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Glyco-pseudopolyrotaxanes: Carbohydrate Wheels Threaded on a Polymer String and Their Inhibition of Bacterial Adhesion

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Abstract: We report glyco-pseudopolyrotaxanes composed of cucurbit[6]uril-based mannose wheels (ManCB[6]) threaded on polyviologen (PV), which not only effectively induce bacterial aggregation, but also exhibit high inhibitory activity against bacterial binding to host cells. Three glyco-pseudopolyrotaxanes (**1**–**3**), which have 10, 5, and 3 ManCB[6] wheels, respectively, on a PV string, were prepared and characterized. Bacterial aggregation assays and hemagglutination inhibition assays illustrated the specific and multivalent interaction between the glyco-pseudopolyrotaxanes and *E. coli* ORN178. Compound **3** was especially effective at inducing bacterial aggregation and showed 300 times higher inhibitory potency than monomeric methyl-α-mannoside (Me-αMan) for ORN178-induced hemagglutination. Furthermore, we demonstrated their inhibitory activities for the adhesion of ORN178 bacteria to urinary epithelial cells as a model of urinary tract infection. Our findings suggest that these supramolecular carbohydrate clusters are potentially useful in antiadhesion therapy.

Keywords: carbohydrates · cluster compounds · cucurbiturils · host–guest systems · rotaxanes · supramolecular chemistry

Introduction

Adhesion of pathogenic organisms to host tissues is a crucial step for the initiation of infectious diseases.[1] It is usually mediated by proteins present on the surface of the pathogenic organism, which bind to the complementary carbohydrates on the surface of the host tissues. These interactions are specific and strong, mainly due to multivalent carbohydrate-protein interactions.[2] Therefore, antiadhesion therapy, in which multivalent carbohydrate clusters with strong binding ability to bacteria are used to prevent the bacterial adhesion to host cells, has been investigated as a potential method to protect humans from pathogenic infection with lower resistance than that of antibacterial chemotherapy.[3,4]

Although a number of multivalent carbohydrate clusters with various scaffolds have been studied to understand and manipulate such multivalent carbohydrate–protein interactions,[5] it is challenging to design adequate multivalent carbohydrate clusters that can interact with complementary proteins in dynamic biological environments, such as cell membranes. As a new direction in the architectural design of carbohydrate clusters, researchers have become interested in developing supramolecular architectures, such as vesicles,[6] nanofibers,[7] and (pseudo)polyrotaxanes[8] as self-assembled dynamic scaffolds, which allow carbohydrate ligands to maximize their interactions with proteins. Especially, pseudopolyrotaxanes, "beads on a string" structures, are known to offer a flexible and dynamic platform for the multivalent display of carbohydrates, that enables the beads freely rotate around, and/or move along the polymer backbone.[8] This adaptability gives carbohydrate ligands an opportunity to find appropriate positions and orientations to optimize interactions with their protein targets. To date, however, their binding or inhibition abilities were mainly evaluated by using proteins in solution, which may not be directly translated to their activity towards inhibition of living organisms. Therefore, studies on the binding/inhibition abilities of such multivalent carbohydrate clusters on a flexi-

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ble and dynamic platform using living organisms such as bacteria are required to examine their potentials in antiadhesion therapy.

Cucurbit[6]uril (CB[6]), a member of the family of macrocyclic cavitands, cucurbit[*n*]uril (CB[*n*], *n* = 5–10), comprising six glycoluril units, has a hydrophobic cavity accessible through two identical carbonyl-fringed portals. It forms stable host–guest complexes with a wide range of neutral or positively charged guest molecules.[9] This property has enabled the synthesis of various (pseudo)polyrotaxanes in which a number of CB[6] "beads" are threaded on various polymer "strings".[10] Among them, water-soluble pseudopolyrotaxanes containing CB[6] threaded on polyviologen (PV), which can be synthesized with a precisely controlled degree of threading, were stable against dethreading without bulky stoppers.[10e,11]

