CHEMISTRY
AN ASIAN JOURNAL

DOI: 10.1002/asia.200600084

Vancomycin Analogues Containing Monosaccharides Exhibit Improved Antibiotic Activity: A Combined One-Pot Enzymatic Glycosylation and Chemical Diversification Strategy

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Abstract: Many natural products contain carbohydrate moieties that contribute to their biological activity. Manipulation of the carbohydrate domain of natural products through multiple glycosylations to identify new derivatives with novel biological activities has been a difficult and impractical process. We report a practical one-pot enzymatic approach with regeneration of cosubstrates to synthesize analogues of vancomycin that contain an *N*-alkyl glucosamine, which exhibited marked improvement in antibiotic activity against a vancomycin-resistant strain of *Enterococcus*.

Keywords: antibiotics · chemoenzymatic synthesis · enzyme catalysis · glycopeptides · vancomycin

Introduction

A diversity of natural products that exhibit a variety of useful biological activities have been isolated and characterized. Many of these bioactive compounds contain sugar units, which are often required for function in vivo, at specific positions on the aglycon core, but their role at the molecular level is not well-understood. Glycosylation of natural products may affect solubility, stability, or molecular recognition associated with the biological target.[1,2] Consequently, development of practical methods for modification of the carbohydrate domain of natural products may lead to a better understanding of the process and identification of new therapeutics.

The glycans on natural products are structurally diverse, with sugar lengths ranging from monosaccharides to oligosaccharides. Though O-linked glycosylation is most common, natural products that contain C- or N-linked glycosylation (e.g., urdamycin and rebeccamycin) are also known. Regardless of the type of linkage, the glycosylated compounds are biosynthesized by glycosyltransferases, which transfer sugars, often late in the biosynthesis, to the aglycon or to elaborate an existing incomplete glycan.

We have been interested in studying the role of glycosylation on glycopeptide antibiotics and the effects of adding sugars to peptide antibiotics. In our previous study of peptide antibiotics, we examined the effects of glycosylating a naturally nonglycosylated natural product, tyrocidine A.[3] Our study and work with Walsh and co-workers resulted in some glycosylated analogues with preservation or improvement of biological selectivity, along with improvements in solubility.[4–6] In this complementary study, we examined the effects of modifying and reducing the sugar complexity of a naturally glycosylated natural product. It is hoped that new antibiotics with a minimized number of sugar units discovered in this study can be prepared on a large scale for further development. Herein we report the use of vancomycin as a model for the development of a practical and efficient chemoenzymatic synthesis of lipoglycopeptide analogues containing a simplified monosaccharide instead of the natural L-vancosaminyl-1,2-D-glucosyl disaccharide.

Vancomycin is a glycopeptide antibiotic used for the treatment of life-threatening methicillin-resistant Gram-positive bacterial infections caused by pathogens such as *Staphylococcus aureus*, *Enterococcus faecium*, and *E. faecalis*.[7,8] It is effective against Gram-positive but not Gram-negative bacteria, owing to the outer membrane of Gram-negative bacte-

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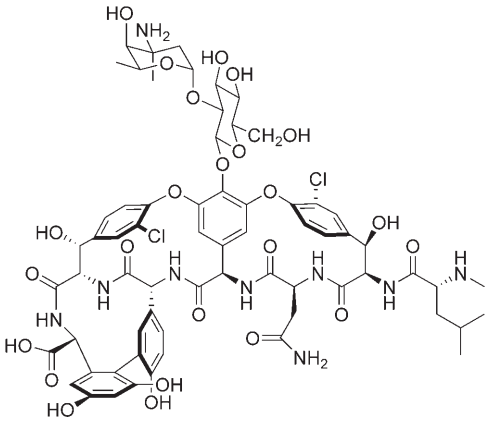
ria acting as a permeability barrier that prevents the glycopeptide from accessing its targets at the periplasmic face of the inner membrane.

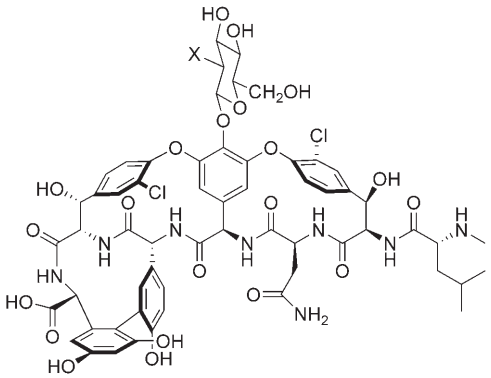
Vancomycin was the first glycopeptide antibiotic to be discovered, and was isolated in the 1950s during the screening of natural products from a soil sample.[9] Since then, other glycopeptide antibiotics (characterized by a heptapeptide core with oxidative cross-linking, a triphenyl ether moiety, and various sites of glycosylation) have been reported, for example, teicoplanin, chloroeremomycin, ristocetin A, and A47934. Isolated from *Amycolatopsis orientalis*, vancomycin functions by binding to the terminal D-Ala–D-Ala dipeptide of peptidoglycan precursors, thereby precluding the maturation of bacterial cell walls.[10–14] Resistance occurs in VanA and VanB bacterial phenotypes when bacteria alter the terminal dipeptide of peptidoglycan precursors to the depsipeptide D-Ala–D-Lac,[15] thus weakening the binding 1000-fold by virtue of the disruption of a hydrogen bond and formation of electrostatic repulsion.[16,17] Furthermore, a VanC phenotype has been observed whereby D-Ala–D-Ser is the terminal dipeptide of peptidoglycan precursors.[18] Over the past decade, the global emergence of vancomycin-resistant enterococci (VRE) and vancomycin-resistant *S. aureus* (VRSA) reveals the need for more potent antibiotics.[19,20] Consequently, the search for vancomycin analogues with improved activity against VRE and VRSA is an apposite and active area of research.

