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ORIGINAL ARTICLE

Tackling vancomycin-resistant bacteria with 'lipophilic–vancomycin–carbohydrate conjugates'

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Vancomycin, a glycopeptide antibiotic, has long been a drug of choice for life-threatening Gram-positive bacterial infections. Vancomycin confers its antibacterial activity by inhibiting bacterial cell wall biosynthesis. However, over the time, vancomycin has also been rendered ineffective by vancomycin-resistant bacteria (VRB). These bacteria developed resistance to it by alteration of cell wall precursor from D-Ala-D-Ala to D-Ala-D-Lac (vancomycin-resistant *Enterococci*, VRE), which leads to manifold reduction in the binding constant and results in the loss of antibacterial activity. Herein, we report various vancomycin–sugar analogs, based on a simple design rationale, which exhibit increased binding affinity to VRB, thereby resensitizing VRB to vancomycin. Optimized vancomycin–sugar conjugate exhibited 150-fold increase in affinity for *N,N′*-diacetyl-Lys-D-Ala-D-Lac compared with vancomycin. This improved binding affinity was also reflected in its antibacterial activity, wherein the MIC value was brought down from 750 to 36 μM against VRE (VanA phenotype). To further sensitize against VRE, we appended lipophilic alkyl chain to optimized vancomycin–sugar conjugate. This lipophilic–vancomycin–sugar conjugate was >1000-fold (MIC = 0.7 μM) and 250-fold (MIC = 1 μM) more effective against VanA and VanB strains of VRE, respectively, compared with vancomycin. Therefore, this synthetically simple approach could lead to the development of new generation of glycopeptide antibiotics, which can be clinically used to tackle VRB infections.
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INTRODUCTION

Infectious diseases are one of the leading causes of death in the world. The problem of infectious diseases is exacerbated by the prevalence of multidrug resistance in bacteria.[1,2] Vancomycin is a glycopeptide antibiotic that has become the drug of last resort to treat life-threatening bacterial infections such as those caused by methicillin-resistant *Staphylococcus aureus* (MRSA).[3] Vancomycin binds to D-Ala-D-Ala terminus of peptidoglycan pentapeptide of the bacterial cell wall, thus inhibiting transpeptidase-catalyzed crosslinking and maturation of the bacterial cell wall.[4] However, bacteria acquired resistance to vancomycin either by alteration of cell wall precursors from D-Ala-D-Ala to D-Ala-D-Lac (vancomycin-resistant *Enterococci*, VRE) or by thickening the cell wall (vancomycin intermediate-resistant *S. aureus*, VISA), and sometimes modifying both (vancomycin-resistant *S. aureus*).[4–6] The alteration of the precursor leads to manifold reduction in the binding constant of vancomycin to its target and thus results in the loss of antibacterial activity.[7,8] This perennial persistence of vancomycin resistance calls for urgent measures to develop more potent analogs. Hence, this persistent threat of drug resistance has triggered the scientific community all over the world to develop various strategies to tackle the problem.[9–16]

Considerable efforts have been adopted toward the development of next-generation glycopeptides to tackle the vancomycin resistance.[17–30] Semisynthetic glycopeptides such as oritavancin, dalbavancin and telavancin containing hydrophobic groups were shown to exhibit improved antibacterial activity against resistant strains with enhanced pharmacological profile.[4,31] Boger and co-workers[32,33] developed vancomycin aglyconamidine to display improved binding affinity to VRE by replacing the amide of vancomycin aglycon with amidine, which showed potent antibacterial activity against VRE strain.

Nitanai *et al.*[34] observed the bridging of water molecule between the carboxylic group of vancomycin and ligand in the crystal structure of vancomycin–ligand complex. This suggests that C-terminus modification of the vancomycin to form direct hydrogen bond with the target peptide could stabilize the structure of vancomycin–ligand complex more effectively and leads to higher activity.[34] Here, we hypothesize that if the C-terminal of the vancomycin (*N*-hydroxyphenylglycine) is extended with a variety of cyclic (cy) and/or acyclic (acy) sugar moieties (which have the ability to form additional feasible hydrogen bonding with the peptides of peptidoglycan), the overall binding constant of vancomycin derivative with the target peptide of VRE could be increased. These vancomycin–sugar conjugates were developed by simple synthetic methodology as described below. The optimized vancomycin–sugar conjugate appended with a lipophilic alkyl chain displayed increased binding affinity of two orders of magnitude and high antibacterial activity against

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vancomycin-resistant bacteria (VRB) (>1000-fold more effective than vancomycin).

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma-Aldrich (St Louis, MO, USA) and SD Fine (Mumbai, India) and used without further purification. Analytical TLC was performed on E Merck TLC plates (Darmstandt, Germany) precoated with silica gel 60 F_{254} (250 μm thickness). Visualization was accomplished using UV light and iodine. Column chromatography was performed on silica gel (60–120 Å pore size). All final compounds were purified by reverse phase HPLC using 0.1% trifluoroacetic acid in water/acetonitrile (0 – 100%) as mobile phase to more than 95% purity. HPLC analysis was performed on a Shimadzu-LC 8 Å Liquid Chromatography instrument (Kyoto, Japan; C_{18} column, 10mm diameter and 250 mm length) with UV detector monitoring at 270 nm. NMR spectra were recorded on Bruker (AV-400, Fallanden, Switzerland) 400 MHz spectrometer in deuterated solvents. HR-MS were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument (CA, USA). UV-absorption measurements were obtained using Thermo-Fisher Scientific UV-10 spectrometer (Madison, WI, USA) for the determination of binding constants. Bacterial strains MRSA ATCC 33591 and Enterococcal strains were obtained from ATCC (Rockville, MD, USA). Tryptic-soy agar media were used for *Staphylococci* and sheep blood agar plates were used for *Enterococci*. Eppendorf 5810R centrifuge was used. TECAN (Infinite series, M200 pro; Grodig, Austria) Plate Reader was used to measure absorbance.

Synthesis and characterization

*Synthesis of 2-bromoethyl 2,3,4,6-tetra-O-acetyl-D-glucopyranose (**1a**).* About 1.0 g of D-glucopyranose pentaacetate was dissolved in 10 ml of dry dichloromethane at 0 °C,[35] and then 1.3 ml (1.2 equivalents) of $BF_3{\cdot}Et_2O$ was added to the reaction mixture dropwise followed by 0.22 ml (1.2 equivalents) of 2-bromoethanol. Thereafter, the reaction mixture was allowed to stir at room temperature for 3 h. After completion of the reaction, anhydrous potassium carbonate (0.53 g, 1.5 equivalents) was added and stirring was continued for further 30 min. Later, the crude solution was extracted with chloroform and purified through silica gel column chromatography (ethyl acetate/hexane 30:70) to get pure **1a** with 79% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.58–4.56 (d, 1H), 4.28–4.08 (m, 6H), 3.85–3.48 (m, 2H), 3.47–3.44 (m, 2H), 2.03 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.5, 100.2, 70.0, 69.3, 68.5, 68.0, 62.0, 61.6, 29.9, 20.9; HR-MS (ESI) calculated for $C_{16}H_{23}BrO_{10}$ $[M+Na]^+$: 477.0372 and found: 477.0351.

Synthesis of 2-azidoethyl 2,3,4,6-tetra-O-acetyl-D-glucopyranose (1b). 0.52 g of **1a** was dissolved in 10 ml of methanol, and then 0.37 g (2.0 equivalents) of sodium azide was added to the reaction mixture. Now, the reaction mixture was refluxed at 70 °C for 24 h. Then, the crude solution was extracted with chloroform and purified through silica gel column chromatography (ethyl acetate/hexane 30:70) to get pure **2b** with 86% yield. FT-IR (NaCl): 2950 cm^{-1} (– CH_2 – asym. str.), 2884 cm^{-1} (– CH_2 sym. str.), 2106 cm^{-1} (– N_3 str.), 1754 cm^{-1} (–OAc C=O str.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.56–4.49 (d, 1H), 4.24–4.00 (m, 6H), 3.52–3.46 (m, 2H), 3.31–3.26 (m, 2H), 2.02 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.3, 99.7, 71.9, 71.0, 70.1, 67.6, 67.4, 60.9, 49.6, 19.7; HR-MS (ESI) calculated for $C_{16}H_{23}N_3O_{10}$ $[M+Na]^+$: 440.1281 and found: 440.1278.

*Synthesis of 2-azidoethyl D-glucopyranose (**1c**).* 0.3 g of **1b** was dissolved in 5 ml of methanol, and then 0.165 g (4.0 equivalents) of sodium methoxide was added to the reaction mixture and kept at room temperature for 2 h with stirring. Thereafter, Dowex resin (strongly acidic) was added to the reaction mixture and pH of the reaction mixture was adjusted to 6. Now, the reaction mixture was filtered and the filtrate was evaporated to get **1c** with quantitative yield. FT-IR (NaCl): 3364 cm^{-1} (–OH str.), 2929 cm^{-1} (– CH_2 – asym. str.), 2885 cm^{-1} (– CH_2 – sym. str.), 2105 cm^{-1} (– N_3 str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.19–4.17 (d, 1H), 3.90–3.85 (m, 1H), 3.68–3.62 (m, 2H), 3.47–3.41 (m, 3H), 3.15–3.08 (m, 2H), 3.04–2.93 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 103.0, 76.9, 76.7, 73.4, 70.1, 67.3, 61.1, 50.4; HR-MS (ESI) calculated for $C_8H_{15}N_3O_6$ $[M+Na]^+$: 272.0859 and found: 272.0844.