Recently, we reported CB[6]-based carbohydrate clusters ("carbohydrate wheels"), which have multiple carbohydrate moieties attached to the periphery of a CB[6] core.[12] Using CB[6]-based mannose, galactose, and glucose wheels (ManCB[6], GalCB[6], and GlcCB[6], respectively), we demonstrated not only their specific and strong multivalent interactions with lectins, but also their formation of host–guest complexes with a fluorescent model drug, which can be delivered to a specific cell by receptor-mediated endocytosis. As a part of our efforts to develop new supramolecular materials for biomedical applications, we now report glyco-pseudopolyrotaxanes composed of CB[6]-based carbohydrate wheels threaded on PV, which not only effectively induce bacterial aggregation, but also exhibit high inhibitory activity against bacterial binding to host cells.

Results and Discussion

Three mannose-pseudopolyrotaxanes **1** (10ManCB[6]@PV), **2** (5ManCB[6]@PV), and **3** (3ManCB[6]@PV), which have 10, 5, and 3 ManCB[6] wheels, respectively, on a PV string, were prepared by simple treatment of the corresponding amounts of ManCB[6] with PV having approximately 11 viologen units (Scheme 1). The formation of the pseudopolyrotaxanes was confirmed by ^{1}H NMR spectroscopy (Figure 1).[10e] The signal of the internal bipyridyl proton A was significantly shifted downfield (from 9.15 ppm to 9.35 ppm), whereas that of the internal bipyridyl proton B was only

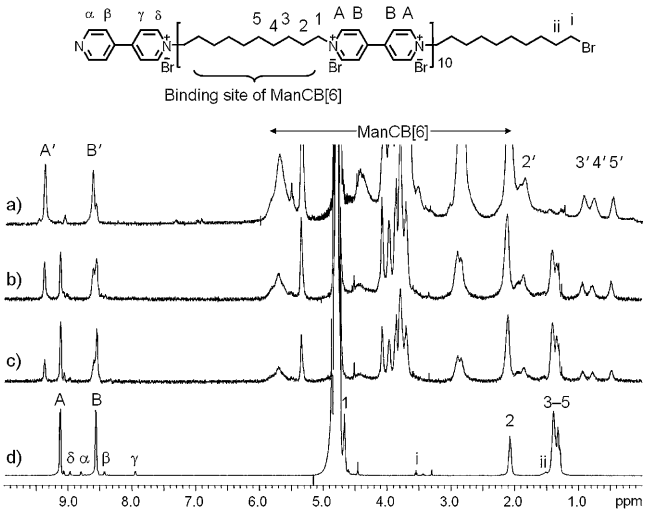
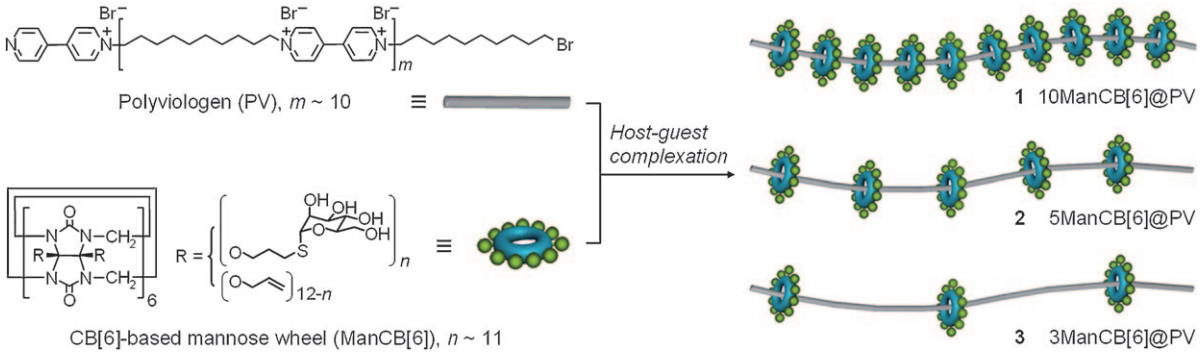


