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Glycosylation of glycopeptides: a comparison of chemoenzymatic and chemical methods

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Abstract—Glycosylating complex molecules remains a major synthetic challenge, making it hard to explore carbohydrate diversity in biologically active glycosylated natural products and their derivatives. In this paper we compare the efficiency of chemical and enzymatic methods for glycosylation of glycopeptides related to vancomycin and describe the parameters that should be considered in designing synthetic approaches to glycosylated natural product derivatives.

1. Introduction

Vancomycin **1** is a glycopeptide antibiotic used to treat methicillin resistant gram positive infections. The emergence of vancomycin resistance has caused considerable alarm, prompting efforts to identify analogues that overcome resistant bacterial strains.[1–3] It has been found that certain types of modifications to the vancosamino sugar of vancomycin increase biological activity against both sensitive and resistant bacterial strains.[4–6] Based on these findings, our laboratory has become interested in exploring the activity of vancomycin analogues in which the vancosamino sugar is replaced with other hexose scaffolds.[7–9] To access a wide range of vancomycin analogues, efficient strategies to glycosylate the vancomycin pseudoaglycone are imperative. Herein, we describe two different approaches to synthesize the vancomycin analogue **5** in which the vancosamino sugar is replaced by its desmethyl analogue, daunosamine. One approach utilizes a chemical glycosylation and the other uses a glycosyltransferase involved in glycopeptide biosynthesis to carry out an enzymatic glycosylation. We consider the efficiency of the two approaches and offer a recommendation for when an enzymatic glycosylation is preferable over traditional chemical glycosylation methods.

2. Results

Both chemical and enzymatic methods require the synthesis of a vancomycin pseudoaglycone acceptor, β-D-glucosyl vancomycin aglycones **2** and **6**, and a sugar donor, sulfoxide **3** and TDP derivative **7**, respectively (Scheme 1). For the chemical glycosylation of the glucosyl 2-OH, the pseudoaglycone must be protected to mask functionalities interfering with the glycosylation reaction (the amino group, the carboxylic acid, the three phenolic and the additional five hydroxyl groups) and to promote solubility in organic solvents at low temperatures. We have developed a method to protect vancomycin and degrade it to the protected pseudoaglycone **2** (Scheme 1).[10,11] The protection of vancomycin **1** was achieved by first converting the amino groups to their *N*-Alloc derivatives with alloc succinimide, followed by alkylation of the carboxylic acid and the phenolic groups with allyl bromide and acetylation of the hydroxyl groups. Finally, the vancosamino sugar was selectively cleaved ($BF_3{\cdot}OEt_2$, PhSH), yielding the protected pseudoaglycone **2** in 58% yield (four steps). In contrast, the synthesis of a suitable acceptor for the enzymatic approach is considerably more straightforward, as pseudoaglycone **6** can be obtained by simple acid degradation of vancomycin **1** in 65% yield (Scheme 1).

The next issue to consider is the synthesis of the sugar donor, either the daunosamine sulfoxide **3** for