*Synthesis of 2-aminoethyl D-glucopyranose (**1d**).* 0.15 g of **1c** was dissolved in water, and then about 0.24 g (1.5 equivalents) of triphenylphosphine was added to the reaction mixture and it was allowed to stir at room temperature for 12 h. Now, the crude solution was extracted with water and dried to get pure **1d** with 75% yield. FT-IR (NaCl): 3322 cm^{-1} (–OH and $-NH_2$ asym., sym. str.), 2929 cm^{-1} (– CH_2 – asym. str.), 2890 cm^{-1} (– CH_2 – sym. str.); ^{1}H NMR (400 MHz, D_2O) δ 4.56–4.55 (d, 1H), 4.20–4.14 (m, 1H), 3.99–3.95 (m, 1H) 3.87–3.74 (m, 4H), 3.35–3.30 (m, 2H), 3.19–3.17 (t, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 104.5, 78.3, 77.9, 75.4, 71.9, 68.1, 59.9, 43.6; HR-MS (ESI) calculated for $C_8H_{17}NO_6$ $[M+H]^+$: 224.1134 and found: 224.1122.

*Synthesis of 2-bromoethyl 2,3,4,6-tetra-O-acetyl-D-galactopyranose (**2a**).* 2.5 g of D-galactose pentaacetate was dissolved in 20 ml of dry dichloromethane at 0 °C, and then 3.63 ml (1.2 equivalents) of $BF_3{\cdot}Et_2O$ was added to the reaction mixture dropwise followed by 0.54 ml (1.2 equivalents) of 2-bromoethanol. Thereafter, the reaction mixture was allowed to stir at room temperature for 3 h. After completion of the reaction, anhydrous potassium carbonate (1.33 g, 1.5 equivalents) was added and stirring was continued for further 30 min. Then, the crude solution was extracted with chloroform and purified through silica gel column chromatography (ethyl acetate/hexane 30:70) to get pure **2a** with 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.53–4.51 (d, 1H), 4.33–4.31 (t, 1H), 4.30–4.06 (m, 3H), 3.83–3.79 (m, 2H), 3.50–3.43 (m, 4H), 2.06 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.7, 100.4, 72.0, 71.2, 69.5, 68.7, 67.2, 61.0, 29.9, 22.1; HR-MS (ESI) calculated for $C_{16}H_{23}BrO_{10}$ $[M+Na]^+$: 477.0372 and found: 477.0351.

*Synthesis of 2-azidoethyl 2,3,4,6-tetra-O-acetyl-D-galactopyranose (**2b**).* 1.0 g of **2a** was dissolved in 20 ml of methanol, and then about 0.729 g (2 equivalents) of sodium azide was added to the reaction mixture. Now, the reaction mixture was refluxed at 70 °C for 24 h. Then, the crude solution was extracted with chloroform and purified through silica gel column chromatography (ethyl acetate/hexane 30:70) to get pure **2b** with 60% yield. FT-IR (NaCl): 2940 cm^{-1} (– CH_2 – asym. str.), 2885 cm^{-1} (– CH_2 – sym. str.), 2102 cm^{-1} (– N_3 str.), 1742 cm^{-1} (–OAc C=O str.); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.55–4.53 (d, 1H), 4.23–3.90 (m, 6H), 3.51–3.45 (m, 2H), 3.31–3.25 (m, 2H), 2.01 (s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3, 101.3, 71.0, 70.9, 68.7, 68.1, 67.1, 61.4, 50.7, 20.8. HR-MS (ESI) calculated for $C_{16}H_{23}N_3O_{10}$ $[M+Na]^+$: 440.1281 and found: 440.1274.

*Synthesis of 2-azidoethyl D-galactopyranose (**2c**).* 0.085 g of **2b** was dissolved in 3 ml of methanol, and then 0.04 g (4.0 equivalents) of sodium methoxide was added to the reaction mixture and kept at room temperature for 2 h with stirring. Thereafter, Dowex resin (strongly acidic) was added to the reaction mixture and pH of the reaction mixture was adjusted to 6. Now, the reaction mixture was filtered and the filtrate was evaporated to get **2c** with 98% yield. FT-IR (NaCl): 3394 cm^{-1} (–OH str.), 2923 cm^{-1} (– CH_2 – asym. str.), 2885 cm^{-1} (– CH_2 – sym. str.), 2105 cm^{-1} (– N_3 str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.12–4.11 (d, 1H), 3.88–3.85 (m, 1H), 3.66–3.63 (m, 2H), 3.45–3.42 (m, 3H), 3.13–3.05 (m, 2H), 3.01–2.93 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d6) δ 103.6, 75.3, 73.5, 70.5, 68.0, 67.1, 60.3, 50.5; HR-MS (ESI) calculated for $C_8H_{15}N_3O_6$ $[M+Na]^+$: 272.0859 and found: 272.0844.

*Synthesis of 2-aminoethyl D-galactopyranose (**2d**).* 50 mg of **2c** was dissolved in water, and then 79 mg (1.5 equivalents) of triphenylphosphine was added to the reaction mixture and was allowed to stir at room temperature for 12 h. Now, the crude solution was extracted with water and dried to get pure **2d** with 75% yield. FT-IR (NaCl): 3329 cm^{-1} (–OH, $-NH_2$ asym. and sym. str.), 2927 cm^{-1} (– CH_2 – asym. str.), 2885 cm^{-1} (– CH_2 – sym. str.); ^{1}H NMR (400 MHz, D_2O) 4.45–4.43 (d, 1H), 4.07–4.01 (m, 1H), 3.95–3.94 (d, 1H), 3.87–3.79 (m, 3H), 3.74–3.66 (m, 2H), 3.58–3.54 (m, 1H), 3.07–3.04 (t, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ/p.p.m.: 103.8, 76.2, 74.2, 71.1, 69.1, 67.9, 61.3, 51.1; HR-MS (ESI) calculated for $C_8H_{17}NO_6$ $[M+H]^+$: 224.1134 and found: 224.1119.

*Synthesis of **3a** and **4a**.* Cellobiose (1 g) or maltose (1 g) was dissolved in 6 ml of Millipore water.[36] Then, 1.2 equivalents of *N*-Boc-1,3-propanediamine was dissolved separately in 10 ml of isopropanol and added to cellobiose or maltose solution dropwise. The reaction mixture was refluxed for 12 h with stirring. Now, the solvent was evaporated to dryness and the residue was washed with ethyl acetate followed by chloroform. Finally, the solid was dried in high vacuum pump. This residue was dissolved in 5 ml of dry methanol and 1.4 equivalents of sodium borohydride was added to it. The reaction was allowed to stir for 12 h at room temperature. After that the reaction mixture was filtered and the filtrate was evaporated to get the pure **3a** or **4a** (86–90%).

***3a** (Cellobiose derivative)*: FT-IR (NaCl): 3362 cm^{-1} (−OH str.), 2930 cm^{-1} (−CH_2− asym. str.), 2881 cm^{-1} (−CH_2− sym. str.), 1690 cm^{-1} (−NHBoc C=O str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.30–4.28 (d, 1H), 4.12–4.08 (d, 2H), 3.69–3.38 (m, 10H), 3.13–2.94 (m, 6H), 1.67–1.58 (d, 2H), 1.37 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.7, 102.8, 76.7, 71.2, 71.1, 70.4, 44.2, 43.9, 36.2, 23.5, 20.6; HR-MS (ESI) calculated for $C_{20}H_{40}N_2O_{12}$ $[M+H]^+$: 501.2659 and found: 501.2653.

***4a** (Maltose derivative)*: FT-IR (NaCl): 3354 cm^{-1} (−OH str., −NH− sym. and asym. str.), 2927 cm^{-1} (−CH_2− asym. str.), 2821 cm^{-1} (−CH_2− sym. str.), 1690 cm^{-1} (−NHBoc C=O str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.82–4.80 (d, 1H), 4.42–4.38 (d, 2H), 3.60–3.38 (m, 10H), 3.13–2.66 (m, 6H), 1.69–1.55 (m, 2H), 1.37 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ/p.p.m.: 171.4, 103.1, 77.2, 70.8, 70.1, 68.6, 48.8, 44.5, 36.9, 23.8, 21.1; HR-MS (ESI) calculated for $C_{20}H_{40}N_2O_{12}$ $[M+H]^+$: 501.2659 and found: 501.2657.

*Synthesis of **3b** and **4b**.* **3a** (1.3 g) or **4a** (1.2 g) was dissolved in 3 ml of methanol, and then 5 ml of 4 N HCl was added to it. The reaction was allowed to stir at room temperature for 4 h. Now, methanol was removed from the reaction mixture and work-up was carried out with chloroform and water. The aqueous layer was collected and dried by using lyophilizer to get the pure **3b** or **4b** with 75% yield.

***3b** (Cellobiose derivative)*: FT-IR (NaCl): 3329 cm^{-1} (−OH, $-NH_2$ sym. and asym. str.), 2929 cm^{-1} (−CH_2− asym. str.), 2885 cm^{-1} (−CH_2− sym. str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.02–4.98 (d, 1H), 4.80–3.44, (m, 12H), 3.06–2.88 (m, 6H), 2.08–1.96 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 102.3, 76.9, 71.3, 71.1, 70.2, 44.2, 44.1, 36.2, 23.5; HR-MS (ESI) calculated for $C_{15}H_{32}N_2O_{10}$ $[M+H]^+$: 401.2135 and found: 401.2159.