Figure 1. ^{1}H NMR spectra of a) **1**, b) **2**, c) **3**, and d) PV.

slightly shifted (from 8.55 ppm to 8.60 ppm) upon formation of the pseudopolyrotaxanes. At the same time, the proton signals of internal methylenes 3–5 were shifted upfield from 1.2–1.5 ppm to 0.5–1.0 ppm, which indicated that the CB[6]-based mannose wheels are located on the internal decamethylene units, not on the internal bipyridyl units, upon formation of the pseudopolyrotaxanes. From the integral ratio of the protons corresponding to the "uncomplexed" units (A and 3–5) and those of the "complexed" units (A′ and 3′–5′), the average number of threaded ManCB[6] wheels on the PV string was determined to be 9.8, 4.7, and 2.8 for **1**, **2**, and **3**, respectively. The resulting glyco-pseudopolyrotaxanes were quite stable in aqueous solution; no appreciable dethreading in the spectra was observed after at least three months in solution. For control experiments, two other glyco-pseudopolyrotaxanes, **4** (3GalCB[6]@PV) containing three galactose wheels and **5** (3GlcCB[6]@PV) containing



Scheme 1. Glyco-pseudopolyrotaxanes **1**, **2**, and **3**.

three glucose wheels were also prepared by following the same procedure except using GalCB[6] and GlcCB[6], respectively, instead of ManCB[6].

To test whether glyco-pseudopolyrotaxanes can specifically and effectively aggregate bacteria, pseudopolyrotaxanes **1–5** were individually added to an opaque solution (2×10^8 CFU mL^{-1}, $OD_{600}=1.0$; CFU = colony-forming units; OD = optical density) of antibiotic-resistant *E. coli* ORN178,[13] which has a mannose-specific binding protein, FimH,[14] on its pili. The bacteria treated with **1**, **2**, or **3** at 60 μM started to aggregate within a few minutes and soon settled down to the bottom of the tube by gravity, which was observed with the naked eye, whereas the bacteria treated with **4** or **5** did not seem to aggregate. It suggests that among the glyco-pseudopolyrotaxanes, only mannose-bearing pseudopolyrotaxanes (**1**–**3**) specifically interact with ORN178.

To obtain quantitative data on the bacterial aggregation, various concentrations of mannose-pseudopolyrotaxanes (**1**–**3**) and ManCB[6] were individually added to the bacterial solutions (2×10^8 CFU mL^{-1}, $OD_{600}=1.0$). After 30 min, the OD_{600} value of each supernatant solution was measured. The percent bacterial aggregation for each compound was calculated by the difference in the OD_{600} values of the supernatant of the bacterial solution before and after treatment with the compounds.[15] As shown in Figure 2 (solid

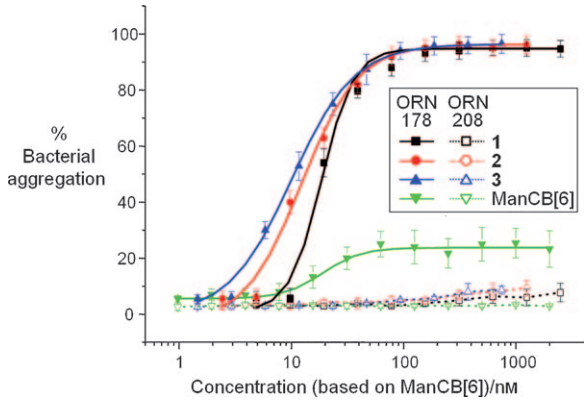


Figure 2. Bacterial aggregation assay. Each value represents the mean ± SD ($n=3$).

lines), a sigmoidal increase in aggregation of ORN178 was observed with increasing concentration of the added mannose-bearing compounds (**1**–**3**, and ManCB[6]). On the other hand, when the experiment was repeated with ORN208,[13] which is deficient of the mannose binding protein FimH, negligible aggregation occurred even at high concentrations of the mannose-bearing compounds (dashed lines, Figure 2), suggesting their ability to specifically bind to ORN178.