The first glycopeptides with lipophilic substituents on the carbohydrate moiety displaying activity against vancomycin-resistant strains were made from vancomycin by reductive animation.[21,22] Since then, semisynthetic glycopeptide analogues with lipophilic portions have been in human clinical trials.[23] Moreover, monoglycosylated lipoglycopeptide analogues of vancomycin have been made by chemoenzymatic synthesis,[24–27] though only one has reported activity against VRE. Additionally, some lipoglycopeptides have been shown to act by direct inhibition of the major transglycosylases of *Escherichia coli* and *S. aureus*.[28–32] To identify new vancomycin analogues with a reduced glycan and to explore the effect of lipidation on antibiotic activity, we report herein a combination of one-pot enzymatic glycosylation and chemical glycan modification as an effective method for synthesizing lipoglycopeptide analogues of vancomycin. These analogues were then examined for antibiotic activity against vancomycin-sensitive and -resistant strains, as well as methicillin-resistant *S. aureus* (MRSA).

We designed a one-pot enzymatic approach to make monoglycosylated glycopeptide antibiotics. This enzymatic methodology is more straightforward and practical compared to lengthy chemical procedures for glycosylation of glycopeptide aglycons. As an initial example, we used vancomycin aglycon as the platform for glycosylation with native and nonnative monosaccharides. GtfE, the biosynthetic enzyme that glucosylates vancomycin aglycon at the phenolic hydroxy group of the $Phegly_4$ residue to produce desvancosaminyl vancomycin (**1**),[33,34] was used in this one-pot method to generate glycopeptide **1** and a glucosamine-containing vancomycin analogue **2** (Figure 1). The synthesis of desvan-



vancomycin



1 X = OH desvancosaminyl vancomycin
2 X = NH_2 glucosamine-containing analogue

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

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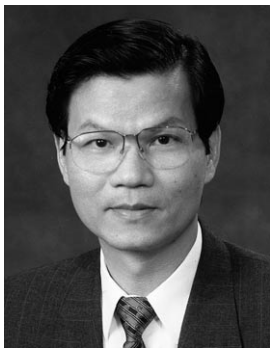

Chi-Huey Wong *received his BS and MS from National Taiwan Univ. and PhD from M.I.T. in 1982. After postdoctoral research at Harvard Univ., he joined Texas A&M Univ. in 1983 and was promoted to Prof. in 1987. He has been Prof. and Ernest W. Hahn Chair in Chemistry at the Scripps Research Institute since 1989, and Director of the Genomics Research Center at Academia Sinica, Taipei since 2003. His research interests involve bioorganic and synthetic chemistry and biocatalysis, focusing on the development of new synthetic methods for carbohydrate-mediated biological recognition.*

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cosaminyl vancomycin in this way is the first application of a one-pot enzymatic strategy with an antibiotic glycosyltransferase. This approach is more efficient than two-step enzymatic procedures with Ep and GtfE, as it decreases product inhibition of GtfE by uridine diphosphate (UDP) and lowers the cost of cosubstrates by regenerating catalytic amounts of nucleotide. Application of the one-pot strategy to make a glucosamine-containing vancomycin analogue is possible owing to the relaxed substrate specificities of Ep[35] and GtfE[36], though greater amounts of cosubstrates and enzymes are required relative to the natural glucose substrate. The glucosamine-containing vancomycin provided an amine as a chemoselective handle for subsequent chemical derivatization. Reductive alkyl- and arylation of the primary amine on the glucosamine moiety would afford a series of vancomycin analogues. Thus, our one-pot enzymatic strategy is designed for generating glycopeptide antibiotics that contain monosaccharides, and coupling this approach with chemical diversification may afford lipoglycopeptide analogues with activity against vancomycin-resistant bacteria. This simple chemoenzymatic approach should achieve modification of the vancomycin glycan by a divergent method that facilitates identification of new antibiotics, and is more amenable to large-scale synthesis than lengthy and complex glycosylation strategies.