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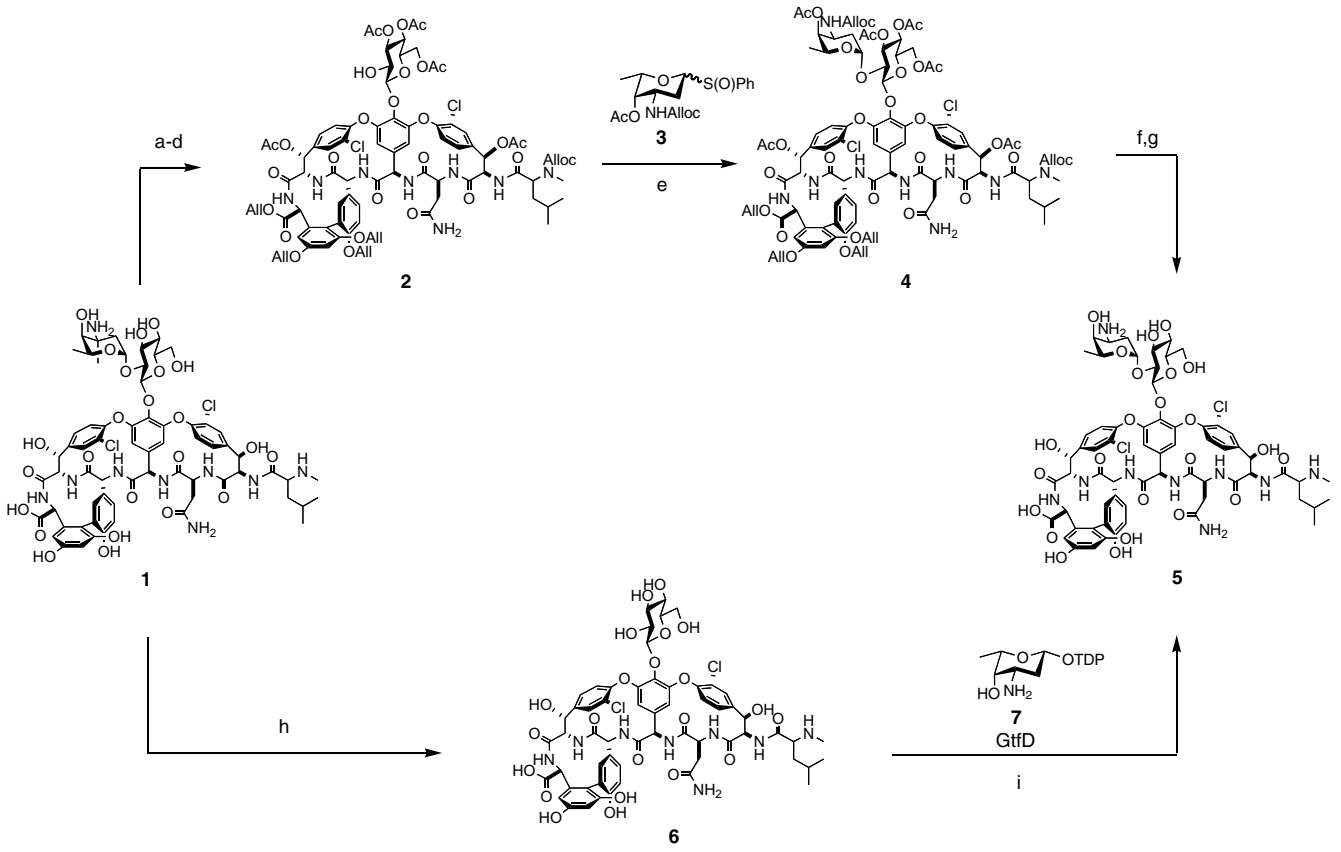
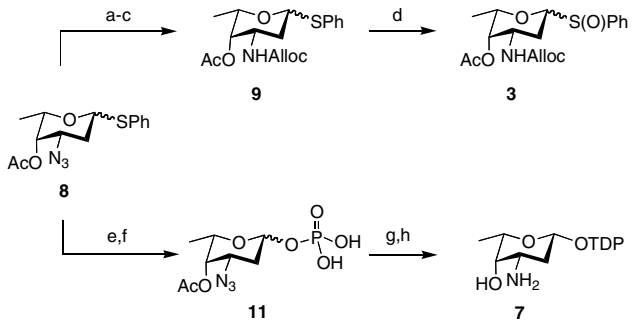


Scheme 1. Chemical and enzymatic routes to **5**, the daunosamine analogue of vancomycin **1**. Reagents and conditions: (a) alloc succinimide, $NaHCO_3$, H_2O/dioxane; (b) 1. AllBr, $NaHCO_3$, DMF; 2. AllBr, Cs_2CO_3, DMF, 82% (three steps); (c) Ac_2O, Py, DMAP, CH_2Cl_2, 91%; (d) $BF_3 OEt_2$, PhSH, CH_2Cl_2, 71%; (e) $BF_3{\cdot}OEt_2$, Tf_2O, Et_2O, CH_2Cl_2, −78 °C → −10 °C, 66%. (f) H_2NNH_2, MeOH/allyl alcohol, 53%; (g) Bu_3SnH, $PdCl_2(PPh_3)_2$, DMF/AcOH, 79%; (h) TFA/H_2O (9:1), 65%; (i) 75 mM Tris (pH = 7.0), 8 mM $MgCl_2$, 2.5 mM TCEP, 1 mg/mL BSA, 10% (v/v) DMSO, 500 μM TDP β-daunosamine **7**, 100 μM vancomycin pseudoaglycone **6**, 5 μM GtfD. The reaction was monitored by HPLC and after 60 min, 70% of the pseudoaglycone was glycosylated.

the chemical glycosylation or TDP β-daunosamine **7** (Scheme 2) for the enzymatic transfer. To facilitate deprotection following chemical glycosylation, we opted to use protecting groups for the sugar donor analogous