The comparative efficiency of mannose-bearing compounds **1**–**3** toward the aggregation of ORN178 (Figure 2, solid lines) revealed that while ManCB[6] caused bacterial aggregation only to a limited extent (20–30%) even at high concentrations, all three mannose-pseudopolyrotaxanes effectively induced bacterial aggregation at $\approx5\times10^{-8}$ M or above (based on ManCB[6]), presumably due to their linear polymeric structure and relatively large size compared to ManCB[6]. The bacterial aggregation efficiency of the mannose-pseudopolyrotaxanes was compared using a half-maximal concentration for aggregation (AC_{50}).[16] The AC_{50} values for **1**, **2**, and **3** were 19, 13, and 10 nM (based on ManCB[6]), respectively, suggesting that three ManCB[6] wheels threaded on a PV string is the most efficient combination for aggregating ORN178 bacteria among the three cases by providing a proper density of ManCB[6] on a PV.

As a control, the same experiment was performed with the "string" (PV) or glyco-pseudopolyrotaxanes bearing galactose (**4**) or glucose wheels (**5**), instead of the mannose-pseudopolyrotaxanes. No aggregation was evident with the naked eye, and less than 10% of bacterial aggregation was observed by OD_{600} with **4**, **5** or PV for both bacteria strains ORN178 and ORN208 (Figure S1, Supporting Information).

The ability of the glyco-pseudopolyrotaxanes to inhibit the bacterial interaction with erythrocytes was investigated by hemagglutination inhibition (HAI) assay. ORN178 is known to agglutinate guinea pig erythrocytes through the interactions between the mannose receptors (FimH) on ORN178 and mannose ligands on guinea pig erythrocytes. The minimal hemagglutinating concentration (MHC) of ORN178 was 0.5×10^8 CFU mL^{-1}. Using this MHC condition, the inhibitory activities of the glyco-pseudopolyrotaxanes for ORN178-induced hemagglutination were investigated. As shown in Table 1, **1** exhibits the lowest minimal inhibitory concentration (MIC), 0.13 μM, among the three glyco-pseudopolyrotaxanes. However, **3** has the highest relative inhibitory potency per mannose residue, 300 (relative to methyl-α-mannoside (Me-αMan) = 1). Parallel with the bacterial aggregation experiment described above, this result indicates that three mannose wheels on the PV string is the optimum number for the inhibition of bacterial interactions among the three cases.

Table 1. Inhibitory potencies of mannose-bearing compounds for ORN178-induced hemagglutination.

	Av number of mannose units	MIC [μM][a]	Relative potency per mannose
Me-αMan	1	2600	1
ManCB[6]	11	2.6	91
1	110	0.13	180
2	55	0.26	180
3	33	0.26	300

[a] Minimum concentration of the compound required to inhibit hemagglutination (see Experimental Section for details).

Encouraged by these results, we then investigated the ability of **3** to prevent bacterial binding to UROtsa cells,[17] urinary epithelial cells. First, ORN178 and ORN208 were directly labeled with fluorescein isothiocyanate (FITC) and then added to UROtsa cells with various bacteria:cell ratios (from 250:1 to 1000:1) as a model of urinary tract infection.

