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Synthesis and Assessment of Globotriose–Chitosan Conjugate, a Novel Inhibitor of Shiga Toxins Produced by *Escherichia coli*

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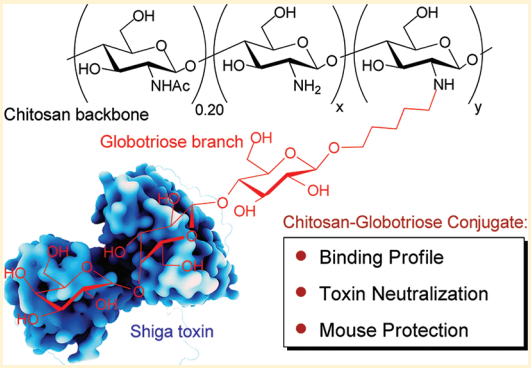
Supporting Information

ABSTRACT: Shiga toxin (Stx)-producing *Escherichia coli* (STEC) causes diarrhea and colitis in humans that can develop into a life-threatening hemolytic uremic syndrome (HUS). Developing efficient means of controlling STEC diseases, for which no drugs or vaccines are currently available, remains a high priority. We report here the construction and development of chitosan conjugates bearing the Stx ligand trisaccharide globotriose to demonstrate their potential as STEC disease treatment agents. The synthesis was accomplished by grafting a globotriose derivative containing an aldehyde-functionalized aglycone to chitosan amino groups. The obtained globotriose–chitosan conjugate bound with high affinity to Stx and efficiently neutralized its toxicity on Vero cells. Moreover, Stx levels in the gut of infected mice receiving oral doses of the conjugate were greatly diminished, enabling the mice to resist a fatal STEC challenge. The conjugate appears to function as a Stx adsorbent in the gut, preventing toxin entry into the bloodstream and consequent development of HUS. As such, the conjugate could act as a novel agent against STEC disease.



INTRODUCTION

Protein–carbohydrate interactions are widespread in living systems and critical in many biological processes such as cellular recognition, immune response, and infection. Glycoligands targeting biologically significant proteins have huge potential for disease diagnosis, prevention, and treatment,[1] but carbohydrate-based drug discovery is currently limited by the low-affinity of interactions between the biomolecules (with dissociation constant (K_d) usually in the millimolar range[2]). Binding strength may be improved by exploiting multivalency, in which multivalent glycoligands bind simultaneously to the proteins of interest.[3] Coupling many monomeric ligands or drugs onto appropriate scaffolds such as polymers can create multivalent systems that achieve not only higher activity but also superior stability and lowered toxicity.[4] The natural polysaccharide chitosan presents, from a pharmacological viewpoint, an ideal scaffold molecule because of its high biocompatibility, biodegradability, and antibacterial activity.[5−8] Combining glycoligands with chitosan exhibiting diverse bioactivities could generate safe and practical agents for pharmaceutical and medical use. We have previously developed a set of chitosan conjugates displaying specific carbohydrate sequences on their backbones that appear to antagonize influenza virus hemagglutinin, *Streptococcus suis* adhesins, and E-selectin.[9−11] Here we present the synthesis and structural characterization of a new member of this group bearing trisaccharide globotriose (globotriose–chitosan conjugate (GC **1**), Chart 1). We also demonstrate the potential of the new conjugate to neutralize Shiga toxin (Stx) and to treat Stx-producing *Escherichia coli* (STEC) infection.

STEC, including strain O157:H7, causes diarrhea and colitis in humans that may develop into a life-threatening hemolytic uremic syndrome (HUS), with a mortality rate of 3–5% in children.[12,13] Frequent global outbreaks of STEC infections (over 70 000 cases annually in the United States alone[14]) present a significant threat to public health. Developing efficient means of controlling such infections and associated diseases remains a high priority, as vaccines and drugs against STEC are currently unavailable. Treatment with antibiotics has proven ineffective and in fact encourages HUS development by causing the release of Stx from injured bacteria in the intestine of patients, making the toxin more available for absorption.[15] Stx, the toxic determinant of STEC, is released by the bacteria into the gut lumen, binds to a glycoligand present on the intestinal epithelial cells, and subsequently invades the blood circulation,

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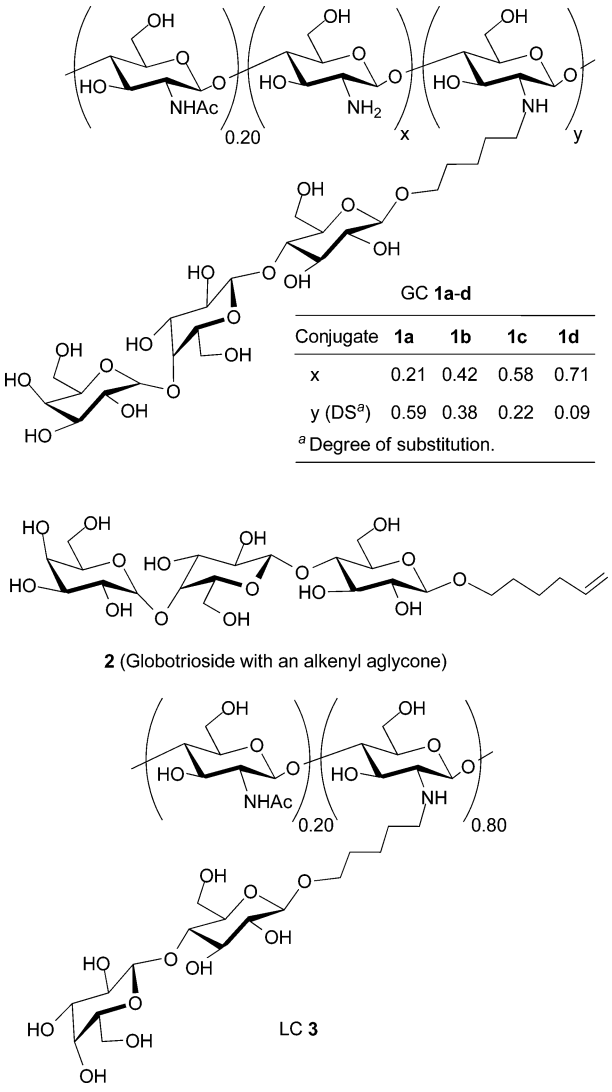
where it targets specific tissues displaying the same sugar entity. Two major antigenic variants of Stx, Stx1 and Stx2, share the typical structure of AB_5 bacterial toxin, comprising a single enzymatic A-subunit and five identical ligand-binding B-subunits (StxB). The B-subunits bind to a cell surface glycan, globotriose [Galα(1,4)Galβ(1,4)Glc], to ferry the holotoxin into the cell. Subsequently, the A-subunit, which functions as an RNA *N*-glycosidase, shuts down eukaryotic protein synthesis.[12,16]