Scheme 2. Preparation of the glycosyl donors **3** and **7**. Reagents and conditions: (a) NaOMe, MeOH, quant.; (b) 1. PPh_3, H_2O/THF, 2. Alloc succinimide, THF, 96% (two steps); (c) Ac_2O, Et_3N, DMAP, CH_2Cl_2, 97%; (d) *m*CPBA, CH_2Cl_2, 96%; (e) NIS, CH_3CN/H_2O, 61%; (f) 1. $(COCl)_2$, DMF, CH_2Cl_2, 2. $Bu_4NH_2PO_4$, CH_3CN, 59% (two steps); (g) TMP morpholidate, tetrazole, Py, 2 days, 49%; (h) 1. MeOH/H_2O/Et_3N, 2. H_2, Pd–C, 67% (two steps).

to the ones used for protecting the aglycone acceptor. The synthesis of daunosamine sulfoxide **3** started from thioglycoside **8**, which is easily accessible from di-*O*-acetyl-L-rhamnal.[12,13] Deacetylation of **8** was followed by Staudinger reduction of the azido group, *N*-protection using alloc succinimide, and reacetylation to afford thioglycoside **9** in 93% yield (four steps). Oxidation with *m*CPBA then gave the sulfoxide donor **3** (96%).

In comparison, the synthesis of the donor substrate for the enzymatic glycosylation is more demanding. In earlier studies, we have purified and characterized the glycosyltransferase GtfD responsible for the transfer of the vancosamine sugar in vancomycin biosynthesis.[14] In analogy to other enzymes transferring 2-deoxy-L-sugars, GtfD only accepts β-configured NDP sugar substrates, which makes the generation of a β-2-deoxy glycosyl phosphate linkage necessary. The synthesis of these substrates is challenging because intermediates can be unstable and the β-isomers are the thermodynamically disfavored anomers. However, we recently reported a method that provides access to a range of NDP β-2-deoxy sugars in a stereoselective manner.[15] As shown in Scheme 2, the lactol derived from thioglycoside **8** is converted to the corresponding α-chloride,

which can be displaced by a phosphate nucleophile in acetonitrile as the solvent, yielding the anomeric phosphate **11** with good β-selectivity (β/α 4:1). The phosphate **11** is then coupled with TMP morpholidate and deprotected to yield the desired TDP sugar donor **7**. Thus both donors can be made readily, but the synthesis of the TDP sugar, was significantly more challenging than the synthesis of the sulfoxide donor until we identified reliable conditions to obtain the required β-anomer.

With the donors and the acceptors in hand, both glycosylation methods were used to generate daunosamine analogue **5**. For the chemical glycosylation, conditions developed for the attachment of the vancosamino sugar to the pseudoaglycone **2** were used (Scheme 1). Briefly, 2 equiv of boron trifluoride diethyletherate and 2 equiv of triflic anhydride were premixed with the protected pseudoaglycone **2** at −78 °C. Then, 4 equiv of the daunosamine sulfoxide **3** were added, and the reaction mixture was slowly warmed to −10 °C, yielding the protected vancomycin analogue **4** in 75% yield. Glycopeptide **4** was deprotected to afford desmethyl vancomycin **5** using a two-step sequence ((1) hydrazine, MeOH, 53%; (2) $PdCl_2(PPh_3)_2$, Bu_3SnH, 79%) as reported for the chemical synthesis of vancomycin, with the only notable difference being that the removal of the daunosamine C-4 acetate occurred more slowly and in slightly lower yield (53% compared to 63% for the deacetylation of the corresponding vancomycin derivative). This result might reflect an influence of the C-3 methyl group on the conformation of the sugar ring, rendering the C-4 acetate of vancosamine more accessible compared to the daunosamine analogue.

The enzymatic glycosylation requires an appropriate enzyme that is able to transfer daunosamine to the 2-position of vancomycin pseudoaglycone **6**. GtfD (vide supra) has been shown to accept a limited number of alternative donors,[14,16] which prompted us to investigate its utility in the synthesis of the daunosamine containing vancomycin analogue **5**. As shown in Figure 1, GtfD was indeed able to transfer daunosamine to vancomycin pseudoaglycone **6**. Using a 5-fold excess of TDP-daunosamine, 70% of the pseudoaglycone was glycosylated within 60 min. The enzyme is stable for prolonged periods of time, and by adjusting conditions we should be able to decrease the amount of TDP-daunosamine.