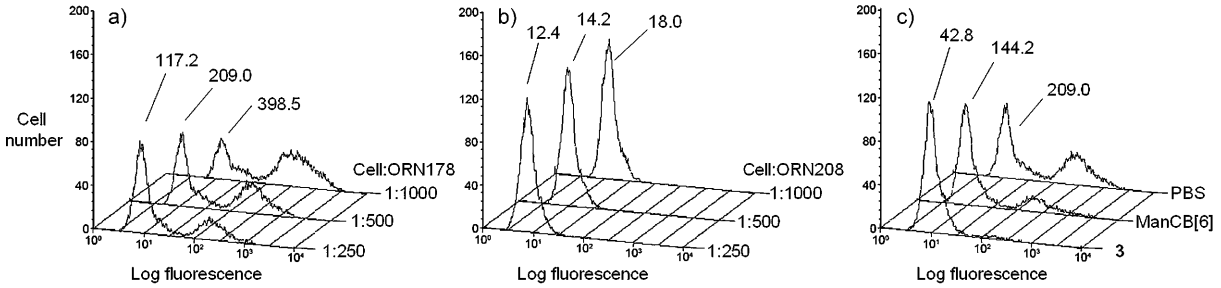


Figure 3. Flow cytometric analysis of binding of a) FITC-labeled ORN178 and b) FITC-labeled ORN208 to human urinary epithelial cells (UROtsa cells). c) Inhibition of FITC-labeled ORN178 attachment to UROtsa cells by **3** (0.10 μM, [ManCB[6]] = 0.30 μM), ManCB[6] (0.30 μM) and PBS as a control at a cell:bacteria ratio of 1:500. Values for mean channel fluorescence are given in each flow cytogram.

The fluorescence intensities of the bacteria-cell complexes were assayed by flow cytometry and the mean channel fluorescence was used as an indicator of the amount of FITC-labeled bacteria bound to the UROtsa cells. As shown in Figure 3a and 3b, the mean channel fluorescence of the UROtsa cells was directly proportional to the number of treated FITC-labeled ORN178, whereas the UROtsa cells treated with FITC-labeled ORN208 showed insignificant fluorescence. This result strongly suggested that the mannose binding protein FimH is involved in the attachment of ORN178 bacteria to UROtsa cells. We then studied the inhibition of bacterial attachment to UROtsa cell by incubating ORN178 bacteria with UROtsa cells in the presence of **3** or ManCB[6]. As illustrated in Figure 3c, **3** showed much higher inhibition activity for bacterial binding to the UROtsa cell than ManCB[6] wheel itself, demonstrating its potential as an anti-adhesion therapeutic agent.[18]

Conclusion

We have synthesized pseudopolyrotaxane-type carbohydrate clusters by threading CB[6]-based carbohydrate wheels on a PV polymer through host–guest interactions, and demonstrated their specific and multivalent interactions with *E. coli* ORN178. The number of carbohydrate wheels threaded on the "string" can be easily controlled by taking advantage of strong host–guest interaction between the wheels and string. Our study demonstrated that the mannose-pseudopolyrotaxanes **1**, **2**, and **3** are much more effective than the mannose wheel (ManCB[6]) in both bacterial aggregation and hemagglutination inhibition, and three ManCB[6] wheels threaded on a PV string is the most efficient combination for these purposes presumably by providing a proper density of the mannose wheel on the string to interact with bacteria. Furthermore, we demonstrated their inhibitory activities for the adhesion of antibiotic-resistant ORN178 bacteria to the urinary epithelial cells as a model of urinary tract infection, which suggests that such a supramolecular strategy for multivalent carbohydrate clusters may offer a viable approach to anti-adhesion therapy.

Experimental Section

General methods: All the reagents and solvents were used as supplied without further purification. Cucurbit[6]uril-based carbohydrate wheels (ManCB[6], GalCB[6], and GlcCB[6])[12] and polyviologen (PV)[10e] were synthesized according to the literature. Methyl-α-D-mannopyranoside (Me-αMan) and fluorescein isothiocyanate (FITC) were purchased from TCI and Sigma, respectively. NMR data were recorded on a DRX500 spectrometer (Bruker). UV/Vis absorption measurements were performed on a Hewlett–Packard 8453 diode array spectrophotometer. Flow cytometry was performed with a FACSCalibur (Becton Dickinson). UV absorption for MTT assay was measured on a Wallac Victor3 1420 multilable counter (Perkin–Elmer). Tetracycline-resistant *Escherichia coli* ORN 178 and ORN 208 strains were kindly donated by Prof. P. E. Orndorff (North Carolina State Univ.). The UROtsa cell line derived from the normal urothelium was kindly donated by Prof. S. H. Garrett (Univ. of North Dakota).