***4b** (Maltose derivative)*: FT-IR (NaCl): 3339 cm^{-1} (−OH, $-NH_2$ sym. and asym. str.), 2928 cm^{-1} (−CH_2− asym. str.), 2886 cm^{-1} (−CH_2− sym. str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 5.02–4.98 (d, 1H), 4.80–3.44 (m, 12H), 3.06–2.88 (m, 6H), 2.08–1.96 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 103.0, 76.5, 71.3, 70.2, 68.5, 49.5, 44.2, 36.2, 23.5; HR-MS (ESI) calculated for $C_{15}H_{32}N_2O_{10}$ $[M+H]^+$: 401.2135 and found: 401.2143.

*Synthesis of **5a** and **6a**.* D-gluconic acid lactone (2 g) or lactobionolactone (1.3 g) was dissolved in 12 ml of methanol, and then about 1.2 equivalents of *N*-Boc-1,3-propanediamine was added to the reaction mixture. Now, the reaction mixture was refluxed for 4 h. Then, methanol was removed by rotary evaporator, the residue was washed with ethyl acetate and finally with chloroform. Later it was kept in high vacuum oven for overnight to get the pure and dry **5a** or **6a**.

***5a** (Gluconic acid lactone derivative)*: Yield: 98%. FT-IR (NaCl): 3329 cm^{-1} (−OH str.), 2933 cm^{-1} (−CH_2− asym. str.), 2882 cm^{-1} (−CH_2− sym. str.), 1687 cm^{-1} (amide-I C=O str.), 1654 cm^{-1} (amide-II −NH− ben.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.48–3.47 (m, 4H), 4.35–3.57 (m, 2H), 3.92–3.07 (m, 4H), 1.51–1.49 (m, 2H), 1.37 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.1, 156.2, 78.2, 73.9, 72.7, 71.8, 70.8, 63.6, 37.5, 36.1, 29.8, 28.6; HR-MS (ESI) calculated for $C_{14}H_{28}N_2O_8$ $[M+Na]^+$: 375.1743 and found: 375. 1726.

***6a** (Lactobionolactone derivative)*: Yield: 72%. FT-IR (NaCl): 3341 cm^{-1} (−OH str.), 2929 cm^{-1} (−CH_2− asym. str.), 2888 cm^{-1} (−CH_2− sym. str.), 1685 cm^{-1} (amide-I C=O str.), 1660 cm^{-1} (amide-II −NH− ben.); ^{1}H NMR (400 MHz, D_2O) δ 4.58–4.56 (d, 1H), 4.41–4.41 (d, 1H), 4.20–4.18 (t, 1H), 4.01–3.55 (m, 10H), 3.31–3.28 (t, 2H), 3.11–3.10 (t, 2H), 1.75–1.68 (m, 2H), 1.44 (s, 9H). ^{13}C NMR (100 MHz DMSO-d_6) δ 171.9, 170.3, 103.1, 81.2, 73.2, 71.4, 69.1, 68.5, 62.2, 49.7, 36.2, 25.9, 21.0; HR-MS (ESI) calculated for $C_{20}H_{38}N_2O_{13}$ $[M+H]^+$: 515.2452 and found: 515.2489.

*Synthesis of **5b** and **6b**.* **5a** (2.56 g) or **6a** (1.35 g) was dissolved in 5 ml of methanol and 5 ml of 4 N HCl was added to it, and then the reaction mixture was kept at room temperature for 4 h with stirring. After completion of the reaction, the solvent was removed to get pure and dry **5b** or **6b**.

***5b** (Gluconic acid lactone derivative)*: Yield: 96%. FT-IR (NaCl): 3335 cm^{-1} (−OH, $-NH_2$ sym. and asym. str.), 2927 cm^{-1} (−CH_2− asym. str.), 2886 cm^{-1} (−CH_2− sym. str.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.23–3.53 (m, 4H), 4.12–3.79 (m, 2H), 2.93–2.87 (t, 4H), 1.92–1.88 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6) δ 174.3, 80.4, 74.0, 72.6, 69.1, 62.9, 60.3, 36.2, 25.1; HR-MS (ESI) calculated for $C_9H_{20}N_2O_6$ $[M+H]^+$: 253.1400 and found: 253.1381.

***6b** (Lactobionolactone derivative)*: Yield: 89%. FT-IR (NaCl): 3297 cm^{-1} (−OH, $-NH_2$ sym. and asym. str.), 2932 cm^{-1} (−CH_2− asym. str.), 2888 cm^{-1} (−CH_2− sym. str.), 1685 cm^{-1} (amide-I C=O str.), 1648 cm^{-1} (amide-II −NH− ben.); ^{1}H NMR (400 MHz, DMSO-d_6) δ 4.58–4.54 (d, 1H), 4.41–4.40 (d, 1H), 4.19–4.19 (t, 1H), 4.0–3.55 (m, 10H), 3.36–3.4 (t, 2H), 3.28–3.30 (t, 2H), 1.69–1.73 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.7, 103.1, 81.3, 73.3, 71.5, 69.1, 68.0, 62.8, 49.6, 36.0, 25.0; HR-MS (ESI) calculated for $C_{15}H_{30}N_2O_{11}$ $[M+H]^+$: 415.1928 and found: 415.1901.

*General protocol for the synthesis of **7a**, **8a** and **9a**.* Vancomycin hydrochloride (150 mg) was dissolved in dry dimethylformamide (1 ml) and dry methanol (1 ml).[37] To this one equivalent of 1-octanal or 1-decanal or 1-dodecanal and 1.2 equivalents of diisopropylethylamine were added. The reaction mixture was stirred at 50 °C for 2 h and then allowed to cool to room temperature before the addition of sodium cyanoborohydride (2.0 equivalents). Then, the reaction mixture was stirred at 50 °C for additional 2 h and allowed to cool to ambient temperature for overnight. The product was purified by preparative reversed-phase HPLC using 0.1% trifluoroacetic acid in H_2O/acetonitrile mixture and then lyophilized to afford trifluoroacetate salt compound **7a** or **8a** or **9a** in 75–80% yield.

***7a*:** Yield: 77%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.44 (s, 1H), 9.18 (s, 1H), 9.08 (s, 1H), 8.98 (bs, 1H), 8.88 (bs, 1H), 8.71–8.51 (m, 2H), 8.09 (bs, 1H), 7.81 (bs, 2H), 7.59–7.45 (m, 4H), 7.31–7.1 (m, 3H), 6.78–6.67 (m, 2H), 6.35–6.24 (dd, 2H), 6.0–5.93 (m, 2H), 5.75–5.65 (m, 2H), 5.36–5.2 (m, 6H), 4.91–4.90 (d, 1H), 4.61–4.42 (m, 4H), 4.18–4.08 (m, 4H), 2.67–2.61 (m, 3H), 1.80–1.75 (m, 1H), 1.66–1.51 (m, 4H), 1.24 (m, 13H), 1.09–1.07 (d, 3H) and 0.91–0.85 (m, 10H). HR-MS: *m/z* 785.8725 (observed) and 785.8578 (calculated for M+2H)$^{2+}$.

***8a*:** Yield: 80%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.45 (s, 1H), 9.20 (s, 1H), 9.08 (s, 1H), 8.97 (bs, 1H), 8.88 (bs, 1H), 8.71–8.53 (m, 2H), 8.12 (bs, 1H), 7.83 (bs, 2H), 7.59–7.45 (m, 4H), 7.34–7.09 (m, 3H), 6.78–6.67 (m, 2H), 6.38–6.24 (dd, 2H), 5.98–5.93 (m, 2H), 5.75–5.63 (m, 2H), 5.36–5.2 (m, 6H), 4.91–4.90 (d, 1H), 4.63–4.42 (m, 4H), 4.19–4.10 (m, 4H), 2.67–2.61 (m, 3H), 1.80–1.75 (m, 1H), 1.66–1.51 (m, 4H), 1.24 (m, 17H), 1.09–1.07 (d, 3H) and 0.92–0.83 (m, 10H). HR-MS: *m/z* 795.7992 (observed) and 795.7578 (calculated for M+2H)$^{2+}$.

***9a*:** Yield: 75%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.41 (s, 1H), 9.20 (s, 1H), 9.12 (s, 1H), 9.01 (bs, 1H), 8.88 (bs, 1H), 8.69–8.53 (m, 2H), 8.25 (bs, 1H), 7.93 (bs, 2H), 7.61–7.45 (m, 4H), 7.33–7.21 (m, 3H), 6.78–6.67 (m, 2H), 6.38–6.24 (dd, 2H), 5.99–5.85 (m, 2H), 5.83–5.63 (m, 2H), 5.36–5.2 (m, 6H), 4.95–4.93 (d, 1H), 4.53–4.42 (m, 4H), 4.21–4.10 (m, 4H), 2.71–2.61 (m, 3H), 1.80–1.77 (m, 1H), 1.66–1.55 (m, 4H), 1.28 (m, 21H), 1.09–1.07 (d, 3H) and 0.91–0.86 (m, 10H). HR-MS: *m/z* 809.7417 (observed) and 809.7365 (calculated for M+2H)$^{2+}$.