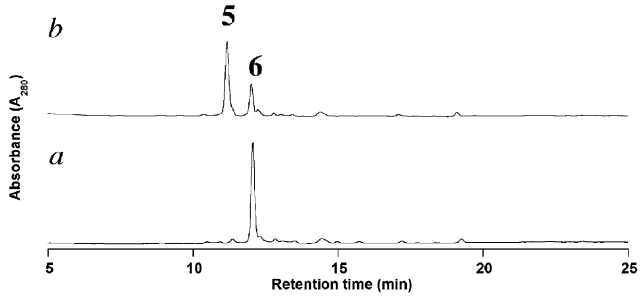


Figure 1. GtfD accepts TDP-daunosamine. HPLC time course showing production of the desmethyl-vancomycin analogue. (a) HPLC trace of the pseudoaglycone, (b) HPLC after incubation with TDP-daunosamine and pseudoglycone.

3. Conclusion

We have compared chemical and enzymatic approaches to glycosylate the vancomycin pseudoaglycone to obtain the novel vancomycin analogue **5**, which contains the 3-desmethyl analogue of vancosamine (daunosamine) as the terminal sugar of the disaccharide portion attached to vancomycin aglycone. The glycosylation reactions are comparable but the overall sequence of reactions is significantly shorter for the route involving the enzymatic glycosylation. Even though the donor sugar synthesis is somewhat more involved, the enzymatic method is more efficient overall for this reaction scale. For unnatural substrates that turn over at a rate of 1 min^{-1}, it is possible to produce 1–5 mg of product overnight, which is more than enough to probe biological activity. For gram quantities of material, the chemical method becomes preferable because it more easily scaled up. In fact, yields are reproducible from 50 to over 500 mg. The major issue with the enzymatic glycosylation method is that the promiscuity of the enzyme is not sufficiently understood yet to assess the scope of the reaction. Daunosamine, the sugar transferred by GtfD in this study, is structurally similar to the natural substrate vancosamine and the transfer rate was acceptable but not exceptional (k_{cat} = 0.3 min^{-1}). Transfer rates more than 10-fold lower than this are not acceptable even for small scale reactions. We conclude that rapid exploration of sugar diversity is better accomplished using enzymes as long as the sugars are closely related to the natural donor. For a broader investigation of sugar donors, chemical methods must be used until we learn how to manipulate the selectivity of glycosyltransferases.

4. Experimental

4.1. General

Vancomycin pseudoaglycone **6** was obtained from vancomycin **1** by acid hydrolysis (TFA/H_2O 9:1, 10 °C, 2 h) and purification by reversed-phase HPLC. TDP daunosamine **7** was synthesized as described.[15] All chemicals were purchased from Aldrich or Sigma and used without further purification. Solvents were reagent grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates precoated with silica gel (250 μm, Sorbent Technologies), with detection by UV and/or spraying with H_2SO_4 (50%). Flash chromatography was carried out on silica gel (60 Å, 32–63 μm), purchased from Sorbent Technologies. Analytical HPLC of reaction mixtures was performed on a Hewlett–Packard 1100 series instrument using a Phenomenex Luna 5 μm C18 column (250 mm × 4.6 mm). Preparative HPLC was performed on a Varian ProStar instrument (flow-rate: 45 mL/min) using a Phenomenex Luna 10 μm C18 column

(250 × 50 mm) or on a Hitachi L6200 instrument (flow-rate: 7.5 mL/min) using a Phenomenex Luna 5 μm C18 column (250 × 21.2 mm). NMR spectra were recorded on Varian Mercury 300 MHz and Inova 400 or 500 MHz spectrometers. Low resolution mass spectra (ESI) were obtained at Princeton University. High-resolution mass spectra (HRMS) were obtained at the University of California at Riverside Department of Chemistry Mass Spectrometry Facility.

4.2. Phenyl 3-allyloxycarbonylamino-4-*O*-acetyl-2,3,6-trideoxy-1-thio-α,β-L-*lyxo*-hexopyranoside 9

To a solution of thioglycoside **7** (770 mg, 2.5 mmol) in MeOH (20 mL) was added K_2CO_3 (380 mg, 1.25 mmol). After stirring for 8 h, the solution was poured into satd aq NH_4Cl, which was extracted with CH_2Cl_2 (3×). The organic layers were washed with brine, dried ($MgSO_4$), filtered and evaporated to give phenyl 3-azido-2,3,6-trideoxy-1-thio-α,β-L-*lyxo*-hexopyranoside (660 mg, quant.) as a colorless syrup.