Preparation of glyco-pseudopolyrotaxanes: Glyco-pseudopolyrotaxane **1** was prepared by stirring a mixture of PV (0.50 mg, 0.10 μM) and ManCB[6] (4.0 mg, 1.0 μM) in water at 50 °C for 2 h. Glyco-pseudopolyrotaxanes **2** and **3** were prepared by the same procedure, except that 5.0 and 3.0 equivalents, respectively, of ManCB[6] were added to the solution. In addition, glyco-pseudopolyrotaxane **4** (3GalCB[6]@PV) and **5** (3GlcCB[6]@PV) were prepared by stirring a mixture of PV (0.50 mg, 0.10 μM) and GalCB[6] and GlcCB[6] (1.2 mg, 0.30 μM), respectively, in water at 50 °C for 1 h. The formation of the complexes was confirmed by ^{1}H NMR spectroscopy.[10e] The average number of threaded CB[6]-based carbohydrate wheels was calculated by integral ratio of the protons corresponding to the "uncomplexed" units (A and 3–5) and those of the "complexed" units (A′ and 3′–5′).

*Data for **1***: ^{1}H NMR (D_2O, 500 MHz, 298 K): $\delta = 9.35$ (m, 39H; A′), 9.04 (m, 4H), 8.70–8.40 (m, 44H), 6.02–5.40 (m, 148H), 5.32 (s, 109H), 4.58–4.16 (m, 118H), 4.08 (s, 109H), 3.96 (s, 108H), 3.91–3.23 (m, 682H), 3.23–2.45 (m, 220H), 2.45–1.97 (m, 222H), 1.97–1.62 (m, 40H), 1.62–1.24 (m, 14H), 1.24–0.56 (m, 79H; 3′ and 4′), 0.48 ppm (s, 39H; 5′).

*Data for **2***: ^{1}H NMR (D_2O, 500 MHz, 298 K): $\delta = 9.35$ (m, 19H; A′), 9.15 (m, 20H) 9.04 (m, 4H), 8.70–8.40 (m, 44H), 6.02–5.40 (m, 75H), 5.32 (s, 55H), 4.58–4.16 (m, 60H), 4.08 (s, 55H), 3.96 (s, 55H), 3.91–3.23 (m, 342H), 3.23–2.45 (m, 108H), 2.45–1.97 (m, 132H), 1.97–1.62 (m, 20H), 1.62–1.24 (m, 74H), 1.24–0.56 (m, 38H; 3′ and 4′), 0.48 ppm (s, 19H; 5′).

*Data for **3***: ^{1}H NMR (D_2O, 500 MHz, 298 K): $\delta = 9.35$ (m, 12H; A′), 9.15 (m, 28H) 9.04 (m, 4H), 8.70–8.40 (m, 44H), 6.02–5.40 (m, 45H), 5.32 (s, 33H), 4.58–4.16 (m, 36H), 4.08 (s, 33H), 3.96 (s, 33H), 3.91–3.23 (m, 206H), 3.23–2.45 (m, 66H), 2.45–1.97 (m, 96H), 1.97–1.62 (m, 12H), 1.62–1.24 (m, 98H), 1.24–0.56 (m, 22H; 3′ and 4′), 0.48 ppm (s, 11H; 5′).

*Data for **4***: ^{1}H NMR (D_2O, 500 MHz, 298 K): $\delta = 9.35$ (m, 11H; A′), 9.13 (m, 28H) 9.00 (m, 4H), 8.70–8.40 (m, 44H), 5.95–5.40 (m, 45H), 4.60–4.20 (m, 69H), 3.99 (s, 33H), 3.90–3.63 (m, 171H), 3.59 (m, 33H), 3.20–2.55 (m, 66H), 2.45–2.00 (m, 96H), 2.00–1.50 (m, 12H), 1.50–1.10 (m, 98H), 1.10–0.66 (m, 22H; 3′ and 4′), 0.48 ppm (s, 11H; 5′).

