kyoto Chromato, Kyoto, Japan). First, water and an aqueous solution containing 1.0 M tetraethylammonium borate were used as eluents, with the concentration of tetraethylammonium borate ranging from 0 to 80%. Then, 10 mM TEAA and acetonitrile were used as eluents, with the concentration of acetonitrile ranging from 0 to 100%. The purified products were analyzed by analytical HPLC after lyophilization, and the purity of the vancomycin derivatives was proven to be more than 98% by HPLC peak integration. All vancomycin derivatives were characterized by LC-MS.

NMR spectroscopy

NMR spectroscopy was achieved by the Korea Basic Science Institute (KBSI, Ochang, Korea). The lyophilized samples were dissolved in 250 μ l of deuterium oxide and 30 μ l of DMSO-d₆ was added. The samples were transferred to the 5 mm thin wall micro-BMS-3 NMR tube (Shigemi, Alison Park, PA, USA). All NMR spectra were recorded at 298 K using Avance II 900 and Avance II 800

spectrophotometer (Bruker-Biospin, Billerica, MA, USA). Conventional two-dimensional experiments ^1H - ^1H correlation spectroscopy, TOCSY (and 70 ms z-filtered DIPSI-2 sequence was used for Hartmann-Hahn mixing), All NMR data were processed using TopSpin 2.1 (Bruker-Biospin) software on a Linux-operating PC workstation.

MIC tests

In vitro MIC assays were performed by culture collection of antimicrobial resistant microbes (CCARM, Korea, <http://www.ccarm.or.kr>) according to the clinical laboratory standards institute (CLSI, Wayne, PA, USA) guidelines. The MICs were measured by microdilution ($0.25 \sim 128 \mu\text{g ml}^{-1}$) in Muller-Hinton broth, supplemented with 2% NaCl for MRSA (CLSI).²⁶ All of the vancomycin analogs were assayed against methicillin-susceptible *S. aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), vancomycin intermediate-resistant *S. aureus* (VISA), vancomycin-resistant *Enterococcus faecalis* (VREF), and vancomycin-susceptible *E. faecalis* (VSEF).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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