This compound (660 mg, 0.25 mmol) was dissolved in THF/H_2O (40 mL, 10:1) and PPh_3 (1.3 g, 5.0 mmol) was added. The reaction was stirred at 60 °C for 2 h and cooled to room temperature. After the addition of a solution of Alloc succinimide (740 mg, 3.7 mmol) in THF (20 mL), the reaction was stirred at room temperature for 3 h, concentrated and purified by flash chromatography (15% EtOAc/CH_2Cl_2) to give phenyl 3-allyloxycarbonylamino-2,3,6-trideoxy-1-thio-α,β-L-*lyxo*-hexopyranoside (770 mg, 96%) as a colorless oil. R_f (15% EtOAc/petroleum ether): 0.25 (α-anomer), 0.30 (β-anomer). To a solution of this compound (770 mg, 2.4 mmol) in CH_2Cl_2 (30 mL) was added triethylamine (1.33 mL, 9.5 mmol) followed by acetic anhydride (0.45 mL, 4.8 mmol) and DMAP (58 mg, 0.48 mmol). The reaction was stirred at room temperature for 1 h and was quenched with methanol. The reaction mixture was evaporated and purified by flash chromatography (10% EtOAc/CH_2Cl_2) to give thioglycoside **9** (840 mg, 97%) as a white solid. R_f (10% EtOAc/CH_2Cl_2): 0.35 (α-anomer), 0.39 (β-anomer). Compound **9**α: ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 7.51–7.23 (m, 5H, ArH), 5.92 (m, 1H, allyl-H), 5.71 (d, $J_{1,2}$ = 5.5 Hz, 1H, H-1), 5.31 (br d, J = 17.5 Hz, 1H, allyl-H), 5.24 (br d, J = 10.5 Hz, allyl-H, 5.20 (s, 1H, H-4), 4.87 (br d, J = 7.6 Hz, 1H, NH), 4.55 (q, $J_{5,6}$ = 6.3 Hz, 1H, H-5), 4.29 (br m, 1H, H-3) 2.25 (ddd, $J_{1,2}$ = 5.5 Hz, $J_{2,3}$ = 13.0 Hz, $J_{2,2'}$ = 13.5 Hz, 1H, H-2), 2.18 (s, 3H, Ac-H), 2.10 (br dd, $J_{2,3}$ = 4.4 Hz, $J_{2,2'}$ = 13.5 Hz, 1H, H-2′), 1.12 (d, $J_{5,6}$ = 6.3 Hz, 3H, CH_3-6). ^{13}C NMR ($CDCl_3$, 500 MHz): δ = 171.4, 155.9, 135.5, 133.4, 131.8, 129.7, 127.8, 118.8, 84.5, 71.8, 66.9, 66.6, 47.4, 32.4, 21.5, 17.6; HRMS (FAB): calculated (M + Na, $C_{18}H_{23}NO_5SNa$) 388.1195; found 388.1184.

4.3. Phenyl 3-allyloxycarbonylamino-4-*O*-acetyl-2,3,6-trideoxy-1-sulfinyl-α,β-L-*lyxo*-hexopyranoside 3

To a solution of thioglycoside **9** (440 mg, 0.12 mmol) in CH_2Cl_2 (30 mL) at −70 °C was added *m*CPBA (345 mg, 60% purity, 0.12 mmol). The reaction was slowly warmed to −20 °C over 45 min and kept at −20 °C for 15 min before being quenched with Me_2S (1.0 mL). The reaction was extracted with satd aq $NaHCO_3$ solution, and the aqueous layer was further extracted with CH_2Cl_2 (3 × 50 mL). The organic layers were combined, dried ($MgSO_4$), filtered and concentrated to a colorless oil. The oil was purified by flash chromatography (60% EtOAc/petroleum ether) to give sulfoxide **3** (435 mg, 96%) as a white solid. R_f (60% EtOAc/petroleum ether): 0.25–0.35. LRMS (ESI): calculated (M + Na, $C_{18}H_{23}NO_6SNa$) 404; found 404.