Data for **5**: ^{1}H NMR (D_2O, 500 MHz, 298 K): $\delta = 9.35$ (m, 11H; A′), 9.13 (m, 28H) 9.00 (m, 4H), 8.70–8.40 (m, 44H), 5.95–5.40 (m, 45H), 4.60–4.16 (m, 69H), 3.90–3.60 (m, 138H), 3.60–3.20 (m, 132H), 3.20–2.55 (m, 66H), 2.45–2.00 (m, 96H), 2.00–1.50 (m, 12H), 1.50–1.05 (m, 98H), 1.10–0.66 (m, 23H; 3′ and 4′), 0.48 ppm (s, 12H; 5′).

Bacterial aggregation test: *E. coli* strains (ORN178 and ORN208) were grown at 37°C in Luria-Bertani (LB) medium (5 mL) containing tetracycline (10 μg mL^{-1}). After 1 day inoculation, strains were recovered by centrifugation at 4000 rpm for 30 min and resuspended in phosphate-buffered saline (PBS, pH 7.3) by gentle pipetting. The bacterial concentrations (2×10^8 CFU mL^{-1}) were adjusted to an optical density (OD) at 600 nm of 1.0. Serially diluted compounds (**1–5** and ManCB[6]; 20 μL) were individually added to the bacterial solutions (1 mL). After 30 min incubation at RT, OD_{600} values of the supernatant solutions were measured. The percent bacterial aggregation (%) was calculated by the difference in OD_{600} values of the supernatant of the bacterial solution before and after the treatment with the compounds. A half-maximal concentration for aggregation (AC_{50}) was used to compare the bacterial aggregation efficiency of the glyco-pseudopolyrotaxanes (**1**–**3**).

Preparation of a 3% guinea pig erythrocyte solution: Guinea pig blood was freshly isolated and stabilized in a 10% citrate–dextrose solution. After centrifugation (15 min, 3000 rpm, 4°C), the erythrocyte sediment was carefully suspended in phosphate buffered saline (PBS, pH 7.3), and settled again by centrifugation as above. This washing procedure was repeated twice. To make a 3% erythrocyte solution, the erythrocytes (3 mL) were suspended in PBS solution (97 mL) and stored at 4°C.

Hemagglutination inhibition (HAI) assay:[19] The hemagglutinating activities of ORN178 and ORN208 were examined by using a 3% guinea pig erythrocyte suspension. Twofold serially diluted solutions (50 μL) of ORN178 or ORN208 were treated in wells of a V-shaped microtiter plate, followed by the addition of erythrocyte suspension (50 μL) and incubation for 1 h at 4°C. The hemagglutination was visualized as a diffuse, feathery appearance on the slope of the well, and the minimum concentration required for hemagglutination (MHC) was determined with the naked eye. The MHC value for ORN178 was 0.5×10^8 CFU mL^{-1} ($OD_{600}=0.25$), whereas ORN208 did not agglutinate erythrocytes at all concentrations. The inhibitory activities for hemagglutination of ManCB[6], **1**, **2**, and **3** and their corresponding sugar (Me-αMan) were examined using a ORN178 strain. A solution of ORN178 (1.0×10^8 CFU mL^{-1}, $OD_{600}=0.5$) was used for the HAI assay. Two-fold serially diluted solutions (25 μL) of compounds (ManCB[6], **1**, **2**, and **3**) and their corresponding sugar (Me-αMan) were prepared, and added to ORN178 solutions (25 μL), and the mixtures were incubated for 1 h at 4°C. The erythrocyte suspension (50 μL) was added to each compound-ORN178 mixture and further incubated for 1 h at 4°C. When the hemagglutination is inhibited by carbohydrate-bearing compounds, the erythrocytes form a distinct button resulting from the settlement of deagglutinated erythrocytes to the bottom of the well. The minimum concentrations of the compounds required to inhibit erythrocyte agglutination (MIC) were determined with the naked eye.