The Stx−globotriose interaction presents an attractive target for antibacterial drug design. Indeed, throughout the past decade, significant effort has been devoted to preparing multivalent globotriose mimics that will neutralize toxins.[17−37] For example, a decameric molecule comprising a glucose core linked by five radially symmetrical arms, with pairs of globotriose at the tips (designated STARFISH by its developers), was able to inhibit Stx-binding at nanomolar concentrations,[17] although the drug conferred no in vivo protection in STEC-challenged mice.[18] Similarly, Synsorb-Pk, a conjugate of globotriose chemically coupled to colloidal silica, displayed promising inhibitory effect against Stx when tested in vitro on human renal adenocarcinoma cells, but clinical trials have failed to establish whether the drug can protect children against Stx-associated HUS.[19,20] In other prominent studies from a same research group, a dendrimer of carbosilane scaffold linked to six globotriose termini (designated SUPER TWIG), and a linear polymer of acrylamide bearing the same sugar branches (termed Gb_3 polymers) were shown to bind Stx with 100000-fold greater affinities than Synsorb-Pk and offered strong protection to mice infected with a fatal STEC strain.[21,22] In addition to the synthetic agents, engineered bacteria displaying globotriose mimics on their surface have also been demonstrated as excellent Stx neutralizers, offering complete protection against lethal toxin doses in mice.[23,24] Extending our work on the development of chitosan-based anti-infective agents,[9,10] we have undertaken the synthesis and bioevaluation of GC **1** as a new class of molecule that targets the Stx−globotriose interaction. Chitosan has been widely employed as the carrier of proteins, nucleic acids, and drugs, especially in the medical and pharmaceutical fields.[5−8] Here we describe a novel chitosan-based agent for use in the prevention and control of STEC disease.

Chart 1. Structures of Globotriose−Chitosan Conjugate (GC 1), Globotrioside 2, and Lactose−Chitosan Conjugate (LC 3)



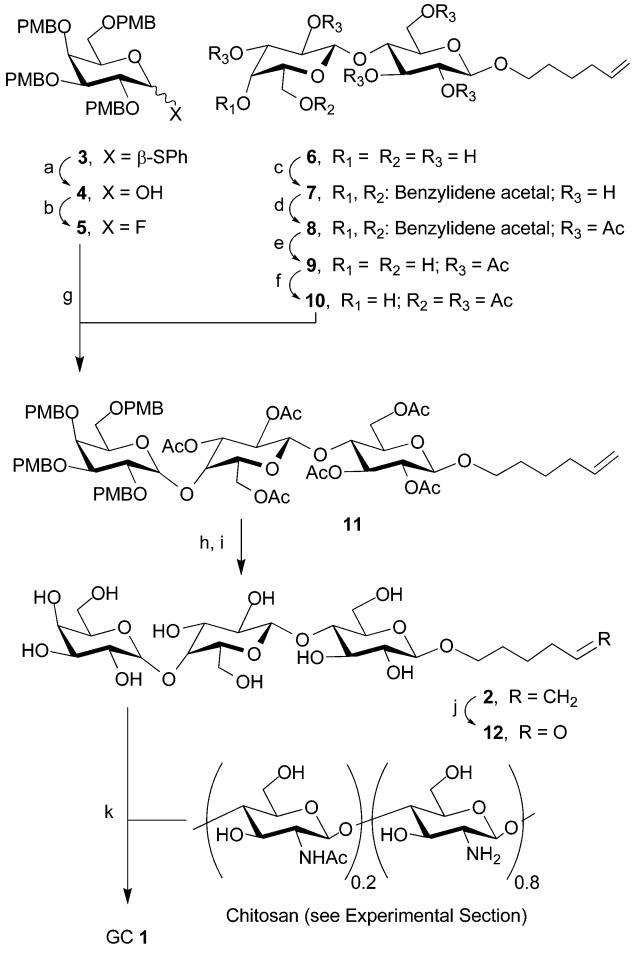
Conjugate	1a	1b	1c	1d
x	0.21	0.42	0.58	0.71
y (DS[a])	0.59	0.38	0.22	0.09

[a] Degree of substitution.

RESULTS AND DISCUSSION

Chemistry. The synthesis commenced with assembly of globotrioside **2** (Chart 1, Scheme 1) bearing an alkenyl spacer that can be easily transformed into an aldehyde group for later conjugation with chitosan amino groups. The challenge in synthesizing globotrioside **2** is to construct the α-linkage between galactose and the lactoside bearing the spacer. Although this can be achieved using a galactosyl donor containing a nonparticipating benzyl group at O-2,[38−41] the general deprotection procedure for benzyl ethers using hydrogenolysis would destroy the C−C double bond in the spacer. Debenzylation by sodium in liquid ammonia is an acceptable alternative;[42] however, because of the dangerous and explosive reaction conditions, we here employed *p*-methoxybenzyl (PMB), a close relative of benzyl, which can be readily cleaved under mild acidic conditions in the presence of weak oxidants such as ceric ammonium nitrate (CAN),[43] as the protecting group. Thus, a glycosyl donor **5** protected with PMB (prepared from thioglycoside **3**,[44] Scheme 1) was coupled with acceptor **10** (obtained from the 5-hexenyl lactoside **6**[45] by sequential manipulations of the protecting groups). The glycosylation of **5** with **10** proceeded with high stereoselectivity in the presence of AgOTf and $SnCl_2$,[46] yielding exclusively the α-linked compound **2** after two steps of the deprotection procedures. Aldehyde **12**, produced by ozonolysis of the C−C double bond of **2**, was directly coupled to chitosan without further purification, as TLC indicated the reaction proceeded quantitatively. Reductive N-alkylation of chitosan with aldehyde **12** afforded GCs **1a−d** in 88−93% yield in an aqueous AcOH solution in the presence of $NaCNBH_3$. The degree of substitution (DS) of the globotriose branch in GC **1** was experimentally adjusted by the molar ratio of aldehyde **12** to the amino group of chitosan. The maximum DS of 59% (GC **1a**) was obtained at 2 equiv of **12** to the amino group. More excessive use of aldehyde **12** was ineffective at further increasing the DS, probably because of the increased steric hindrance of the conjugate (Table S1, Supporting Information). As a negative control of the bioevaluation of GC **1**, a lactose-branched chitosan conjugate (LC **3**, Chart 1) was similarly synthesized by coupling an aldehyde from lactoside **6** to chitosan. Unlike GC **1a** (DS = 59%), LC **3** was fully N-substituted

Scheme 1. Synthesis of GC 1[a]



[a]Reagents and conditions: (a) NBS, acetone/H_2O (10:1); (b) DAST, THF, 93% overall yield (steps a and b); (c) $PhCH(OMe)_2$, *p*-TsOH, DMF, 53%; (d) Ac_2O, DMAP, pyridine; (e) TFA (aq wt 90% solution), CH_2Cl_2, 70% overall yield (steps d and e); (f) AcCl, pyridine, 84%; (g) AgOTf, $SnCl_2$, 4 Å molecular sieves, Et_2O/CH_2Cl_2 (2:1), 95% from compound **10**; (h) CAN, $MeCN/H_2O$ (9:1); (i) NaOMe/MeOH (0.05 M), 72% overall yield (steps h and i); (j) O_3, Me_2S, MeOH, quantitatively; (k) $NaBH_3CN$, AcOH (aq wt 5% solution), 88–93% for GCs **1a–d**.