4.4. Protected desmethyl vancomycin 4

The pseudoaglycone **2** (808 mg, 0.459 mmol) was azeotroped with toluene (3 × 1 mL), dissolved in CH_2Cl_2 (20 mL) and cooled to −78 °C. $BF_3{\cdot}OEt_2$ (113 μL, 0.918 mmol) was added followed by triflic anhydride (77.2 μL, 0.122 mmol). A solution of the sulfoxide **3** (315 mg, 0.826 mmol) in a mixture of CH_2Cl_2 (6 mL) and Et_2O (8 mL) was added to the reaction dropwise over 15 min. The reaction was allowed to warm up to −10 °C over 1 h and then quenched with a mixture of methanol (400 μL) and DIPEA (400 μL). The solvents were evaporated and the residue was purified by flash chromatography (gradient 2.5–4% MeOH/CH_2Cl_2) to afford the protected desmethyl vancomycin **4** (608 mg, 66%) as a white solid. R_f (5% MeOH/CH_2Cl_2): 0.28. HRMS (FAB) calculated (M + Na, $C_{97}H_{109}Cl_2N_9O_{34}Na$) 2036.6352; found 2036.6319.

4.5. Desmethyl vancomycin 5

4.5.1. Deprotection of 4. The protected **4** (392 mg, 0.194 mmol) was dissolved in a solution of 3% hydrazine in allyl alcohol/MeOH (15 mL, 1:2). The reaction was stirred at room temperature for 24 h and then quenched by the addition of acetic acid (2 mL). The solution was concentrated to a slurry and diluted with butanol (30 mL), which was extracted with water and aq NH_4Cl solution (3 × 25 mL). The butanol layer was concentrated to a white solid and purified by flash chromatography (gradient 12–16% MeOH/CH_2Cl_2) to give the deacetylated glycopeptide (182 mg, 53%) as a white solid. R_f (20% MeOH/CH_2Cl_2): 0.29. ^{1}H NMR (CD_3OD, 500 MHz): see supporting information. HRMS (FAB): calculated (M + Na, $C_{85}H_{97}Cl_2N_9O_{28}Na$) 1784.5718; found 1784.5664.

To a solution of this compound (160 mg, 91 μmol) in DMF/acetic acid (4.0 mL, 1:1) was added $PdCl_2(PPh_3)_2$ (25.5 mg, 31 μmol). The reaction was degassed and put under argon. To this mixture was added Bu_3SnH (2.5 mL) in 100 μL portions every 5 min until all the material had been added. The crude reaction mixture was precipitated in acetone (80 mL). The mixture was centrifuged and decanted to give a white solid, which was suspended in water (5 mL) and kept at 4 °C overnight. The suspension was filtered and the filtrate was concentrated and purified by reversed-phase HPLC

(gradient 0–20% acetonitrile/water with 0.1% TFA over 30 min, flow rate 7.5 mL/min and UV detection at 285 nm) to afford desmethyl vancomycin **6** (110 mg, 79%) as a white solid. R_f (6:6:1:2 $CHCl_3$:MeOH:H_2O: satd aq NH_3) = 0.30. ^1H NMR (CD_3OD, 500 MHz): see supporting information. HRMS (FAB): calculated (M + H, $C_{65}H_{74}Cl_2N_9O_{24}$) 1434.4224, found 1434.4138.

4.5.2. Enzymatic glycosylation of pseudoaglycone 6. Vancomycin pseudoaglycone **6** was enzymatically glycosylated with TDP-β-daunosamine **7** using the vancomycin biosynthetic glycosyltransferase GtfD to yield the doubly glycosylated vancomycin analogue. The reaction conditions were as follows: 75 mg/mL Tris (pH = 7.0), 8 mM $MgCl_2$, 2.5 mM TCEP, 1 mM BSA, 10% (v/v) DMSO, 500 μM TDP-β-daunosamine **7**, 100 μM vancomycin pseudoaglycone **6**, 5 μM GtfD, 37 °C. The reaction was monitored by HPLC and after 60 min, 70% of the pseudoaglycone was glycosylated.

Kinetic characterization of daunosaminyl transfer by GtfD GtfD activity was monitored using a previously reported HPLC based assay.[14] To characterize the ability of GtfD to transfer TDP-β-daunosamine **7** to vancomycin pseudoaglycone **5**, initial velocity experiments were performed in triplicate using a variety of sugar concentrations ranging from 25 to 500 μM. The reaction conditions were as follows: 75 mM Tris (pH = 7.0), 8 mM $MgCl_2$, 2.5 mM TCEP, 1 mg/mL BSA, 10% (v/v) DMSO, 100 μM vancomycin pseudoaglycone, 5 μM GtfD, 37 °C, 10 min. The data were fit to the Michaelis Menten model which resulted in the kinetic parameters K_{cat} = 0.28 min^{-1} and K_m = 43.1 μM.

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