Results

Design for Synthesis of Monoglycosylated Lipoglycopeptide Vancomycin Analogues

Although enzymatic[36] and chemical[37–40] approaches have been used to glycosylate vancomycin aglycon, we pursued enzymatic synthesis as a means to make a monoglycosylated vancomycin intermediate that could be modified directly to lipoglycopeptide analogues (Figure 2). This chemoenzymatic strategy[24–27,41] is straightforward and practical as it circumvents protection[37] (and the corresponding deprotection) of the peptide aglycon required in chemical procedures, yet provides access to a wider range of analogues than could be obtained by only enzymatic methods. Compared to previous enzymatic strategies, we decided to use a one-pot approach with regeneration of cosubstrate (Scheme 1) for the enzymatic glycosylation to minimize product inhibition and lower cosubstrate cost. We envisioned that this one-pot method could provide sufficient quantities of product for divergent chemical modification and biological testing.

Improving the Thermal Stability of GtfE

Before attempting the one-pot glycosylation method (described below; Scheme 1) the GtfE reaction was investigated and optimized. Vancomycin aglycon, prepared by HF-mediated cleavage of the disaccharide,[42] was glycosylated enzymatically with GtfE by using UDP–glucose as the glycosyl donor (Figure 3a). Previous work has shown that GtfE has optimal activity at 37 °C.[43] However, it became evident that GtfE loses its catalytic activity at 37 °C after about 12 h (Figure 3b). Therefore, chemical thermal stabilizers were explored to prolong enzymatic activity and to achieve respectable product formation. Polyols, including ethylene glycol, glycerol, and several sugars, were tested as additives to extend GtfE activity (Figure 3b). Such polyols are known to stabilize protein native structures in certain cases when used as cosolvents.[44–50] In general, additives with more hydroxy groups or higher concentrations of those with fewer such

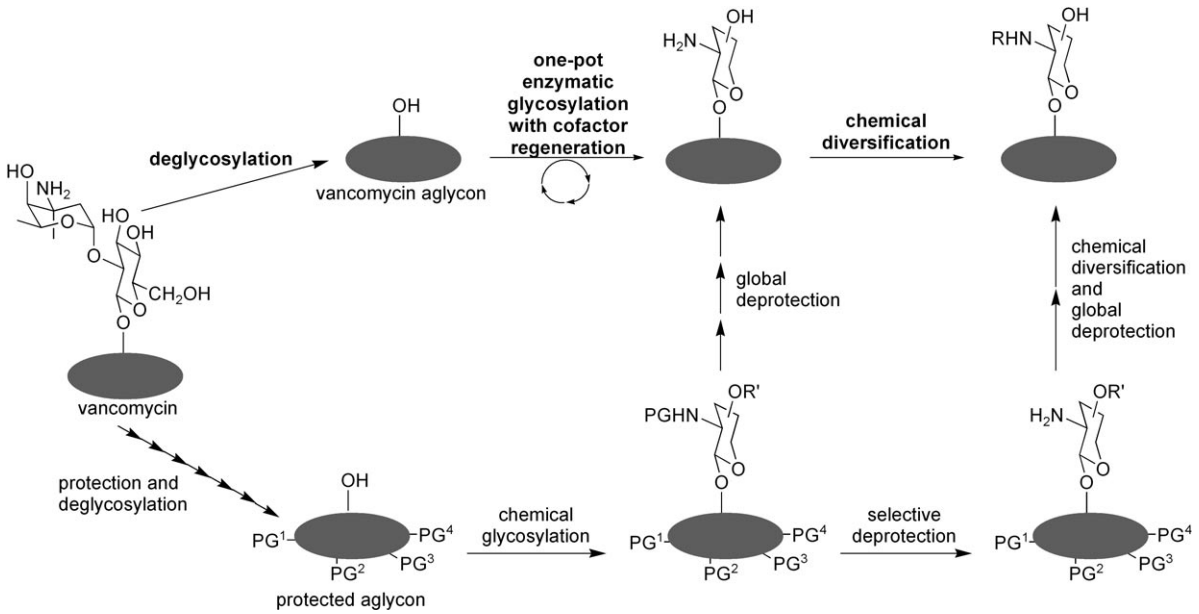

Figure 2. Comparison of enzymatic and chemical approaches for glycosylation of vancomycin aglycon, with our strategy highlighted in bold. Chemical diversification of the glycan provides access to lipoglycopeptide analogues. PG = protecting group.