*Synthesis of vancomycin–sugar conjugates (**1–9**).* Either vancomycin hydrochloride or **7a–9a** is respectively dissolved in dry dimethylformamide (1 ml) or dry dimethyl sulfoxide (1 ml).[38] To this two equivalents of compounds bearing primary amine group (**1d**, **2d**, **3b**, **4b**, **5b** and **6b**), 1 ml of dry dimethylformamide was added. The reaction mixture was cooled to 0 °C, and 0.22 ml (1.5 equivalents) of 0.45 M *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate solution in DMF was added followed by 58 μl of diisopropylethylamine (5.0 equivalents). The reaction mixture was then allowed to warm to room temperature and stirred for 8–12 h. The products were purified by preparative reversed-phase HPLC to more than 95% using 0.1% trifluoroacetic acid in H_2O/acetonitrile mixture and then lyophilized to afford tris-(trifluoroacetate) salts of final compounds (47–54 μmol, 70–80%).

*Vancomycin–sugar conjugate (**1**; Van-cyGlu)*. Yield: 72% (48.2 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.35–9.34 (d, 1H), 9.07–8.95 (m, 3H), 8.82 (bs, 1H), 8.68 (bs, 1H), 8.45–8.44 (m, 2H), 7.97–7.85 (m, 2H), 7.62–7.44 (m, 6H), 7.33–7.31 (d, 1H), 7.20–7.18 (d, 2H), 7.05–7.04 (m, 1H), 6.77–6.62 (m, 2H), 6.35–6.23 (m, 1H), 5.96–5.87 (m, 1H), 5.76–5.59 (m, 1H), 5.49–5.43 (m, 1H), 5.36–5.02 (m, 6H), 4.99–4.65 (m, 4H), 4.57–4.35 (m, 2H), 4.22–4.02 (m, 2H), 3.69–3.66 (m, 2H), 3.07–2.96 (m, 4H), 2.59 (bs, 2H), 2.19–2.11 (m, 2H), 1.91–1.87 (m, 2H), 1.75–1.74 (m, 4H), 1.07–1.05 (d, 3H) and 0.91–0.85 (m, 4H). HR-MS: *mlz* 828.2645 (observed) and 828.2436 (calculated for M+2H)$^{2+}$.

*Vancomycin–sugar conjugate (**2**; Van-cyGlu)*. Yield: 70% (47 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H), 9.0–8.97 (d, 2H), 8.68 (bs, 1H), 8.45–8.44 (d, 2H), 7.91–7.86 (t, 2H), 7.61–7.44 (m, 7H), 7.34–7.32 (d, 2H), 7.20–7.18 (t, 2H), 7.04 (bs, 1H), 6.77–6.64 (m, 3H), 6.35–6.27 (dd, 2H), 5.92–5.74 (m, 3H), 5.60 (s, 1H), 5.45–5.08 (m, 9H), 4.90–4.89 (d, 2H), 4.71–4.58 (m, 3H), 4.45–4.38 (m, 3H), 4.22–4.12 (m, 3H), 4.02–4.00 (t, 1H), 3.78–3.43 (m, 8H), 3.18–3.16 (d, 2H), 2.59 (bs, 2H), 2.18–2.12 (m, 2H), 1.91–1.88 (m, 2H), 1.75–1.53 (m, 5H), 1.29 (bs, 3H), 1.07–1.06 (d, 3H) and 0.91–0.86 (m, 7H). HR-MS: *m/z* 828.2641 (observed) and 828.2436 (calculated for M+2H)$^{2+}$.

*Vancomycin–sugar conjugate (**3**; Van-β-1cyGlu–4acyclic-glucose (acyGlu))*. Yield: 78% (52.3 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.36 (s, 1H), 9.06–9.02 (d, 2H), 8.67 (bs, 1H), 8.47–8.30 (m, 3H), 8.09 (bs, 1H), 7.84 (bs, 1H), 7.65–7.45 (m, 7H), 7.34–7.31 (d, 1H), 7.22–7.20 (m, 2H), 7.08 (bs, 1H), 6.77–6.69 (m, 2H), 6.53 (bs, 1H), 6.38 (s, 1H), 6.22 (s, 1H), 5.98–5.57 (m, 3H), 5.59 (s, 1H), 5.49–5.45 (m, 2H), 5.38–5.34 (m, 2H), 5.27–5.10 (m, 6H), 5.02–4.57 (m, 7H), 4.50–4.22 (m, 6H), 4.04–4.01 (t, 2H), 3.88–3.87 (d, 1H), 3.70–3.53 (m, 4H), 3.18–3.12 (m, 3H), 3.08–2.88 (m, 4H), 2.17–2.12 (m, 1H), 1.91–1.53 (m, 7H), 1.30 (s, 3H), 1.26–1.24 (t, 2H), 1.07–1.06 (d, 3H) and 0.91–0.85 (m, 7H). HR-MS: *m/z* 916.8140 (observed) and 916.8427 (calculated for M+2H)$^{2+}$.

*Vancomycin–sugar conjugate (**4**; Van-α-1cyGlu–4acyGlu)*. Yield: 80% (54 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.34 (s, 1H), 9.05–9.02 (d, 2H), 8.64 (bs, 1H), 8.43–8.33 (m, 3H), 8.09 (bs, 1H), 7.82 (bs, 1H), 7.62–7.39 (m, 7H), 7.34–7.31 (d, 1H), 7.22–7.18 (m, 2H), 7.02 (bs, 1H), 6.75–6.63 (m, 2H), 6.53 (bs, 1H), 6.41 (s, 1H), 6.18 (s, 1H), 6.01–5.59 (m, 3H), 5.55 (s, 1H), 5.49–5.45 (m, 2H), 5.35–5.31 (m, 2H), 5.17–5.10 (m, 6H), 5.02–4.57 (m, 6H), 4.45–4.18 (m, 6H), 3.95–3.91 (t, 2H), 3.88–3.84 (d, 1H), 3.65–3.53 (m, 4H), 3.18–3.12 (m, 3H), 3.08–2.92 (m, 4H), 2.17–2.12 (m, 1H), 1.91–1.53 (m, 7H), 1.28 (s, 3H), 1.26–1.24 (t, 2H), 1.07–1.06 (d, 3H) and 0.91–0.85 (m, 7H). HR-MS: *m/z* 916.8127 (observed) and 916.8427 (calculated for M+2H)$^{2+}$.

*Vancomycin–sugar conjugate (**5**; Van-acyGlu)*. Yield: 75% (50.3 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.35 (s, 1H), 9.07–9.00 (m, 3H), 8.68 (bs, 1H), 8.45 (bs, 1H), 7.97–7.85 (m, 2H), 7.62–7.44 (m, 6H), 7.33–7.18 (dd, 2H), 6.77–6.51 (m, 2H), 6.35–6.23 (m, 1H), 5.96–5.87 (m, 1H), 5.76–5.59 (m, 1H), 5.49–5.43 (m, 1H), 5.36–5.04 (m, 6H), 4.99–4.65 (m, 4H), 4.57–4.35 (m, 2H), 4.22–4.01 (m, 2H), 3.69–3.66 (m, 2H), 3.07–2.96 (m, 4H), 2.59 (bs, 2H), 2.19–2.11 (m, 1H), 1.75–1.54 (m, 4H), 1.3 (s, 3H), 1.07–1.05 (d, 2H) and 0.91–0.85 (m, 7H). HR-MS: *m/z* 842.7744 (observed) and 842.7641 (calculated for M+2H)$^{2+}$.

*Vancomycin–sugar conjugate (**6**; Van-β-1cyGlu–4acyGlu)*. Yield: 72% (48.2 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H), 9.02–8.94 (m, 4H), 8.69 (bs, 1H), 8.53–8.46 (m, 2H), 8.07–8.05 (t, 1H), 7.85 (s, 1H), 7.68–7.45 (m, 10H), 7.33–7.18 (m, 3H), 7.09–7.08 (d, 1H), 6.77–6.66 (m, 3H), 6.48 (bs, 1H), 6.37–6.22 (dd, 2H), 5.94–5.93 (d, 1H), 5.80–5.75 (m, 2H), 5.61 (s, 1H), 5.45–5.43 (d, 1H), 5.34–5.17 (m, 6H), 5.09 (bs, 1H), 4.92–4.91 (d, 1H), 4.68–4.66 (d, 1H), 4.46–4.35 (m, 2H), 4.24–4.21 (d, 2H), 4.02–3.96 (d, 2H), 3.70–3.67 (d, 1H), 3.57–3.44 (m, 3H), 2.9 (bs, 1H), 2.81–2.76 (q, 2H), 2.68–2.62 (m, 4H), 2.15–2.08 (m, 2H), 1.91–1.89 (d, 2H), 1.75–1.55 (m, 7H), 1.30 (s, 3H), 1.07–1.06 (d, 3H) and 0.92–0.85 (m, 7H). HR-MS: *m/z* 923.8035 (observed) and 923.8346 (calculated for M+2H)$^{2+}$.