(DS of lactose branch of 80%), probably because the aldehyde formed from **6** is considerably smaller than aldehyde **12**. In contrast to water-insoluble chitosan, both GCs **1a–d** and LC **3** demonstrated excellent aqueous solubility (≥1.0 mg/mL, pH 7.2) owing to the incorporated hydrophilic sugar branches.

GC **1** and LC **3** were characterized by ^{1}H NMR spectroscopy, CHN elemental analysis, and gel permeation chromatography (GPC) analysis. The structural compositions were verified by ^{1}H NMR identification of several baseline-separated signals of globotriose or lactose moiety, alkyl linker, and acetyl group of chitosan backbone, as illustrated in Figure 1. The DS values, determined by integral ratio of the branch-derived peaks to chitosan Ac peaks, closely matched those obtained from elemental analysis. Moreover, the successive increase in molecular weight (M_w) of chitosan and GCs **1d–a** (measured by GPC equipped with a laser light scattering (LLS) detector) further verified that the sugar units had been properly incorporated (Supporting Information). However, an attempt at further characterization by ^{13}C NMR spectroscopy failed because

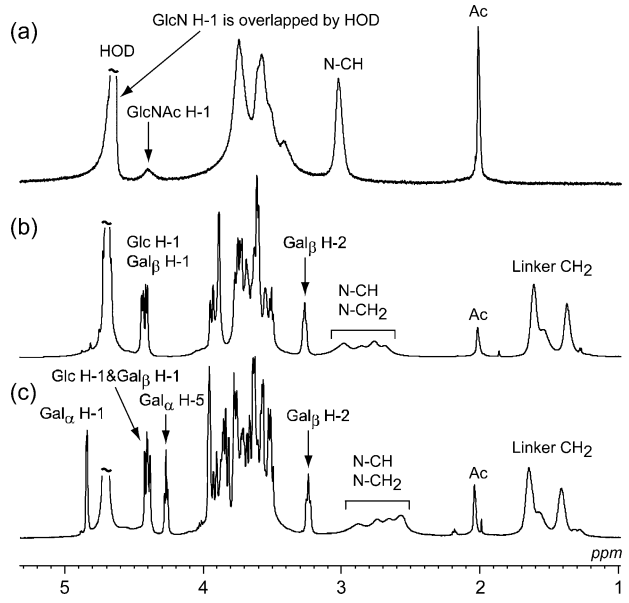


Figure 1. ^{1}H NMR spectra (600 MHz, 27 °C) of (a) chitosan (DCl/D_2O), (b) LC **3** (D_2O), and (c) GC **1a** (D_2O). Abbreviations are as follows: Gal_α, *α*-linked Gal in **1a**; Gal_β, *β*-linked Gal in **1a** or **3**.

of the rigid chitosan backbone[47] and increased macromolecular size of the conjugates.

Bindings with StxB. To establish the binding activity of the globotriose tethered to chitosan scaffold, interactions between GC **1** and Stx1B or Stx2B were analyzed by a BIAcore biosensor system based on the surface plasmon resonance (SPR) technique.[48] The recombinant histidine-tagged Stx1B or Stx2B was covalently immobilized onto an Ni^{2+} activated sensor chip.[49] The buffer containing various concentrations of GCs **1a–d** was poured over the sensor chip surface, and the binding affinity (K_d) of the conjugates was determined. As illustrated in Figure 2, GCs **1a** and **1b**, with higher DS values than **1c** and **1d**, bind significantly to both Stx1B and Stx2B, demonstrating that binding activity is highly DS-dependent. This phenomenon is due to the multivalency effect (multivalent glycosystems containing high densities of sugar moiety frequently display increased binding to their target proteins[50−53]). The affinities of GC **1a** to both toxin subunits (Table 1, K_d of 0.05 and 0.43 μM for Stx1B and Stx2B, respectively) are comparable to those reported for Gb_3 polymers (K_d of 0.34 and 0.68 μM for Stx1B and Stx2B, respectively[22]). In contrast, no activities were detected for LC **3**, verifying that the globotriose branches of GC **1** are responsible for the observed specific bindings. Low affinity interactions between Stx and a methyl globotrioside (K_d in the millimolar range) have been reported.[54] Here, as evidenced by the SPR study, the monomeric compound **2** does not noticeably bind at 162 μM or less. The high attraction of GC **1a** and **1b** to both toxin B-subunits (K_d = 0.05−1.41 μM) demonstrates that multivalent presentation of globotriose ligands on a chitosan backbone encourages strong binding. However, differences in the binding preference of GC **1** to Stx1B and Stx2B were observed; **1c** and **1d** bound Stx1B more effectively than Stx2B. This affinity variance is reflected in the K_d values; the K_d values of GCs **1a–d** for Stx1B were of similar order of magnitude (0.05−0.12 μM), whereas those for Stx2B increased markedly as DS decreased (0.43−12.55 μM). Previous reports have demonstrated that Stx1 has greater preference for globotriose than does Stx2.[55,56] Consistent with

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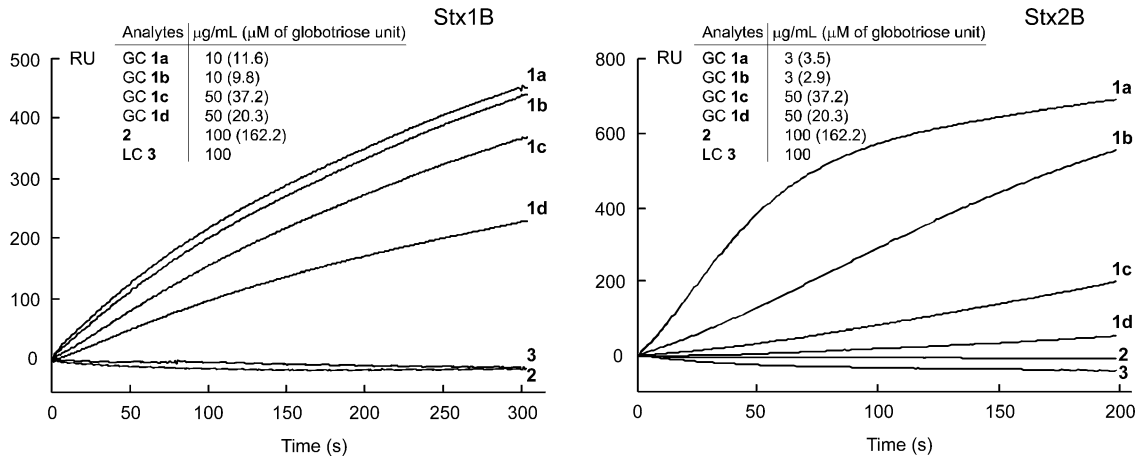

Figure 2. Representative sensorgrams for binding of GC **1** to Stx B-subunits (Stx1B and Stx2B) analyzed by a BIAcore biosensor system. The Stx1B or Stx2B with a histidine tag was covalently immobilized onto an Ni^{2+} activated sensor chip. The analyte (GC **1**, compound **2**, or LC **3**) in running buffer at the specified concentration was poured over the sensor chip surface. Data are expressed as resonance unit (RU) from the time at which the analyte was introduced (0 s) to time at which the binding plateau was reached.