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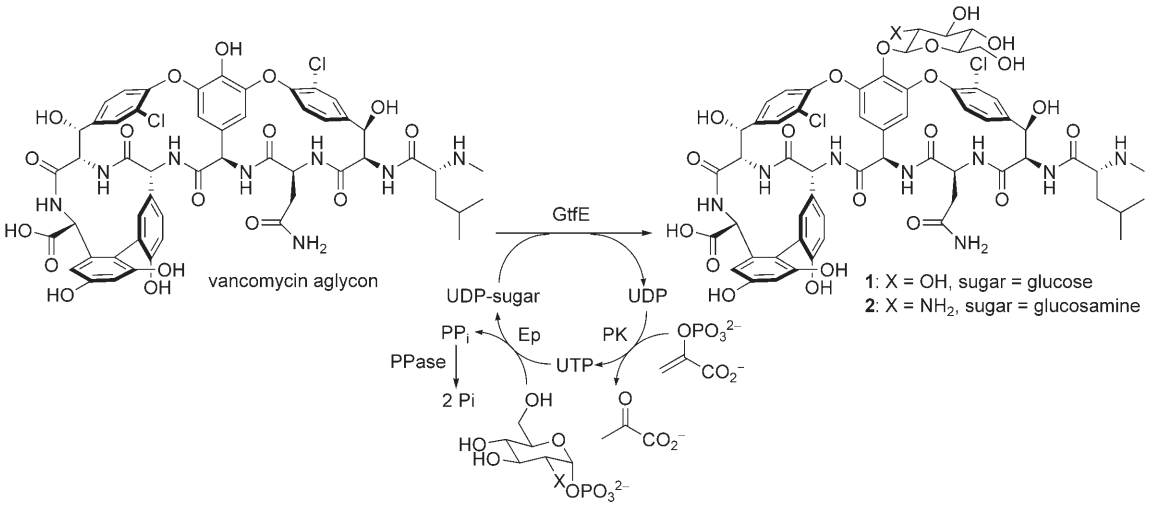

Scheme 1. One-pot enzymatic glycosylation of vancomycin aglycon to desvancosaminyl vancomycin **1** and analogue **2** with glycosyltransferase (GtfE), thymidylyltransferase (Ep), pyruvate kinase (PK), and inorganic pyrophosphatase (PPase). UDP = uridine 5′-diphosphate, UTP = uridine 5′-triphosphate.

groups were better thermal stabilizers. Other additives such as alcohols (methanol, ethanol, *iso*-propanol, *n*-propanol, *tert*-butanol) and detergents (tween 80 and BRIJ35) were also tried, but they did not improve the reaction (data not shown). With the knowledge that some polyols could extend the activity of GtfE to 48 h, we included such a thermal stabilizer in our one-pot approach.

One-pot Conditions for the Synthesis of Desvancosaminyl Vancomycin

As a one-pot enzymatic approach had not yet been reported for an antibiotic glycosyltransferase, we used the glucosylation of vancomycin aglycon as an initial prototype to test our enzymatic one-pot procedure. We considered that the synthesis of desvancosaminyl vancomycin from the aglycon could be reconstituted in vitro by using a one-pot approach with four enzymes (Scheme 1). Starting from α-D-glucose-1-phosphate (Glc-1-P), thymidylyltransferase (Ep) condenses Glc-1-P with uridine 5′-triphosphate (UTP) to form uridine 5′-diphosphoglucose (UDP-Glc) and inorganic pyrophosphate (PP_i). Since the equilibrium of this reaction lies towards Glc-1-P and UTP, the desired UDP-Glc is sequestered by hydrolysis of PP_i with inorganic pyrophosphatase into two equivalents of inorganic phosphate (P_i). UDP-Glc then acts as substrate for GtfE for glucosylation of vancomycin aglycon to desvancosaminyl vancomycin, along with release of uridine 5′-diphosphate (UDP). Pyruvate kinase then converts UDP into UTP by using one equivalent of phosphoenolpyruvate (PEP) as the phosphoryl donor.

Although all enzymes except GtfE have optimal activities near pH 7.5, GtfE has a very strict requirement for pH 9.[43] For this reason, the one-pot enzymatic synthesis of glycopeptide **1** was best carried out by using pH 9 tricine buffer with glycerol as a thermal stabilizer. Furthermore, Ep requires a divalent metal cation,[51] and, among those evaluated, including Ca, Ni, Co, and Zn, magnesium and manganese gave the optimal yield for coupling Ep and GtfE (data not shown). For the one-pot procedure, magnesium chloride was used to satisfy the prerequisite for a divalent metal.

Unlike previous reports of generating **1** enzymatically through two steps (by using Ep and GtfE sequentially),[36] this one-pot procedure uses catalytic amounts of UTP (0.125 equiv per equiv of aglycon) and only a slight excess of cosubstrates (1.25 equiv each of Glc-1-P and PEP). Substoichiometric amounts of UTP and regeneration of UTP by pyruvate kinase are advantageous for minimizing product inhibition of GtfE by UDP.

Modification of One-Pot Conditions for Introduction of Glucosamine

With the optimized conditions for one-pot glycosylation with Glc-1-P, we were encouraged to use our method to incorporate a nonnatural glucose analogue that could serve as a substrate for Ep and GtfE and provide a chemoselective handle for chemical lipophilic modification. Previous work has shown the general applicability of using Ep for the synthesis of UDP–sugars and GtfE for nonnatural glycosylation of vancomycin aglycon.[52] We envisioned that our one-pot method could provide sufficient quantities of product for divergent chemical modification and biological testing. We chose glucosamine as the desired glycan, as lipophilic modification of the primary amine of the vancosamine moiety of vancomycin was shown to yield lipoglycopeptide antibiotics with activity against VRE.[21,22] A glucosamine-containing vancomycin derivative with a lipophilic modification has also been reported with VRE activity.[27] Furthermore, the corresponding sugar-1-phosphate and UDP–sugar are known substrates of Ep[35] and GtfE,[36] respectively.