*Lipophilic–vancomycin–sugar conjugate (**7**; VanC$_8$-β-1cyGlu–4acyGlu)*. Yield: 80% (54 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H), 9.03–8.99 (d, 2H), 8.69 (bs, 1H), 8.48–8.46 (d, 2H), 8.14–8.06 (m, 2H), 7.84–7.39 (m, 9H), 7.35–7.06 (m, 4H), 6.78–6.66 (m, 2H), 6.48 (bs, 1H), 6.37–6.22 (dd, 2H), 5.90–5.62 (m, 5H), 5.36–5.10 (m, 8H), 4.91 (bs, 1H), 4.61–4.60 (d, 2H), 4.46–4.45 (d, 2H), 4.37–4.35 (d, 2H), 4.24–4.22 (d, 3H), 4.11–4.08 (t, 3H), 2.79–2.78 (d, 2H), 2.70–2.66 (m, 2H), 2.33–2.31 (m, 2H), 2.19 (bs, 1H), 2.00–1.97 (m, 1H), 1.80–1.65 (m, 5H), 1.59–1.53 (m, 3H), 1.36 (s, 3H), 1.25 (m, 13H), 1.10–1.08 (d, 3H) and 0.92–0.84 (m, 10H). HR-MS: *m/z* 979.8707 (observed) and 979.9411 (calculated for M+2H)$^{2+}$.

*Lipophilic–vancomycin–sugar conjugate (**8**; VanC$_{10}$-β-1cyGlu–4acyGlu)*. Yield: 77% (51.6 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.35 (s, 1H), 9.04–9.00 (d, 3H), 8.68 (bs, 1H), 8.48–8.47 (d, 2H), 8.18–8.06 (m, 3H), 7.72 (bs, 2H), 7.55–7.45 (m, 4H), 6.78–6.65 (m, 3H), 6.38–6.22 (dd, 2H), 5.96–5.75 (m, 3H), 5.67–5.62 (m, 2H), 5.35–5.11 (m, 8H), 4.93–4.92 (d, 1H), 4.64–4.59 (m,1H), 4.46–4.33 (m, 2H), 4.25–4.09 (m, 3H), 3.94 (bs, 1H), 3.71–3.67 (m, 2H), 3.63–3.46 (m, 4H), 2.80–2.78 (m, 3H), 2.62 (bs, 3H), 2.17–1.98 (m, 2H), 1.81–1.54 (m, 8H), 1.36 (s, 3H), 1.27–1.24 (m, 17H), 1.10–1.08 (d, 3H) and 0.92–0.84 (m, 10H). HR-MS: *m/z* 993.8801 (observed) and 993.9676 (calculated for M+2H)$^{2+}$.

*Lipophilic–vancomycin–sugar conjugate (**9**; VanC$_{12}$-β-1cyGlu–4acyGlu)*. Yield: 77% (51.6 μmol). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.33 (s, 1H), 9.04–8.99 (d, 2H), 8.69 (bs, 1H), 8.48–8.47 (d, 2H), 8.14–8.05 (m, 2H), 7.84 (s, 2H), 7.67 (bs, 3H), 7.54–7.45 (m, 4H), 7.30–7.21 (m, 3H), 7.07 (bs, 1H), 6.78–6.69 (m, 3H), 6.37–6.22 (dd, 2H), 5.92 (bs, 2H), 5.80–5.75 (m, 3H), 5.63–5.62 (d, 2H), 5.36–5.10 (m, 7H), 4.91–4.90 (d, 1H), 4.61–4.60 (d, 2H), 4.46–4.45 (d, 2H), 4.37–4.35 (d, 2H), 4.24–4.20 (m, 2H), 4.12–4.09 (t, 2H), 3.71–3.66 (m, 4H), 2.81–2.78 (m, 3H), 2.67–2.66 (m, 1H), 2.33–2.32 (m, 2H), 2.00–1.97 (d, 1H), 1.80–1.64 (m, 4H), 1.58–1.53 (m, 3H), 1.36 (s, 3H), 1.24 (m, 21H), 1.09–1.08 (d, 3H) and 0.92–0.83 (m, 10H). HR-MS: *m/z* 1007.4024 (observed) and 1007.9941 (calculated for M+2H)$^{2+}$.

Biological studies

In vitro anti-bacterial activity

Minimum inhibitory concentration. All vancomycin derivatives were assayed in a modified microdilution broth format.[39] All the derivatives were serially diluted using autoclaved Millipore water. Bacteria, to be tested, were grown for 6 h in the suitable media. Overnight grown bacteria contained ~ 10^9 CFU ml^{-1} (determined by spread plating method), which was then diluted to 10^5 CFU ml^{-1} using suitable media. Fifty microliters of serially diluted compound was added to a 96-well plate containing 150 μl media containing bacterial solution. Two controls were made: one containing 150 μl of media and 50 μl of compound of every concentration and the other containing 200 μl of media containing bacterial solution. The plate was then incubated at 37 °C for a period of 24 h and the OD value was measured at 620 nm using TECAN (Infinite series, M200 pro) Plate Reader. Each concentration had triplicate values and the whole experiment was carried out at least two times and the MIC value was determined by taking the average of triplicate OD values for each concentration and plotting it against concentration. The data were then subjected to sigmoidal fitting. From the curve, the MIC value was determined, as the point in the curve where the OD is similar to that of control having no bacteria.

Titration binding assays with model ligands (*N,N′*-diacetyl-Lys-D-Ala-D-Ala and *N,N′*-diacetyl-Lys-D-Ala-D-Lac). The binding constants for vancomycin, vancomycin–sugar conjugates (**1–6**) and lipophilic–vancomycin–sugar conjugate (**8**) with the model ligands *N,N′*-diacetyl-Lys-D-Ala-D-Ala and *N,N′*-diacetyl-Lys-D-Ala-D-Lac were determined using UV-absorption difference measurements. UV scans were run with a baseline correction that consisted of 0.02 M sodium citrate buffer (pH = 5.1) and measured the range from 200 to 345 nm. A solution of test compounds (100 μM in 0.02 M sodium citrate buffer) was placed into a quartz UV cuvette (1 cm path length) and the UV spectrum recorded versus a reference cell containing 0.02 M sodium citrate buffer. UV spectra were recorded after each addition of a solution of *N,N′*-diacetyl-Lys-D-Ala-D-Ala (0.05–5.0 equivalents) or *N,N′*-diacetyl-Lys-D-Ala-D-Lac (0.05–40.0 equivalents) in 0.02 M sodium citrate buffer. The absorbance value at the λ_{max} (279 nm) was recorded and the running change in absorbance, $\delta A_{x\ equiv}$ ($A_{initial} - A_{x\ equiv}$), was measured. The number of ligand equivalents was plotted versus δ*A* to afford the ligand binding titration curve. The break point of this curve is the saturation point of the system and its xy coordinates were

determined by establishing the intersection of the linear fits of the pre- and postsaturation curves. $\delta A_{saturation}$ was calculated and used to determine the concentration of free ligand in the solution at each titration point after saturation. δA was plotted against δA/free ligand concentration to give a Scatchard plot from which the binding constants were determined.

RESULTS

Synthesis and characterization

In the synthetic strategy used for preparing vancomycin–sugar conjugates, sugar moieties (cy or/and acy) bearing a linker with a primary amine group (propylene imine or ethylene imine) were coupled to the carboxyl group of vancomycin (Figure 2; compounds **1**–**6**) via amide bond formation by using *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate as the coupling reagent. These vancomycin derivatives were purified by reversed-phase HPLC to more than 95% purity in 70–80% yield and characterized by ^{1}H NMR spectroscopy and HR-MS.

First, we synthesized vancomycin–sugar conjugates (compounds **1** and **2**) containing cyclic-glucose (cyGlu) and cyclic-galactose (cyGal) where sugar moiety is connected to ethylene imine linker, through ether linkage (Scheme 1). To synthesize compounds **1** and **2**, D-hexopyranose pentaacetate was first coupled with 2-bromoethanol in $BF_3{\cdot}Et_2O$-catalyzed reaction, and then the bromo compound (**1a** or **2a**) was treated with sodium azide in methanol to get azido compound (**1b** or **2b**). After deacetylation, the azido compound (**1c** or **2c**) was subjected to Staudinger reduction to afford 2-aminoethyl D-hexopyranose (**1d** or **2d**). Then, 2-aminoethyl D-hexopyranose is coupled to the carboxyl group of vancomycin to give vancomycin–sugar conjugates **1** and **2**.