Preparation of FITC-labeled bacteria:[20] *E. coli* strains (ORN 178 or ORN 208) were grown at 37°C in Luria-Bertani (LB) medium (5 mL) containing tetracycline (10 μg mL^{-1}). After 1 day inoculation, strains were recovered by centrifugation at 4000 rpm for 30 min and resuspended in 0.15 M NaCl/0.1 M sodium carbonate solution (pH 9.0; 1 mL) by gentle pipetting. A solution of FITC (1 mg mL^{-1}; 5 mL), freshly prepared in the buffer solution, was added to the bacterial suspension, which was then incubated for 1 hr at 4°C in the dark. The bacteria were recovered by centrifugation at 4000 rpm for 15 min, resuspended by gentle pipetting in phosphate-buffered saline (PBS; 20 mL) containing 0.05% of Tween 20, and pelleted again by centrifugation as above. This washing procedure was repeated until the fluorescence of supernatant was not detected, and then the labeled bacteria were resuspended to a final concentration of 5×10^8 CFU mL^{-1}, which was adjusted by OD_{600} value. The solutions of the labeled bacteria were utilized immediately or stored at −20°C until further use.

In vitro anti-adhesion test:[21] UROtsa cells were cultured in Dulbecco's Modified Eagle Media (DMEM, low glucose) supplemented with glucose (1 mg mL^{-1}), heat-inactivated fetal bovine serum (FBS; 5%) and penicillin/streptomycin (PS; 1%). Cells were grown at 37°C in humidified air containing 5% CO_2. UROtsa cells were seeded on a 6-well plate (1×10^6 cells per well) and cultured for 24 h. After washing out the culture media, the FITC-labeled bacteria were added to the UROtsa cells with bacteria:cell ratios of 1000:1 to 250:1. After 1 h incubation, unbound bacteria were removed by gentle pipetting with PBS and the remaining bacteria-cells complexes were collected by trypsin treatment and centrifugation (1000 rpm, 3 min). After resuspending with PBS, the samples were assayed by flow cytometry. Mean channel fluorescence was used as an indicator of the amount of FITC-labeled bacteria bound to UROtsa cells. For the inhibition of bacterial attachment by carbohydrate clusters, FITC-labeled ORN178 (5×10^8 CFU mL^{-1}) was incubated with **3** (0.10 μM), ManCB[6] (0.30 μM) or PBS alone for 30 min. The mixtures were added on the UROtsa cells (1×10^6 cells per well) and incubated further for 1 h at 37°C. After removing the unbound bacteria by gentle pipetting with PBS, the remaining bacteria-cells complexes were recovered by trypsinization and centrifugation (1000 rpm, 3 min). After resuspending with PBS, the samples were assayed by flow cytometry.

In vitro cytotoxicity against UROtsa cells: UROtsa cells were seeded in a 96-well plate at a density of 1×10^4 cells per well in DMEM (200 μL, low glucose) containing glucose (1 mg mL^{-1}), FBS (5%) and PS (1%) and incubated in a humidified CO_2 (5%) atmosphere at 37°C for 24 h. The cell culture medium was replaced with a fresh one (200 μL) containing **1**, **2**, **3**, PV and ManCB[6] (20 μL). The cells were incubated for 2 d at 37°C. Subsequently, the cells were incubated with fresh media (200 μL) containing methylthiazolyldiphenyltetrazolium bromide (MTT, 20 μL, 5 mg mL^{-1}) for an additional 4 h at 37°C, and then the medium was gently removed. The purple, water insoluble crystals formed by live cells remaining at the bottom of the wells were dissolved with DMSO (200 μL) and the solution was gently shaken for 10 min. UV absorption of the solution at 590 nm was measured by a multi-well plate reader.

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