Table 1. Binding Affinity of GC 1 to Stx B-Subunits (Stx1B and Stx2B) and Neutralizing Activity of GC 1 against Stx-Induced Cytotoxicity in Vero Cells

		K_d (μM)[a]		IC$_{50}$ (μg/mL)[b]	
sample	DS (%)	Stx1B	Stx2B	Stx1	Stx2
GC **1a**	59	0.05	0.43	0.02 ± 0.002 (0.14 μM)	0.09 ± 0.01 (0.10 μM)
GC **1b**	38	0.07	1.41	0.23 ± 0.05 (0.23 μM)	2.58 ± 0.8 (2.53 μM)
GC **1c**	22	0.10	7.89	0.89 ± 0.09 (0.66 μM)	ND[c]
GC **1d**	9	0.12	12.55	ND[c]	ND[c]

[a] K_d was obtained by SPR analysis. [b] IC$_{50}$ was calculated as the mean of data obtained from at least three individual cytotoxicity assays: μg/mL of sample ± standard deviation (μM globotriose moiety). [c] Not determined.

these results, sufficiently high densities of globotriose unit in GC **1** were required for effective binding to Stx2 in our present study.

Neutralizations of Stx-Induced Cytotoxicity. We next performed in vitro cytotoxicity assays to determine the extent to which GC **1** can protect host cells from lethal doses of Stx. As shown in Figure 3, Stx-sensitive Vero cells[32] incubated with GC **1** were effectively protected against the lethal effects of Stx1 or Stx2. Consistent with the binding results of the SPR study, neutralization of both toxins by GC **1** depended heavily on the DS values. GC **1a** was the most potent cytotoxin inhibitor among the tested samples, suggesting that effective neutralization is related to the high-affinity binding of GC **1a** to the toxin B-subunits. Although the inhibitory potency of GC **1a** against Stx1 (IC$_{50}$ = 0.14 μM; Table 1) is lower than that of a previously reported Stx neutralizer, Gb_3 polymer (IC$_{50}$ = 0.05 μM[22]), GC **1a** is approximately 8-fold more inhibitory against Stx2 (IC$_{50}$ = 0.10 μM) than the Gb_3 polymer (IC$_{50}$ = 0.82 μM). The superiority of GC **1a** in neutralizing Stx2 also becomes apparent in comparisons with other potent antagonists such as STARFISH (IC$_{50}$ = 1.58 μM[17]) and SUPER TWIG (IC$_{50}$ = 0.17 μM[21]). Such potency probably arises from the macromolecular size of the chitosan backbone (5000 KDa) and the

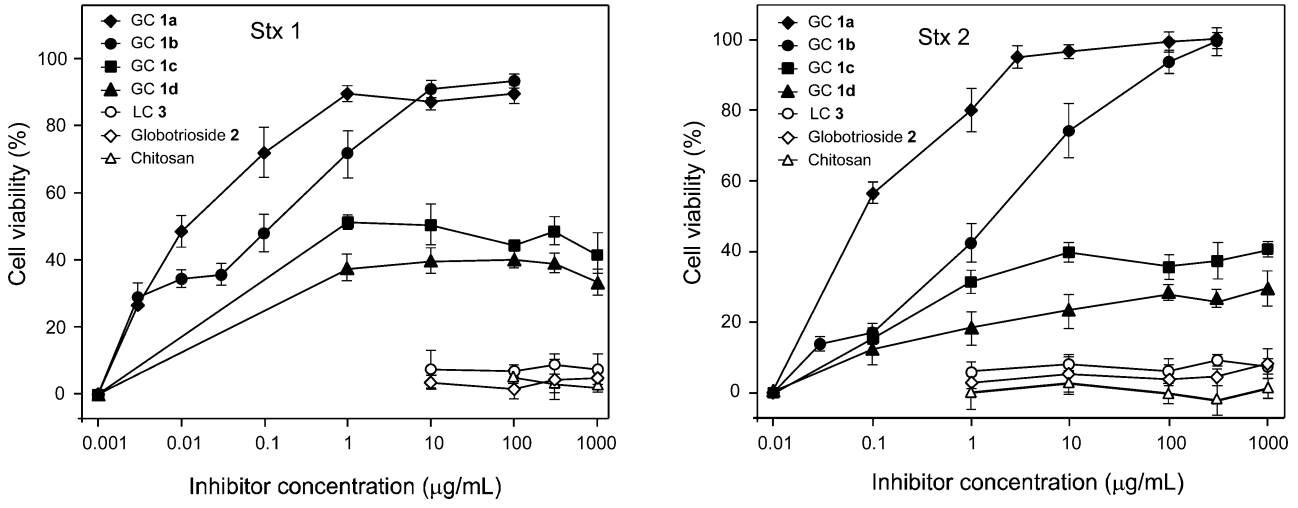

Figure 3. Neutralization of Stx-induced cytotoxicity. Vero cells were incubated with the samples (GC **1**, LC **3**, globotrioside **2**, or chitosan) in the presence of lethal doses of Stx1 or Stx2. Cell viability is expressed relative to that in a toxin-free environment. All experiments were repeated at least three times, and data are presented as the mean ± standard deviation.

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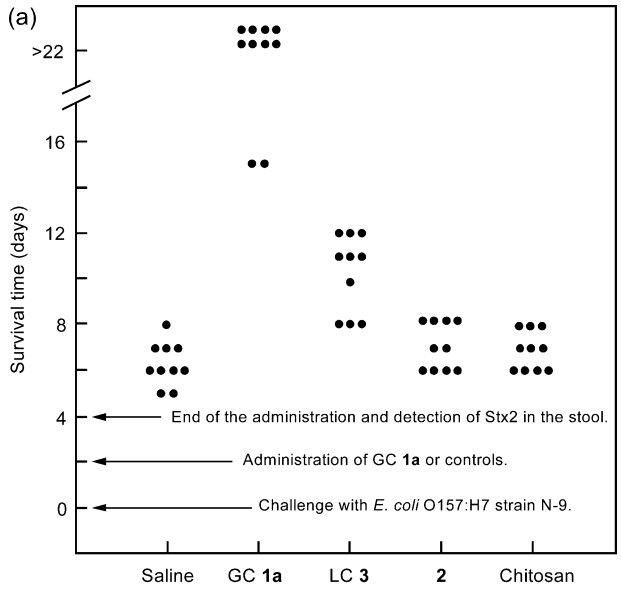
highly branched globotriose units. Efficient neutralization of Stx2 is particularly important in clinical practice, as this variant is more toxic than Stx1 and more relevant to HUS development.[57,58] In this context, GC **1a** is a highly promising STEC disease treatment agent. In addition, exposing Vero cells to the negative controls (chitosan, globotrioside **2**, or LC **3**) or to GC **1c** or **1d** (with considerably lower DS values) resulted in no effective neutralizations of any of the toxins. None of the treatments affected cell viability in the absence of the toxins (data not shown).