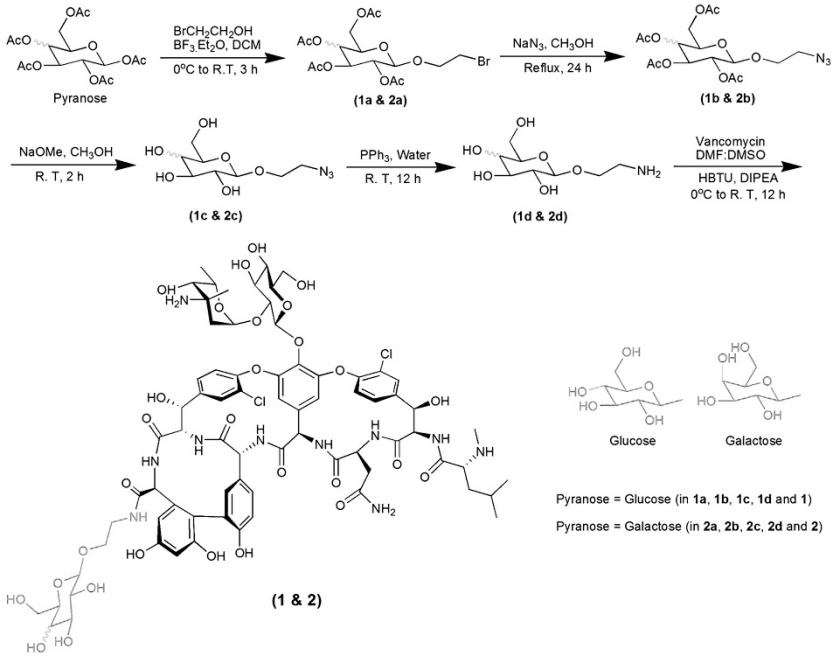
Then, we sought to incorporate disaccharides such as cellobiose and maltose to vancomycin to find whether the number and orientation of hydroxyl groups affects the binding efficiency or not. To do so, we synthesized compounds **3** and **4**, which contain both cyGlu and acyGlu moieties of two different conformations such as β1–4 (β-1cyGlu–4acyGlu) and α1–4 (α-1cyGlu–4acyGlu), respectively. Here, we performed Schiff's base formation with *N*-Boc-1,3-propanediamine, followed by reduction of imine derivative of disaccharide (cellobiose or maltose) to obtain compounds **3a** and **4a**. Now, compounds **3a** and **4a** were subjected to deprotection in the presence of acid to give *N*-Boc free compounds **3b** and **4b**. Then, these compounds were coupled to the carboxylic group of vancomycin to give vancomycin–sugar conjugates **3** and **4** (Scheme 2). For both the compounds (**3** and **4**), the acyclic sugar moiety is connected to a propylene imine linker through secondary amine.

Next, we synthesized compound **5** comprising only acyGlu moiety and compound **6** containing a cyGal and acyGlu moieties (β-1cyGal–4acyGlu), wherein the acyclic sugar moiety for both the compounds is connected to a propylene imine linker through amide bond. To synthesize compounds **5** and **6**, δ-gluconolactone or lactobionolactone was subjected to nucleophilic ring opening reaction with *N*-Boc-1,3-propanediamine, which give *N-Boc-1,3*-propanediamine derivatized sugar derivatives (**5a** or **6a**) followed by deprotection of *N*-Boc, to give compounds **5b** or **6b**, which were finally coupled to vancomycin to give vancomycin–sugar conjugates **5** and **6** (Scheme 3).

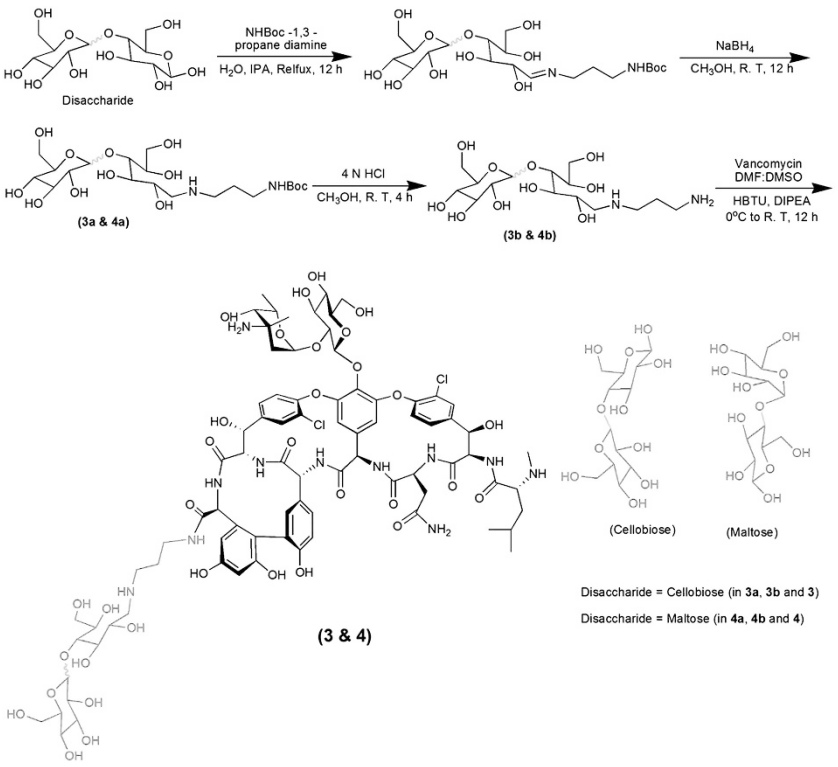
Finally, after optimizing the sugar moiety, we have incorporated lipophilicity to vancomycin. Here, we performed *N*-alkylation of vancomycin through Schiff's base formation using 1-octanal, 1-decanal and 1-dodecanal, followed by reduction to give compounds **7a**, **8a** and **9a**, respectively. These *N*-alkylated vancomycin derivatives were coupled to **6b** to give lipophilic–vancomycin–sugar conjugates **7**, **8** and **9** (Figure 2 and Scheme 4).

In vitro antibacterial activities

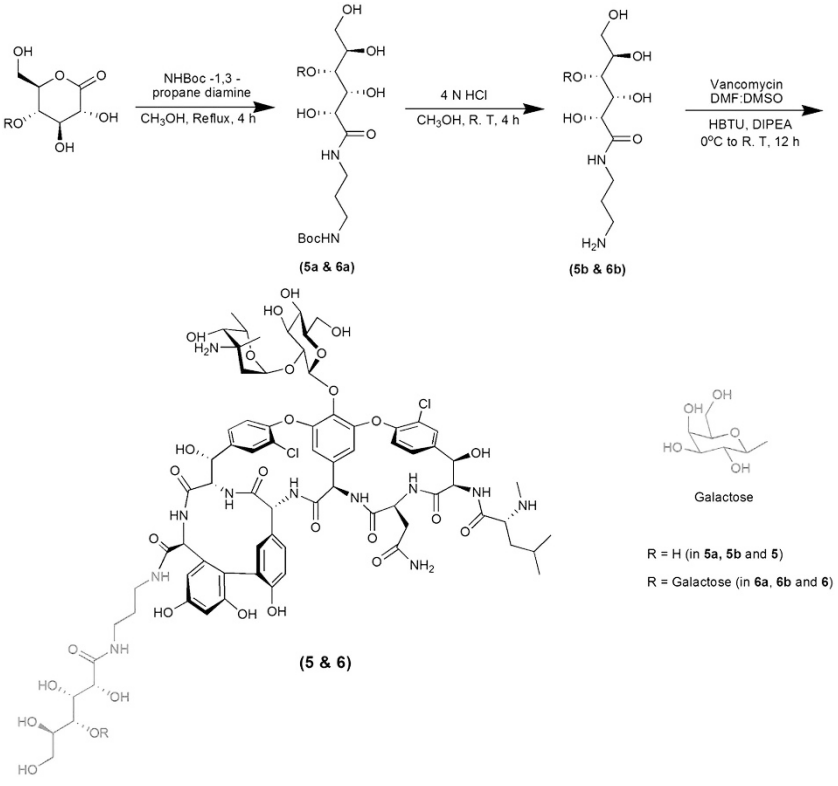
The antibacterial activities of vancomycin and its derivatives were evaluated by determining the MICs against MRSA, VISA and *Enterococci* (VRE; VanA and VanB phenotypes). The results are



Scheme 1 Synthesis of vancomycin–sugar conjugates **1** and **2.** $BF_3{\cdot}Et_2O$, boron trifluoride diethyl etherate; DIPEA, *N,N*-diisopropylethylamine; HBTU, *N,N,N′,N′*-tetramethyl-*o*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate; NaN_3, sodium azide; NaOMe, sodium methoxide; PPh_3, triphenylphosphine. A full color version of this figure is available at *The Journal of Antibiotics* journal online.



Scheme 2 Synthesis of vancomycin–sugar conjugates **3** and **4**. IPA, isopropanol; $NaBH_4$, sodium borohydride. A full color version of this figure is available at *The Journal of Antibiotics* journal online.



Scheme 3 Synthesis of vancomycin–sugar conjugates **5** and **6**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