The one-pot synthesis of vancomycin analogue **2** is analogous to that developed for desvancosaminyl vancomycin,

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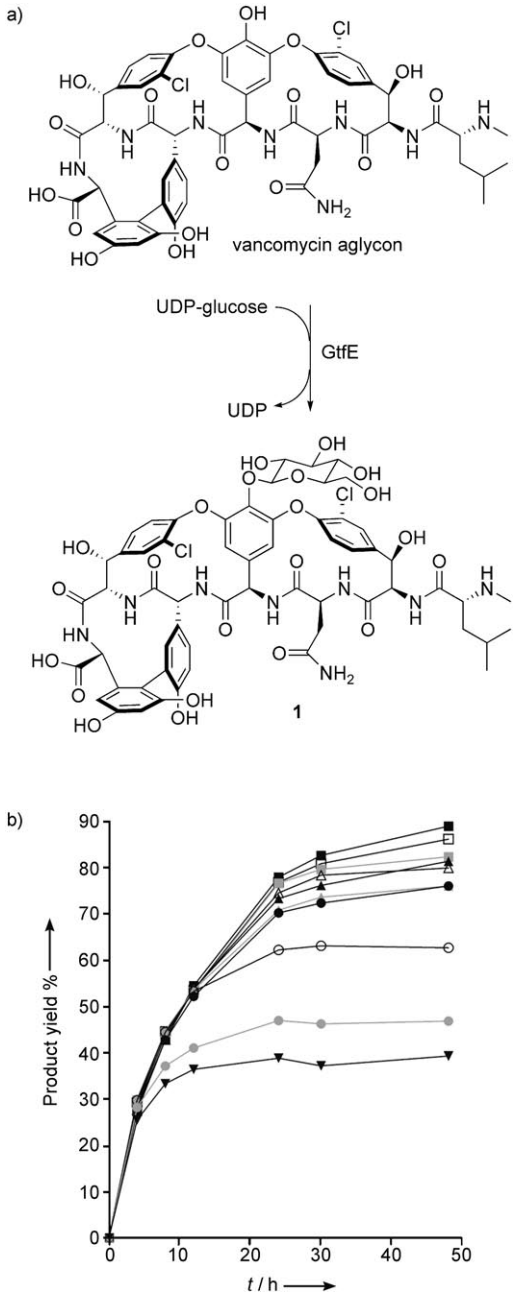
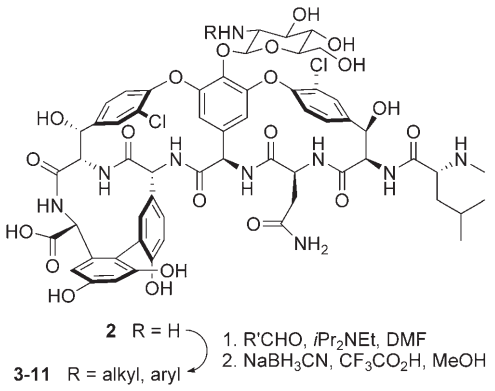


Figure 3. Prolonged enzymatic activity of a) the GtfE reaction was observed by b) addition of polyol thermal stabilizers. ■ Sucrose, □ glycerol (1.5 M), ■ ethylene glycol (2 M), ▲ sorbitol, △ trehalose, ▲ lactose, ● glucose, ○ maltose, ● ethylene glycol, ▼ none. All polyols were tested at 0.5 M unless noted otherwise.

with α-D-glucosamine-1-phosphate ($GlcNH_2$-1-P) replacing Glc-1-P, thereby changing the sugar nucleotide substrate of GtfE to uridine 5′-diphosphoglucosamine (UDP-$GlcNH_2$; Scheme 1). Earlier work showed that Ep accepts $GlcNH_2$-1-P as it does Glc-1-P.[35] Previous kinetic studies of GtfE with several NDP–sugar substrates demonstrated the substrate flexibility of GtfE.[36] These studies report a relative specificity constant (k_{cat}/K_M) for UDP-Glc that is 13 times that for UDP-$GlcNH_2$, with the difference due completely to a reduction in k_{cat}. To obtain the favorable conversion of vancomycin aglycon to glucosamine-containing vancomycin analogue **2**, the one-pot method was modified for the new substrate with additional amounts of cosubstrates and enzymes. Two equivalents of cosubstrates were required in this method, which is still an advance over the five equivalents applied to two sequential enzymatic reactions with Ep and GtfE.

Chemical Diversification of Glucosamine-Containing Vancomycin

As we predicted, replacing glucose with glucosamine provided a chemoselective handle for chemical diversification. Selective reductive alkyl- and arylation of the primary amine on the glucosamine moiety with a series of aldehydes was achieved by imine formation followed by reduction with sodium cyanoborohydride (Scheme 2). Alkyl chains with even numbers of carbon atoms from 6 to 18, benzyl, and 4-chloro-4′-methylbiphenyl substituents were appended to the primary amine of glycopeptide **2**.