In Vivo Protection of Mice against Challenge with STEC. Encouraged by the results from the binding studies and cytotoxicity assays, we proceeded to assess the in vivo effect of GC **1a**, the most promising candidate among the samples. Mice with protein calorie malnutrition (PCM) that are susceptible to infection by O157:H7 *E. coli* and subsequent development of HUS were challenged with strain N-9, a bacterial isolate producing both Stx1 and Stx2,[59] on day 0. Infection was confirmed in the early morning of day 2 by enzyme-linked immunosorbent assay (ELISA), which detected significant amounts of Stx2 in the fecal samples. GC **1a** and the controls (saline, LC **3**, globotrioside **2**, and chitosan) were intragastrically administered twice daily for 3 consecutive days (days 2−4), and the survival times were monitored (Figure 4a). All mice in the control groups died within 12 days from toxic neurological damage; however, 8 of the 10 mice treated with GC **1a** remained alive and healthy after 22 days ($P < 0.01$), demonstrating clearly that GC **1a** can protect mice against fatal challenge with STEC (none of the treatments affected the survival times of germ-free mice, data not shown). Moreover, Stx2 was reduced below the detectable level (<12 pg/mg, Figure 4b) in stool samples of the survivors on day 4, whereas the stools of control groups continued to harbor high levels of the toxin (>37 pg/mg). These results indicate that the toxin had been quickly and efficiently eliminated from the gut by excretion of the toxin−GC **1a** binding complex early in the treatment (toxin quantification by ELISA was not affected by GC **1a** presence in the stools, data not shown). In previous reports, Synsorb-Pk conferred no protection against Stx2 in mice infected with an O91:H21 *E. coli* strain.[32] STARFISH protected mice from lethal doses of Stx1 but not Stx2,[18] whereas Gb_3 polymers and SUPER TWIG ensured the survival of mice inoculated with an O157:H7 strain producing both Stx1 and Stx2.[21,22] Here we have engineered an additional compound, GC **1a**, which actively inhibits Stx2 in vivo. The therapeutic potential of GC **1a** in the treatment of STEC disease is highlighted by the clinical importance of Stx2 over Stx1. Interestingly, PCM mice treated with LC **3** survived moderately longer than mice inoculated with the other controls (median survival time of approximately 10 days following LC **3** treatment versus 6 days for other controls). Miyagawa and colleagues have recently reported that a multivalent conjugate of lactose attached to a polyacrylamide backbone neutralizes Stx1 with very low activity, presumably via specific binding between the lactose unit (the internal part of globotriose) and the toxin.[28] Although a similar result was not obtained from our SPR and cytotoxicity assays, LC **3** most likely adsorbed the toxins in the gut via a weak lactose−toxin interaction, conferring weak protection by partially preventing the entry of toxins into the blood circulation.



(b) Stx2 content in stool of the mice on day 4:

Treatment	Stx2 ± SE (pg/mg of fecal pellets)[a]
Saline	50.9 ± 5.3
GC **1a**	< 12[b]
LC **3**	46.2 ± 6.2
Globotrioside **2**	37.5 ± 6.8
Chitosan	47.3 ± 4.4

[a] Fecal samples of 3 mice in each group were examined by ELISA respectively. All the detections were repeated at least 3 times and the means were calculated. SE represents standard deviation. [b] The limit of detection was tested to be 12 pg/mg.

Figure 4. Protection of mice against lethal challenge with *E. coli* O157:H7. The mice were challenged with the bacterial strain N-9 on day 0. The presence of infection was established by the detection of Stx2 in the stool on the early morning of day 2 (quantified by ELISA). Subsequently the mice orally received GC **1a** or control samples (saline, LC **3**, globotrioside **2**, or chitosan) twice daily for 3 consecutive days (days 2−4). (a) Survival time of each group of 10 mice is shown. (b) Stx 2 content in the stool on day 4, quantified by ELISA.

CONCLUSIONS

We have constructed a new class of Stx neutralizers, designated GC **1**, and have demonstrated their therapeutic potential in the treatment of STEC disease for which no drugs are currently available. We emphasize that GC **1a**, carrying the highest density of globotriose moiety among our developed compounds, effectively inhibits not only Stx1 but also the more clinically significant Stx2 both in vitro and in vivo. This finding is of particular importance to clinical practice, especially since the well-studied Stx neutralizers, FISHSTAR and Synsorb-Pk, have not shown activities against Stx2 in vivo. Additionally, chitosan backbone as the carrier of biological components could provide therapeutic advantage in terms of safety and efficacy, as has been illustrated by delivering DNA or drugs such as insulin and famotidine.[7] In particular, chitosan is recognized as an effective dietary supplement[60] and has been approved as a food additive in many countries, including Japan and Korea.[61] The established safety of chitosan further enhances

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the potential for GC **1a** as an STEC treatment agent, acting via removal of free Stx from the gut of patients.

EXPERIMENTAL SECTION

Materials. Chitosan was obtained from Yaizu Suisankagaku Industry Co., Ltd. The M_w and polydispersity index (PDI = M_w/M_n) were 5000 kDa and 1.8, respectively, as measured by a GPC system equipped with a LLS detector. The deacetylation degree was 80% (80% GlcN, 20% GlcNAc), as determined by the ^{1}H NMR spectrum. Compounds **3** and **6** were synthesized by methods described previously.[44,45] Stx1 and Stx2 were prepared from *Escherichia coli* O157:H7 as described by Takeda et al.[62,63] The purity of the toxins was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) and amino acid analysis (data not shown). Recombinant C-terminal histidine-tagged Stx1B and Stx2B were prepared from *E. coli* BL21(DE3) according to the published procedures.[22] Vero cells were maintained in DMEM supplemented with 10% fetal calf serum and seeded in 96-well plastic microplates for cytotoxicity assay. The O157:H7 *E. coli* N-9 strain is a clinical isolate from patients with development of HUS. The specific pathogen free C57BL/6 mice (female and 3-week-old) were used in the protection experiment. All chemicals and solvents were of regent grade and used as supplied.

Measurements. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX 600 spectrometer at 600 and 150 MHz, respectively. FAB and MALDI-TOF mass spectra were obtained with a JEOL JMS-HX 110 and a Bruker BIFLEX III mass spectrometer, respectively, using *m*-nitrobenzyl alcohol (NBA) or 2,5-dihydroxybenzoic acid as the matrix. Elemental analysis was performed with a Yanaco CHN corder MT-6. The M_w and PDI of chitosan and the conjugates were measured by a GPC system (CTO-20A, Shimadzu Co., Ltd.) equipped with a multiangle LLS detector (DAWN HELEOS-II, Wyatt Technology Corp.) in acetate buffer solutions (pH 4.5). The purity of conjugates (>95%) was established by the combined analyses of GPC, ^{1}H NMR spectroscopy, and CHN elemental analysis. TLC was performed on the Merck silica gel glass plates 60F_{254}. Optical rotation was measured on a Perkin-Elmer polarimeter 343. Medium pressure liquid chromatography (MPLC) was carried out using a Yamazen 700E pump and a Yamazen model UV-10V detector with columns packed with Merck silica gel N60 (40−63 mm). SPR analysis was performed by a BIAcore biosensor system (BIAcore 3000, BIAcore Co., Ltd.).