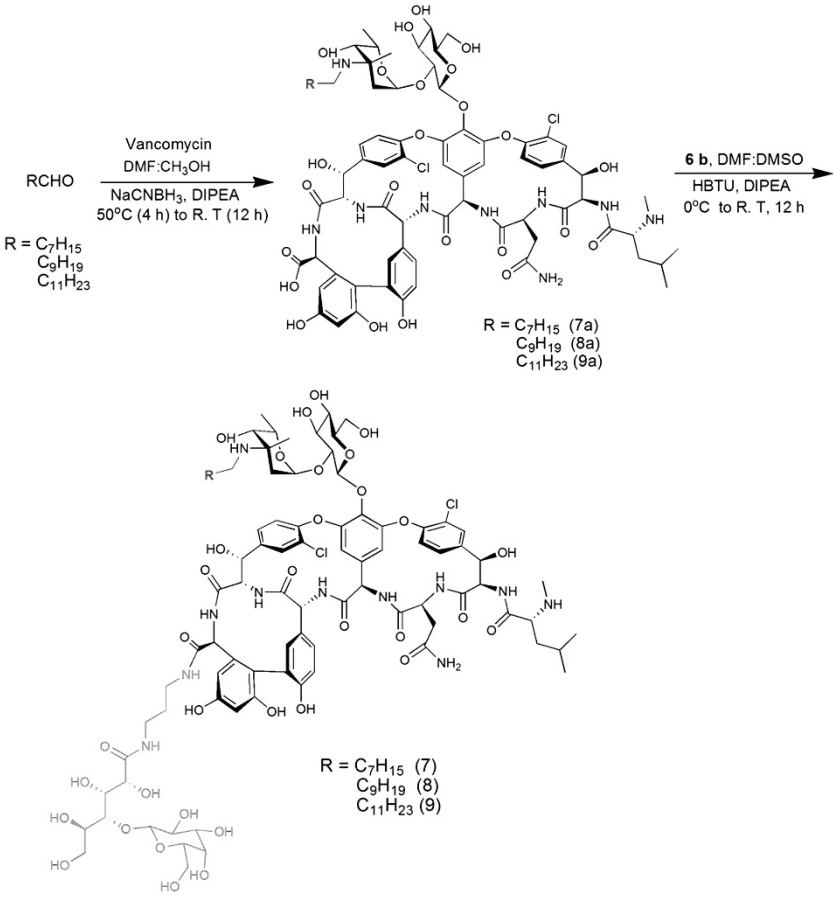
summarized in Table 1. Against MRSA, all these compounds showed similar or slightly better efficacy than vancomycin. Compounds **1** (cyGlu) and **2** (cyGal) exhibited much improved antibacterial activity toward VISA (MIC ~2 μM) in comparison with vancomycin (MIC of 13 μM). Incorporation of an acyclic moiety and replacing the C-2 oxy spacer with C-3 amine spacer yielded compounds **3** (β-1cyGlu–4acyGlu) and **4** (α-1cyGlu–4acyGlu). Compounds **3** and **4** were around two-fold more active than compounds **1** and **2** against VISA, indicating the importance of disaccharide moieties (additionally the importance of the open form of the sugar) toward antibacterial activity. However, all of these compounds (**1–4**) were found to be inactive against both the strains of VRE.

It was envisioned that incorporation of amide bond might aid in additional hydrogen bonding interactions. Thus, compounds **5** and **6** were designed and synthesized. The open monosaccharide analog, **5**, showed little increase in activity against VISA (MIC of 0.9 μM) in comparison with **3** and **4**. The open disaccharide analog, **6**, on the other hand displayed even better activity against VISA with an MIC value of 0.3 μM. In comparison with vancomycin, compounds **5** and **6** showed 15- and 40-fold more activity against VISA. When tested against VRE (VanA phenotype, *E. faecium*), however, compounds **5** and **6** exhibited MICs of 54 and 36 μM, respectively, whereas the MIC for vancomycin was found to be 750 μM (Table 1). Compounds **5** and **6** also showed much improved activity against VanB phenotype of VRE (*E. faecalis*) with the MICs of 60 and 30 μM, respectively, whereas

Table 1 ***In vitro*** **antibacterial activity and binding affinities of the compounds**

	MIC (μM)				*Association constant (K_a, M^{-1})*	
Compound	*MRSA*	*VISA*	*VREm*	*VREs*	*Susceptible*	*Resistant*
Vancomycin	0.63	13	750	250	1.1×10^5	5.0×10^2
1	1.4	2.0	>100	>100	1.4×10^5	9.1×10^2
2	1.2	2.1	>100	>100	1.2×10^5	8.5×10^2
3	1.0	1.0	>100	>100	0.5×10^5	12×10^2
4	1.0	1.0	>100	>100	0.8×10^5	11×10^2
5	0.6	0.9	54	60	2.2×10^5	6.3×10^4
6	0.4	0.3	36	30	2.1×10^5	8.8×10^4
7	0.4	0.3	2.0	6.2	ND	ND
8	0.2	0.2	0.7	1.0	6.0×10^5	6.0×10^4
9	0.3	0.2	0.8	1.0	ND	ND
7a	0.5	0.4	25	13.1	ND	ND
8a	0.3	0.3	14	6.2	ND	ND
9a	0.3	0.3	6.9	3.7	ND	ND
Dalbavancin	0.1	ND	18	2.0	ND	ND
Telavancin	0.5	ND	5	4.0	ND	ND

Abbreviations: MRSA (ATCC 33591), methicillin-resistant *Staphylococcus aureus*; ND, not determined; resistant, *N,N'*-diacetyl-Lys-D-Ala-D-Lac (model ligand for VRE); susceptible, *N,N'*-diacetyl-Lys-D-Ala-D-Ala (model ligand for susceptible bacteria); VISA, vancomycin-intermediate-resistant *Staphylococcus aureus* was generated from MRSA after treating with vancomycin for 52 passages;[30] VREm, vancomycin-resistant *Enterococcus faecium* (VanA phenotype, ATCC 51559); VREs, vancomycin-resistant *Enterococcus faecalis* (VanB phenotype, ATCC 51575). Dalbavancin and telavancin data was taken from literature.[40,41]



Scheme 4 Synthesis of lipophilic–vancomycin–sugar conjugates **7**, **8** and **9**. $NaCNBH_3$, sodium cyanoborohydride. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

vancomycin was active at 250 μM. Compound **6** having the best activity against VRE (VanA phenotype, 36 μM) among compounds **1–6** turned out to be the highlight of this study.

To sensitize VRE toward such compounds further, lipophilic–vancomycin–sugar conjugates were developed, wherein lipophilic alkyl chains were incorporated into compound **6.** The antibacterial activities of lipophilic–vancomycin–sugar conjugates were also evaluated against vancomycin-resistant strains (VRE and VISA). All the compounds showed better activity against VISA in comparison with compounds **1–6** and the best activity was achieved for lipovancomycin–sugar conjugate containing decyl and dodecyl chains (compounds **8** and **9**). Intermediate compounds (**7a–9a**) showed similar or slightly better activity than vancomycin against MRSA. Against VRE (VanA phenotype), compounds **7a–9a** had the MIC values ranging from 6.9 to 25 μM, which is 30- to 108-fold more active than vancomycin. Whereas lipophilic–vancomycin–sugar conjugates (**7–9**) exhibited MIC values of 0.7–2 μM, which is 350- to >1000-fold higher than vancomycin (Table 1). The MIC_{90} values of telavancin and dalbavancin (Figure 1) against VRE (VanA phenotype) were found to be 4 and 18 μM, respectively, which are less active than compounds (**7–9**) (Table 1).[40,41]

Binding affinities

To prove our hypothesis, we had evaluated the binding constants of vancomycin–sugar conjugates **1–6** using UV-difference spectroscopy[42,43] against both sensitive and resistant model ligands: *N,N′*-diacetyl-Lys-D-Ala-D-Ala and *N,N′*-diacetyl-Lys-D-Ala-D-Lac, respectively, and the results are displayed in Table 1 (Supplementary Information and Supplementary Figures S1–S7). The binding affinities of compounds **5** and **6** were found to be two-fold higher than vancomycin against *N,N′*-diacetyl-Lys-D-Ala-D-Ala, whereas compounds **1–4** exhibited binding affinities similar to vancomycin. When