Scheme 2. Chemical modification of glycopeptide analogue **2** by selective reductive alkyl- and arylation of the primary amine on the glucosamine moiety. $R = C_{2n}H_{4n+1}$ ($n = 3$–9), Bn, 4-chloro-4′-methylbiphenyl; $R' = C_{2n-1}H_{4n-1}$ ($n = 3$–9), Ph, 4′-chlorobiphenyl.

Biological Activity Assays of Vancomycin Analogues

Minimal inhibitory concentration (MIC) tests were performed on all synthesized vancomycin derivatives. The derivatives were purifed by reverse-phase HPLC prior to MIC determination with MRSA (ATCC 33591), vancomycin-sensitive *E. faecalis* (VSE; ATCC 29212), and VRE (ATCC 51575, VanB). The MRSA and VSE strains were tested at concentrations of 0.1 to 10 μM, and the VRE strain was tested at 0.5 to 50 μM (except vancomycin, which was tested to 2 mM). All derivatives displayed activity against the vancomycin-sensitive strains, and derivatives containing alkyl chains of 12, 14, and 16 carbon atoms showed the best activity against the VRE strain (Table 1).

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Table 1. MIC values of synthesized glycopeptides against methicillin-resistant *S. aureus* (MRSA), vancomycin-sensitive *E. faecalis* (VSE), and vancomycin-resistant *E. faecalis* (VRE).

Glycopeptide	MRSA MIC [μM]	VSE MIC [μM]	VRE MIC [μM]
vancomycin	1	2	1000
vancomycin aglycon	1	5	>50
2 R = H	2	10	>50
3 R = C_6H_{13}	2	5	>50
4 R = C_8H_{17}	1	2	50
5 R = $C_{10}H_{21}$	0.5	0.5	10
6 R = $C_{12}H_{25}$	1	0.5	2
7 R = $C_{14}H_{29}$	2	1	1
8 R = $C_{16}H_{33}$	2	2	2
9 R = $C_{18}H_{37}$	5	10	50
10 R = CH_2Ph	2	5	50
11 R = chlorobiphenyl	0.5	0.5	10

Discussion

We have developed the first one-pot enzymatic method with an antibiotic glycosyltransferase for the glycosylation of vancomycin aglycon (Scheme 1). This approach is more convenient and direct than traditional chemical or stepwise enzymatic glycosylation of glycopeptide aglycon structures. Chemical methods[37–39] involve lengthy procedures for protecting the aglycon prior to glycosylation, followed by further deprotection steps (Figure 2). Also, reports of chemical glycosylation of vancomycin aglycon include extensive and difficult syntheses of the sugar donor, due to the poor reactivity of the aromatic hydroxy group of $Phegly_4$. However, the advantage is that chemical synthesis can, in principle, generate a large diversity of glycans, as illustrated in the use of the reactivity-based one-pot glycosylation method[40], whereas enzymatic methods are limited by substrate specificity. The limitation of the diversity of glycans that can be incorporated enzymatically is lessened in our approach by coupling the enzymatic glycosylation with chemical diversification.[24–27,41] Furthermore, this one-pot approach regenerates the nucleotide cosubstrate, which is an improvement over sequential two-step enzymatic methods with Ep and GtfE, as smaller amounts of cosubstrates and substrates are required.

Key conditions for the one-pot synthesis of desvancosaminyl vancomycin include the use of a thermal stabilizer for prolonged turnover by GtfE, pH 9 buffer for optimal GtfE activity, and magnesium for the divalent-metal requirement of Ep. Despite this slight improvement, the conditions have yet to be optimized for use on a larger scale. Owing to the relaxed substrate specificity of the Ep and GtfE enzymes, modification of this one-pot procedure resulted in the incorporation of glucosamine to generate derivative **2**. This methodology, with slight modification, should also be amenable to other glucose derivatives that are substrates for Ep and GtfE. Furthermore, the substrate specificity of Ep has been expanded by structure-based protein engineering,[53] and directed evolution or structure-based engineering of GtfE would create the potential for more glycopeptide antibiotic analogues.

As mentioned previously, an alternative to direct enzymatic incorporation of a particular glycan is chemical modification of a precursor glycan after enzymatic glycosylation. This approach was used to generate monoglycosylated lipoglycopeptide analogues of vancomycin. The primary amine present on the glucosamine moiety of vancomycin derivative **2** served as a chemoselective site of direct chemical diversification (i.e. no protection of **2** was necessary before modification). Selective reductive alkyl- and arylation of this primary amine over the secondary amine in the peptide backbone was performed with a variety of aldehydes to create a series of monoglycosylated vancomycin derivatives **3**–**11**. Initially a reported procedure was followed for the reductive alkylation,[21,22] though these conditions resulted in mixtures of mono- and dialkylated products with mostly unreacted starting material. A more recent method at lower temperature with acidic conditions during the reduction proved to be very selective for the primary amine and resulted in nearly complete conversion into the desired products (Scheme 2).[54] With this improved procedure, we were able to prepare a number of analogues with different alkyl chain lengths in order to examine their antibiotic activities.