Synthesis of 2,3,4,6-Tetra-*O*-(*p*-methoxybenzyl)-D-galactopyranosyl Fluoride (5). To a solution of phenyl 2,3,4,6-tetra-*O*-(*p*-methoxybenzyl)-1-thio-*β*-D-galactopyranoside (**3**, 6.0 g, 7.97 mmol) in acetone/water (10:1, 130 mL) at 0 °C was added *N*-bromosuccinimide (NBS, 5.7 g, 32.03 mmol). The mixture was allowed to return to room temperature, stirred for 1 h, diluted with ethyl acetate, washed successively with a 10% aqueous solution of potassium carbonate, water, and brine, and then dried ($MgSO_4$). The solvent was evaporated and the resultant syrup was purified by MPLC (1:1 → 4:1 ethyl acetate/*n*-hexane) to give compound **4** as a white solid, which was directly dissolved in dry tetrahydrofuran (THF, 50 mL). The solution was cooled to −20 °C, and (diethylamino)sulfur trifluoride (DAST, 1.2 mL, 9.36 mmol) was added. The mixture was allowed to return to room temperature, stirred for 30 min, and then cooled again to −20 °C. The reaction was quenched by methanol, and the solvent was evaporated to give a residue, which was purified by MPLC (1:2 ethyl acetate/*n*-hexane) to afford a light yellow syrup of fluoride **5** (4.9 g) in 93% yield. ^{1}H NMR ($CDCl_3$): *δ* 7.33−6.87 (24H, m, aromatic protons), 5.54 (0.5H, dd, *J* = 2.6 and 53.7 Hz, *α*H-1), 5.15 (1H, dd, *J* = 7.0 and 53.1 Hz, *β*H-1), 4.88−4.35 (12H, m, PhC$\underline{H}_2$), 4.09−3.86 (6H, m, C$\underline{H}$O of sugar ring), 3.84−3.81 (18H, m, PhOC$\underline{H}_3$), 3.50 (3H, m, H-6). MALDI-TOF MS (*m*/*z*) calcd for $C_{38}H_{43}FO_9$: 662.29. Found 685.28 $[M + Na]^+$.

Synthesis of 5-Hexenyl 4,6-*O*-Benzylidene-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside (7). A stirred mixture of 5-hexenyl *β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside (**6**, 12.2 g, 28.74 mmol), benzaldehyde dimethyl acetal (14 mL, 93.28 mmol), and *p*-toluenesulfonic acid monohydrate (250 mg, 1.32 mmol) in *N*,*N*-dimethylformamide (DMF, 125 mL) was heated to 55 °C, and the stirring was continued overnight under reduced pressure. After the mixture was returned to room temperature, triethylamine (3 mL) was added to the mixture. The solvent was removed, and purification of the residue by MPLC (15:1 chloroform/methanol) afforded compound **7** (7.8 g, 53%) as a white solid. ^{1}H NMR (DMSO-d_6): *δ* 7.37−7.27 (5H, m, aromatic protons), 5.79 (1H, m, C$\underline{H}$=CH$_2$), 5.58 (1H, s, PhC$\underline{H}$=), 5.02−4.92 (2H, m, CH=C$\underline{H}_2$), 4.39 (1H, d, *J* = 7.7 Hz, H-1′), 4.19 (1H, d, *J* = 7.8 Hz, H-1), 3.82−3.23 (14H, m, C$\underline{H}$O of sugar ring and OC$\underline{H}_2$CH$_2$CH$_2$CH$_2$CH=CH$_2$), 2.03 (2H, m, OCH$_2$CH$_2$CH$_2$C$\underline{H}_2$CH=CH$_2$), 1.54 (2H, m, OCH$_2$-C$\underline{H}_2$CH$_2$CH$_2$CHCH$_2$), 1.41 (2H, m, OCH$_2$CH$_2$C$\underline{H}_2$CH$_2$CH=CH$_2$). MALDI-TOF MS (*m*/*z*) calcd for $C_{25}H_{36}O_{11}$: 512.23. Found 535.48 $[M + Na]^+$.

Synthesis of 5-Hexenyl 2,3-Di-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-Dnoside (9). A solution of compound **7** (6.7 g, 13.07 mmol), 4-dimethylaminopyridine (DMAP, 35 mg, 0.29 mmol), and acetic anhydride (40 mL) in pyridine (80 mL) was stirred at 60 °C overnight. The mixture was allowed to return to room temperature, poured into cold water, and then extracted with ethyl acetate. The organic layer was washed in turn with saturated aqueous solution of sodium bicarbonate and brine, dried ($MgSO_4$), and concentrated to give the crude compound **8**, which was directly used in the next step without further purification. Thus, to a stirred solution of **8** in dichloromethane (180 mL) at 0 °C was added a 90% aqueous solution of trifluoroacetic acid (TFA, 20 mL). The stirring was continued at 0 °C for 3 h. The mixture was diluted with dichloromethane, washed successively with water and saturated aqueous solution of sodium bicarbonate, dried ($MgSO_4$), and evaporated. Purification of the residue by MPLC (5:1 ethyl acetate/*n*-hexane) yielded compound **9** as a white powdery material (5.8 g, 70%). ^{1}H NMR ($CDCl_3$): *δ* 5.79 (1H, m, C$\underline{H}$=CH$_2$), 5.21−5.16 (2H, m, H-3 and H-2′), 5.02−4.93 (2H, m, CH=C$\underline{H}_2$), 4.93−4.90 (2H, m, H-2 and H-3′), 4.48 (1H, d, *J* = 7.8 Hz, H-1′), 4.47 (1H, m, H-6$_a$), 4.46 (1H, d, *J* = 7.9 Hz, H-1), 4.13 (1H, t, *J* = 3.7 Hz, H-4′), 4.10 (1H, dd, *J* = 11.9, 5.4 Hz, H-6$_b$), 3.93 (1H, m, H-6′$_a$), 3.84 (3H, m, H-6′$_b$, H-4 and OC$\underline{H}_2$CH$_2$CH$_2$CH$_2$CH=CH$_2$), 3.64 (1H, m, H-5), 3.59 (1H, t, *J* = 5.3 Hz, H-5′), 3.49 (1H, m, OC$\underline{H}_2$CH$_2$CH$_2$CH$_2$CH=CH$_2$), 3.03 (1H, br, 4′-OH), 2.62 (1H, br, 6′-OH), 2.15-2.03 (17H, m, COC$\underline{H}_3$ and OCH$_2$CH$_2$CH$_2$C$\underline{H}_2$CH=CH$_2$), 1.58 (2H, m, OCH$_2$C$\underline{H}_2$CH$_2$CH$_2$CHCH$_2$), 1.43 (2H, m, OCH$_2$CH$_2$C$\underline{H}_2$CH$_2$CH=CH$_2$). MALDI-TOF MS (*m*/*z*) calcd for $C_{28}H_{42}O_{16}$: 634.25. Found 657.78 $[M + Na]^+$.