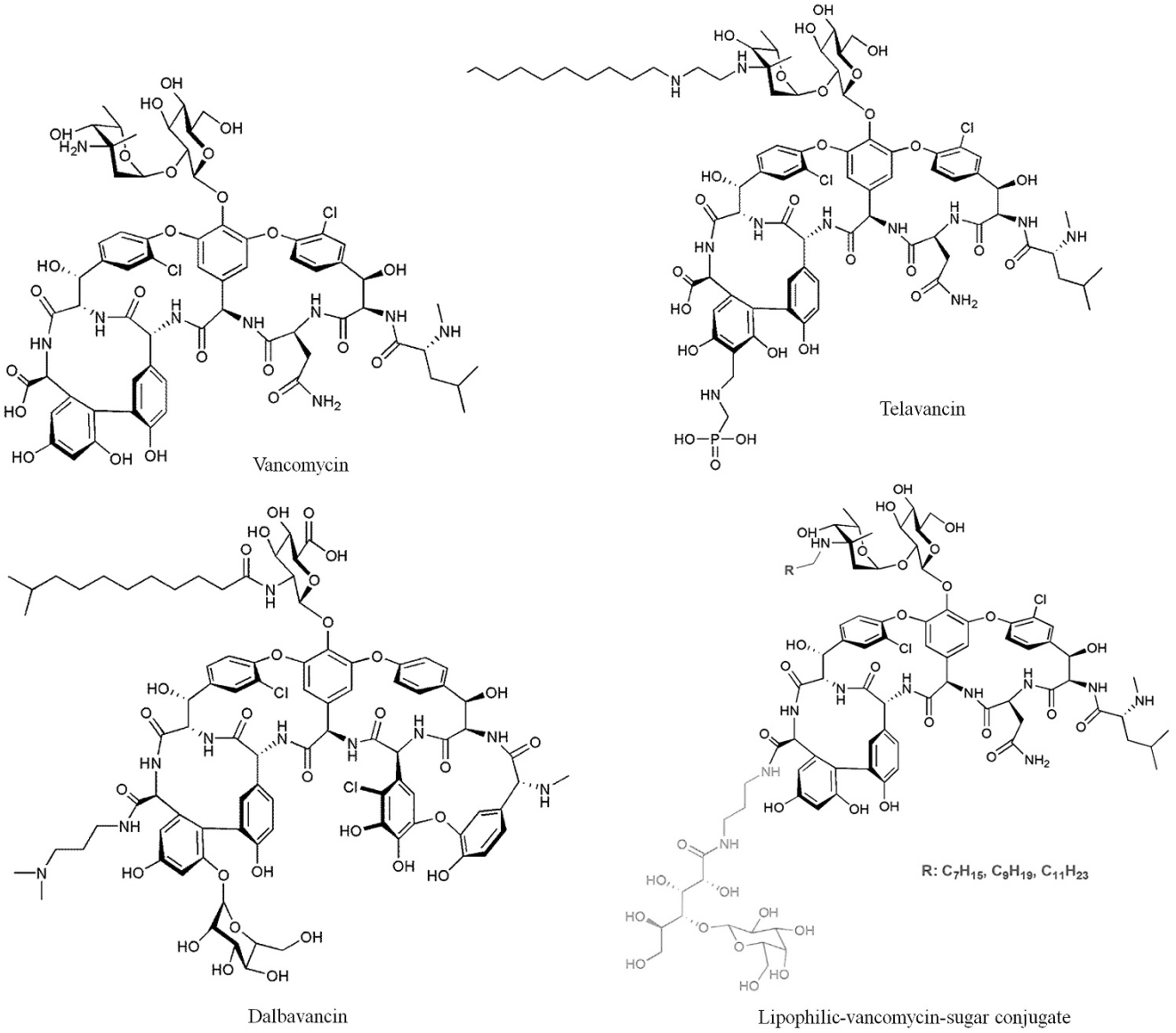


Figure 1 Structures of vancomycin, telavancin, dalbavancin and lipophilic–vancomycin–sugar conjugates. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

evaluated against *N,N′*-diacetyl-Lys-D-Ala-D-Lac, compounds **1–4** displayed low binding affinities similar to vancomycin. The binding affinities of derivatives **5** and **6** against *N,N′*-diacetyl-Lys-D-Ala-D-Lac, on the other hand, were 125-fold ($6.3\times10^4\,M^{-1}$) and 170-fold ($8.8\times10^4\,M^{-1}$) higher than vancomycin ($5\times10^2\,M^{-1}$), respectively. This result is a clear proof of our initial hypothesis. We had also evaluated the binding affinities of lipophilic–vancomycin–sugar conjugate **8** for both sensitive and resistant model ligands. As the presence of alkyl chain has no effect on the interaction with the peptides, the binding affinities of compound **8** were found to be similar to compound **6** (Table 1 and Supplementary Information; Supplementary Figure S8).

DISCUSSION

In an attempt to develop novel therapeutics to conquer bacterial resistance, much attention has been focused on developing semisynthetic glycopeptide antibiotics. Successful designs in the field have focused on improving binding affinity of vancomycin analogs to VRB.[32,44] In this report, we have adopted, in our design strategy, a very simple chemical approach to enhance binding affinity to the target peptides.

To enhance the binding affinity of the compounds with modified peptidoglycan of resistant bacteria, we have incorporated various sugar moieties at the C-terminus of vancomycin backbone. Initially we had incorporated cyGlu and cyclic galactose moieties via the anomeric–OH group of the sugars. Significant improvement in activity against VISA was observed independent of the orientation of OH moieties in the sugars. Upon replacement of monosaccharide by a disaccharide, a little improvement in activity was observed. Although it cannot be conclusively said, what brings about this improvement, it is surmised that the additional OH groups or the open structure of the first sugar in the disaccharides bring about some sort of a favorable interaction. However, as activity against VRE was not achieved in these compounds, incorporation of amide bonds was envisioned. Significant improvement in antibacterial activity was observed in compounds **5** and **6** (containing the newly incorporated amide bond). The acyclic compound **5** differed from compound **1** in the presence of an amide bond over ether and the presence of a C-3 spacer over C-2 spacer. This small difference was significant in restoring the activity against VRE. This significant improvement in activity might be attributed to the favorable H-bonding interactions provided by the amide bond. On comparing the activity of compounds **3** and **4** with that of compound **5**, it becomes clear that the presence of amide bonds is more important than the presence of additional OH groups. However, the presence of extra OH groups is beneficial after the amide bond has been incorporated, as was concluded upon comparing activity of compound **6** with that of compound **5**. The importance of the amide bond toward increase in activity was well demonstrated in the results portrayed by an experiment determining the association constants. Compounds **5** and **6** had binding constants >100- and ~150-fold higher than that of compounds **1–4** and vancomycin, respectively. Similar observations were reported recently by Slusarz *et al.*[45] in a theoretical simulation, wherein vancomycin derivatives modified with non-cyclic sugar moieties not only had more conformational freedom than cyclic sugar vancomycin derivatives but also moved closer to the peptidoglycan layer to have some favorable interactions.

Additionally, to increase the activity against VRE further, we had incorporated a lipophilic aliphatic moiety to the optimized vancomycin–sugar conjugate (Figures 1 and 2). This appendage brings about an additional property of enhanced bacterial membrane interaction to the molecules. It has been shown in the literature too that inclusion of lipophilicity to glycopeptides leads to enhanced anti-bacterial activity against VRE.[30,46–52] *N*-Alkylation of compound **6** through Schiff's base formation using long-chain aldehydes (varying from octyl to dodecyl) followed by reduction yielded lipophilic–vancomycin–sugar conjugates. The antibacterial activities of these lipophilic–vancomycin–sugar conjugates were compared with the activities of second generation of glycopeptides such as telavancin and dalbavancin. Compound **8** was 7-, 25- and >1000-fold more active against VRE compared with telavancin, dalbavancin and vancomycin, respectively. The binding

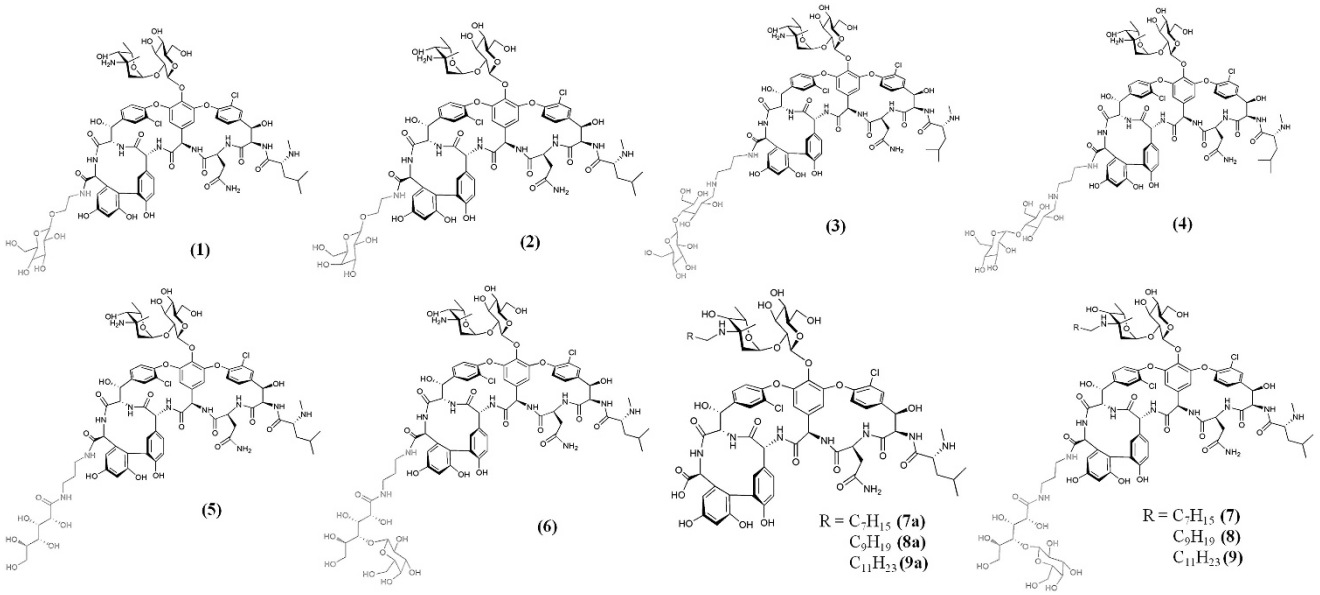


Figure 2 Structures of vancomycin–sugar conjugates (**1–6**), lipophilic–vancomycin derivatives (**7a–9a**) and lipophilic–vancomycin–sugar conjugates (**7–9**). A full color version of this figure is available at *The Journal of Antibiotics* journal online.

affinity of compound **8** with model ligands simulating the peptides from both sensitive and resistant strains was found to be similar to compound **6.** This was expected as both the compounds bear the same sugar moiety, which aids in binding with the peptides. Therefore, this superior anti-bacterial activity of lipophilic–vancomycin–sugar conjugates can be attributed to the collective action on cell wall biosynthesis and bacterial cell membrane. The approach we have reported here is a first of its kind and can bring about the development of many more such interesting molecules.

In conclusion, we have developed a simple synthetic strategy to enhance vancomycin binding affinity to the target peptides of vancomycin-resistant bacteria. This improved binding affinity significantly resulted in the high antibacterial activity of the compounds against VISA and VRE, thus successfully overcoming vancomycin resistance. This strategy paves a way for a rational approach in the development of novel glycopeptide antibiotics. We believe that this approach would be a beneficial extension to clinically approved glycopeptide antibiotics for the treatment of infections caused by vancomycin-resistant bacteria.

CONFLICT OF INTEREST

The authors declare no conflict of interest. JNCASR has filed a patent application based on the work described in the manuscript.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)