The antibiotic activities of the synthesized glycopeptides were tested by in vitro MIC assays. Three Gram-positive strains were assayed: the MRSA, VSE, and VRE strains (Table 1). All the tested vancomycin derivatives showed antibiotic activity against the MRSA strain, with the glucosamine-containing derivative with the decyl chain **5** and the *p*-chlorobiphenyl analogue **11** having the best MIC values. All derivatives also displayed activity against the VSE strain in vitro, although glucosamine-containing derivative **2** and octadecyl derivative **9** have the least favorable MIC values. For the VRE (VanB) strain, MIC values for alkylated derivatives **3**–**9** displayed a trend of optimal alkyl chain lengths. Derivatives that contain dodecyl, tetradecyl, and hexadecyl chains (**6**–**8**) have the best antibiotic activity, whereas those with longer or shorter alkyl chains have diminished activity against VRE. For the aryl derivatives, the *p*-chlorobiphenyl derivative **11** has some activity, but that of the smaller benzyl derivative **10** was only modest.

Although the exact mechanism of action of glycopeptides **3**–**11** remains to be determined, it is possible that these glycopeptides with lipophilic chains are direct inhibitors of transglycosylase. Some vancomycin analogues that contain a lipophilic group on the disaccharide moiety were shown to inhibit transglycosylation by binding with decreased affinity to the substrate (i.e. the D-Ala–D-Ala terminus of peptidoglycan precursor lipid II), while also binding directly to the transglycosylation complex.[29–32] Further inhibition studies are required to establish the mechanism underlying the biological activity of the lipoglycopeptides synthesized here.

Conclusions

The combined approach of one-pot enzymatic glycosylation and chemical modification reported herein has been an ef-

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fective and convenient means of synthesizing lipoglycopeptide analogues of vancomycin. These derivatives contain a reduced glycan (monosaccharide instead of disaccharide, thus better candidates for large-scale synthesis), yet maintain activity against MRSA and VSE, and some have striking activity against VRE. The one-pot enzymatic glycosylation strategy should also be applicable to other glycopeptide antibiotic peptide scaffolds with the proper biosynthetic antibiotic glycosyltransferase. This chemoenzymatic method may be useful for creating analogues of other glycosylated natural products, for example, other glycopeptides, polyketides, and hybrid nonribosomal peptide/polyketide compounds.

Experimental Section

Purification of Ep and GtfE

Plasmids encoding His-tagged Ep (gift from Prof. Jon S. Thorson, University of Wisconsin) and His-tagged GtfE (gift from Prof. Christopher T. Walsh, Harvard Medical School) were expressed in *E. coli* (BL21(DE3)) and purified by affinity chromatography as described previously.[34–36]

Effect of Chemical Thermal Stabilizers on GtfE Activity

A variety of polyols were tested in enzymatic reactions with GtfE in an attempt to prolong the activity of GtfE at 37 °C. Ethylene glycol, glucose, lactose, maltose, sorbitol, sucrose, and trehalose (all 0.5 M) were added to reaction mixtures containing tricine (75 mM), $MgCl_2$ (2 mM), KCl (5 mM), tris-(2-carboxyethyl)phosphine (2 mM), bovine serum albumin (BSA; 1 mg mL^{-1}), vancomycin aglycon (2 mM), UDP-Glc (2 mM), and GtfE (500 nM) at pH 9.0. The reaction mixtures were incubated at 37 °C. Aliquots were removed after 4, 8, 12, 24, 30, and 48 h, quenched with an equal volume of cold methanol, and analyzed by reverse-phase (RP) HPLC (C_{18}). Ethylene glycol and glycerol were also tested at 2.0 and 1.5 M, respectively.

Syntheses

1: The one-pot enzymatic reaction mixtures at pH 9.0 contained the following: tricine (50 mM), $MgCl_2$ (1 mM), glycerol (10% *v*/*v*), tris-(2-carboxyethyl)phosphine (2 mM), BSA (0.2 mg mL^{-1}), UTP (0.25 mM), Glc-1-P (2.5 mM), phosphoenolpyruvate (PEP; 2.5 mM), vancomycin aglycon (2 mM),[42] inorganic pyrophosphatase (4 U mL^{-1}), Ep (50 nM), GtfE (500 nM), pyruvate kinase (4 U mL^{-1}). Reaction mixtures of up to 20 mL were incubated at 37 °C and monitored by RP-HPLC. Typically over 85% substrate conversion was observed after 48 h. Reactions were quenched with an equal volume of cold methanol, and any precipitate was removed by centrifugation. The supernatant was concentrated by rotary evaporation and lyophilization. The desired glycopeptide **1** was purified by preparative RP-HPLC and characterized by HRMS (ESI-TOF): *m/z* calcd for $C_{59}H_{63}Cl_2N_8O_{22}$: 1305.3428 $[M+H]^+$; found: 1305.3436.

$GlcNH_2$-1-P: α-D-glucosamine-1-phosphate was synthesized from 2-deoxy-2-trifluoroacetamido-1,3,4,6-tetra-*O*-acetyl-D-glucose according to a published procedure for the synthesis of 2-deoxy-α-D-glucose-1-phosphate,[55] except that the MacDonald reaction was done in neat crystalline phosphoric acid at 55 °C for 8 h under vacuum.