Synthesis of 5-Hexenyl 2,3,6-Tri-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranoside (10). To a solution of compound **9** (5.7 g, 8.98 mmol) in dry pyridine (80 mL) at −20 °C was added dropwise acetyl chloride (665 *μ*L, 9.36 mmol). The mixture was allowed to return to room temperature and stirred for 2 h. Water (30 mL) was added to the mixture, and the solvent was removed by evaporation. The resultant residue was dissolved in ethyl acetate, washed in turn with water and brine, dried ($MgSO_4$), and evaporated. Purification of the residue by MPLC (1:1 → 4:1 ethyl acetate/*n*-hexane) afforded a white powdery material of compound **10** (5.1 g) in 84% yield. ^{1}H NMR ($CDCl_3$): *δ* 5.79 (1H, m, C$\underline{H}$=CH$_2$), 5.22−5.16 (2H, m, H-3 and H-2′), 5.03−4.95 (2H, m, CH=C$\underline{H}_2$), 4.93−4.89 (2H, m, H-2 and H-3′), 4.50 (1H, m, H-6$_a$), 4.47 (1H, d, *J* = 7.8 Hz, H-1′), 4.46 (1H, d, *J* = 7.8 Hz, H-1), 4.30 (2H, m, H-6′$_{a,b}$), 4.11 (1H, dd, *J* = 11.9, 5.3 Hz, H-6$_b$), 4.01 (1H, t, *J* = 4.2 Hz, H-4′), 3.86 (1H, m, OC$\underline{H}_2$CH$_2$CH$_2$CH$_2$CH=CH$_2$), 3.79 (1H, t, *J* = 9.4 Hz, H-4), 3.72 (1H, t, *J* = 6.5 Hz, H-5′), 3.62 (1H, m, H-5), 3.48 (1H, m, OC$\underline{H}_2$CH$_2$CH$_2$CH$_2$CH=CH$_2$), 2.43 (1H, d, *J* = 5.1 Hz, 4′-OH), 2.14-2.04 (20H, m, COC$\underline{H}_3$ and OCH$_2$CH$_2$CH$_2$C$\underline{H}_2$CH=CH$_2$), 1.58 (2H, m, OCH$_2$C$\underline{H}_2$CH$_2$CH$_2$CH=CH$_2$), 1.43 (2H, m, OCH$_2$CH$_2$C$\underline{H}_2$CH$_2$CH=CH$_2$). ^{13}C NMR ($CDCl_3$): *δ* 170.8, 170.4, 170.2, 170.1, 169.5, 169.3, 138.5, 114.7, 101.0, 100.6, 76.3, 73.3, 72.7, 72.6, 72.1, 71.7, 69.9, 69.5, 66.7, 62.2, 61.9, 33.3, 28.8, 25.1, 20.9, 20.8, 20.7, 20.6, 20.5. MALDI-TOF MS (*m*/*z*) calcd for $C_{30}H_{44}O_{17}$: 676.26. Found 699.41 $[M + Na]^+$.

Synthesis of 5-Hexenyl 2,3,4,6-Tetra-*O*-(*p*-methoxybenzyl)-*α*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranoside (11). To a

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stirred mixture of silver triflate (1.1 g, 4.28 mmol), tin chloride (0.8 g, 4.22 mmol), powdered 4 Å molecular sieves (1.5 g), and compound **10** (1.5 g, 2.22 mmol) in dry ether/dichloromethane (2:1, 35 mL) at 0 °C was added dropwise a solution of compound **5** (4.5 g, 6.79 mmol) in dry ether (15 mL). The reaction mixture was stirred at 0 °C under nitrogen atmosphere overnight, diluted with dichloromethane, and filtered (Celite). The filtrate was washed in turn with saturated aqueous solution of sodium bicarbonate and brine, dried ($MgSO_4$), and evaporated. The resulting residue was purified by MPLC (1:2 ethyl acetate/*n*-hexane) to afford compound **11** as a white solid (2.8 g, 95% on the basis of compound **10**). ^{1}H NMR ($CDCl_3$): δ 7.31−6.78 (16H, m, aromatic protons), 5.79 (1H, m, C$\underline{H}$=CH_2), 5.20−5.17 (2H, m, H-3′ and H-2), 5.02−4.96 (2H, m, CH=C$\underline{H_2}$), 4.89 (1H, dd, J = 7.8, 9.5 Hz, H-2′), 4.85−4.32 (13H, m, H-1″, H-1′, H-1, PhC$\underline{H_2}$, sugar C$\underline{H}$O), 4.25 (1H, m, H-6′$_a$), 4.12−3.58 (21H, m, sugar C$\underline{H}$O, PhOC$\underline{H_3}$ and OC$\underline{H_2}$$CH_2CH_2CH_2CH$=$CH_2$), 3.48 (1H, m, OC$\underline{H_2}$$CH_2CH_2CH_2CH$=$CH_2$), 3.40 (1H, m, H-6′$_b$), 2.13−1.90 (20H, m, COC$\underline{H_3}$ and $OCH_2CH_2CH_2C\underline{H_2}CH$=$CH_2$), 1.58 (2H, m, $OCH_2C\underline{H_2}CH_2CH_2CH$=$CH_2$), 1.43 (2H, m, $OCH_2CH_2C\underline{H_2}CH_2CH$=$CH_2$). ^{13}C NMR ($CDCl_3$): δ 170.8, 170.4, 170.2, 170.1, 169.5, 169.3, 159.3, 159.2, 159.1, 159.0, 138.5, 131.2, 130.0, 129.8, 129.7, 129.0, 114.7, 113.8, 113.7, 113.6, 113.5, 101.5, 101.1, 100.7, 76.7, 76.6, 76.4, 76.3, 75.8, 75.6, 75.7, 73.3, 72.7, 72.6, 72.3, 72.1, 71.7, 69.9, 69.5, 66.7, 62.2, 61.9, 61.2, 55.3, 33.3, 28.8, 25.1, 20.9, 20.8, 20.7, 20.6, 20.5. MALDI-TOF MS (m/z) calcd for $C_{68}H_{86}O_{26}$: 1318.54. Found 1341.65 $[M + Na]^+$.