2: Enzymatic reaction mixtures of up to 20 mL were incubated at 37 °C and pH 9.0 with the following: tricine (50 mM), $MgCl_2$ (1 mM), glycerol (10% *v*/*v*), tris-(2-carboxyethyl)phosphine (2 mM), BSA (0.2 mg mL^{-1}), UTP (2 mM), $GlcNH_2$-1-P (2 mM), PEP (2 mM), vancomycin aglycon (1 mM),[42] inorganic pyrophosphatase (4 U mL^{-1}), Ep (250 nM), GtfE (5 μM), pyruvate kinase (20 U mL^{-1}). When more than 80% yield of product was observed, typically after about 48 h at 37 °C, the reactions were quenched with an equal volume of cold methanol and subjected to centrifugation to remove any precipitate. The supernatant was concentrated by rotary evaporation and lyophilization. The desired vancomycin derivative **2** was purified by preparative RP-HPLC (C_{18}) and characterized by HRMS (ESI-TOF): *m/z* calcd for $C_{59}H_{64}Cl_2N_9O_{21}$: 1304.3588 $[M+H]^+$; found: 1305.3588.

Chemical diversification of **2**: Vancomycin derivative **2** (1.3 mg, 1 μmol) was dissolved in dry *N*,*N*-dimethylformamide (DMF; 20 μL). A solution of the aldehyde (1.3 μmol) in dry DMF (10 μL) was added to the glycopeptide. *N*,*N*-diisopropylethylamine (2 μmol) was added to the reaction mixture, which was stirred at room temperature for 1 h. Then sodium cyanoborohydride (1 μmol) in dry MeOH (5 μL) was added, and the mixture was diluted with dry MeOH (10 μL). Trifluoroacetic acid (3 μmol) was added, and stirring was continued for another hour at room temperature. Products **3**-**11** were purified by preparative RP-HPLC (C_{18}) and characterized by HRMS (ESI-TOF). **3**: m/*z* calcd for $C_{65}H_{76}Cl_2N_9O_{21}$: 1388.4527 $[M+H]^+$; found: 1388.4512; **4**: *m/z* calcd for $C_{67}H_{80}Cl_2N_9O_{21}$: 1416.4840 $[M+H]^+$; found: 1388.4512; **5**: *m/z* calcd for $C_{69}H_{84}Cl_2N_9O_{21}$: 1444.5153 $[M+H]^+$; found: 1444.5147; **6**: *m/z* calcd for $C_{71}H_{88}Cl_2N_9O_{21}$: 1472.5466 $[M+H]^+$; found: 1472.5449; **7**: *m/z* calcd for $C_{73}H_{92}Cl_2N_9O_{21}$: 1500.5779 $[M+H]^+$; found: 1500.5749; **8**: *m/z* calcd for $C_{75}H_{96}Cl_2N_9O_{21}$: 1528.6092 $[M+H]^+$; found: 1528.6102; **9**: *m/z* calcd for $C_{77}H_{100}Cl_2N_9O_{21}$: 1556.6405 $[M+H]^+$; found: 1556.6416; **10**: *m/z* calcd for $C_{66}H_{70}Cl_2N_9O_{21}$: 1394.4058 $[M+H]^+$; found: 1394.4066; **11**: *m/z* calcd for $C_{72}H_{73}Cl_3N_9O_{21}$: 1504.3981 $[M+H]^+$; found: 1504.3984.

MIC Assays of Glycopeptide Antibiotics

In vitro MIC assays were performed according to benchmarks of the National Committee for Clinical Laboratory Standards. The glycopeptides were assayed against MRSA (ATCC 33591), vancomycin-sensitive *E. faecalis* (ATCC 29212), and vancomycin-resistant *E. faecalis* (ATCC 51575). The MRSA and VSE strains were tested in 96-well plates in serial 2-fold dilutions (0.1–10 μM), and the VRE strain was tested in serial 2-fold dilutions (0.5–50 μM), except for vancomycin, which was tested to 2 mM. The *S. aureus* strain was grown overnight at 35 °C in nutrient broth and then diluted 1:10000 in Mueller–Hinton broth for the assay. The *E. faecalis* strains were grown overnight at 35 °C in Brain Heart Infusion (BHI) broth and diluted 1:10000 in new BHI broth for the assay. The plates were incubated at 35 °C overnight, and the concentrations necessary for complete inhibition of bacterial growth were determined visually.

Acknowledgements

This work was supported by the National Institutes of Health (C.-H.W.) and National Science Foundation Predoctoral and Norton B. Gilula Graduate Student fellowships (D.A.T.). We are grateful to Prof. Christopher T. Walsh for discussions regarding this work. We thank the Thorson and Walsh laboratories for the Ep and GtfE constructs and the Ghadiri laboratory for MRSA and *E. faecalis* strains.

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Received: March 20, 2006
Published online: August 8, 2006

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