Synthesis of 5-Hexenyl α-D-Galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (2). A mixture of compound **11** (2.5 g, 1.89 mmol) and ceric ammonium nitrate (9 g, 16.42 mmol) in acetonitrile/water (9:1, 100 mL) was stirred at 45 °C for 20 h. After returning to room temperature, the mixture was evaporated to remove the solvent. The resultant residue was dissolved in a mixture of chloroform and a saturated aqueous solution of sodium bicarbonate. The organic layer was separated, washed with brine and water, dried ($MgSO_4$), and evaporated. Purification of the residue by MPLC (15:1 chloroform/methanol) afforded a demethoxybenzylated intermediate, which was dissolved in a solution of sodium methoxide in methanol (0.05 M, 50 mL). The mixture was stirred at room temperature for 16 h, neutralized with Dowex 50 (H^+), filtered, and evaporated. The resulting residue was purified by MPLC (65:25:4 chloroform/methanol/water) to afford **2** as a white solid (802 mg, 72%). $[\alpha]_D$ +36.2° (c 0.22, H_2O). ^{1}H NMR (D_2O): δ 5.81 (1H, m, C$\underline{H}$=CH_2), 4.99−4.89 (2H, m, CH=C$\underline{H_2}$), 4.85 (1H, d, J = 3.7 Hz, H-1″), 4.41 (1H, d, J = 7.8 Hz, H-1′), 4.38 (1H, d, J = 8.0 Hz, H-1), 4.26 (1H, t, J = 6.4 Hz, H-5″), 3.95−3.47 (18H, m, sugar C$\underline{H}$O and OC$\underline{H_2}$$CH_2CH_2CH_2CH$=$CH_2$), 3.20 (1H, t, J = 8.5 Hz, H-2), 2.07 (2H, m, $OCH_2CH_2CH_2C\underline{H_2}CH$=$CH_2$), 1.61 (2H, m, $OCH_2C\underline{H_2}CH_2CH_2CH$=$CH_2$), 1.46 (2H, m, $OCH_2CH_2C\underline{H_2}CH_2CH$=$CH_2$). ^{13}C NMR ($D_2O$, 27 °C): δ 139.7, 114.4, 103.3, 101.9, 100.3, 78.7, 77.3, 75.4, 74.8, 74.5, 72.9, 72.2, 70.9, 70.8, 70.5, 69.1, 68.9, 68.6, 60.5, 60.4, 60.1, 32.7, 28.2, 24.4. FAB-MASS (m/z) calcd for $C_{24}H_{42}O_{16}$: 586.25. Found 587 $[M + H]^+$.

Synthesis of GCs 1a−d. Ozone was successively bubbled through a stirred solution of compound **2** (15 mg/mL) in methanol at −78 °C until TLC (9:4:2 ethyl acetate/2-propanol/water) indicated no starting material left. Nitrogen was subsequently bubbled through the solution for 10 min to remove the remaining ozone. After addition of dimethyl sulfide (2 equiv), the reaction mixture was stirred at room temperature overnight followed by evaporation to give the corresponding crude aldehyde **12** as a white solid, which was directly subjected to the next coupling reaction with chitosan. Thus, to a stirred aqueous 5 wt % acetic acid solution of chitosan at 9.0 mg/mL was added dropwise a solution of **12** in the same solvent (30 mg/mL). The mixture was stirred for 1 h at room temperature, and then sodium cyanoborohydride (2 equiv) was added. After the mixture was stirred for 24 h, the acidic solution was neutralized with 1 N aqueous solution of sodium hydroxide. The mixture was subjected to dialysis using a cellulose membrane tube (MWCO = 10 kDa). After filtration to remove the precipitates, lyophilization of the filtration gave GC **1** as a white amorphous solid. This procedure was performed for yielding GCs **1a−d**, respectively. The details of reactants, yield, and structural parameter of the products are summarized in Supporting Information (Table S1). ^{1}H NMR (D_2O) of GC **1a**: δ 4.84 (1H, d, J = 3.6 Hz, H-1″ of the branch), 4.41−4.37 (2H, m, H-1′ and H-1 of the branch), 4.25 (1H, brt, J = 6.3 Hz, H-5″ of the branch), 3.93−3.45 (26.7H, m, sugar C$\underline{H}$O and linker OC$\underline{H_2}$), 3.19 (1H, br, H-2 of the branch), 2.82−2.50 (3.4H, brm, H-2 of GlcN and NC$\underline{H_2}$), 1.98 (1H, br, COC$\underline{H_3}$), 1.55 (4H, br, linker C$\underline{H_2}$), 1.34 (2H, br, linker C$\underline{H_2}$). GCs **1b−d** have the similar ^{1}H NMR data. The elemental analysis data of GCs **1a−d** are listed in Supporting Information (Table S2).

Synthesis of Lactose−Chitosan Conjugate (LC 3). LC **3** was prepared in a similar procedure as described for GC **1**. Ozonolysis of compound **6** (40 mg, 94.2 μmol) and subsequent N-alkylation of chitosan (10 mg, 58.9 μmol, 47.1 μmol of GlcN) afforded 28 mg (56.2 μmol) of LC **3** (80% DS, 95% yield). ^{1}H NMR (D_2O): δ 4.42 (1H, d, J = 7.8 Hz, H-1′), 4.38 (1H, d, J = 7.8 Hz, H-1), 3.93−3.45 (19.5H, m, sugar ring C$\underline{H}$O and linker OC$\underline{H_2}$), 3.25 (1H, br, H-2′), 2.88−2.66 (3H, br, H-2 of GlcN and NC$\underline{H_2}$), 2.01 (0.75H, br, COC$\underline{H_3}$), 1.59 (4H, br, linker C$\underline{H_2}$), 1.36 (2H, br, linker C$\underline{H_2}$). Anal. Calcd for $(C_8H_{13}NO_5)_{0.20}(C_{23}H_{41}NO_{15})_{0.80}\cdot 0.23H_2O$: C, 47.81; H, 7.14; N, 2.79. Found: C, 47.59; H, 7.25; N, 2.78.

Binding Studies. Binding assays were carried out by a BIAcore biosensor system based on the SPR technique.[48] The histidine-tagged Stx1B or Stx2B was covalently immobilized on a Ni^{2+} activated sensor chip that was precoated with nitrilotriacetic acid. Bindings of the analytes (GC **1**, globotrioside **2**, or LC **3**) in the running buffer to the immobilized toxin subunits were evaluated by the assay system, according to a standard procedure.[49] Briefly, serial dilutions of the analytes in running buffer were injected over the sensor chip surface at a flow rate of 20 mL/min for at least 3 min to reach the binding plateau. The response units (RUs) were monitored and the binding kinetics were analyzed by Scatchard plot, using the standard BIAevaluation software (BIAcore).

Cytotoxicity Assays. Vero cell cytotoxicity assays were performed according to published protocols.[21,22] Briefly, subconfluent Vero cells in a 96-well plate were incubated with Stx1 or Stx2 (10 pg/mL) in the absence or presence of the samples (GC **1**, globotrioside **2**, LC **3**, or chitosan) at different concentrations for 72 h. The relative number of living cells was determined by a cell counting kit.

Mouse Protection Experiments. Mouse models with high sensitivity to O157:H7 *E. coli* strain were built by PCM according to the published protocol.[59] The mice (3-week-old) were fed a low-protein diet (5 wt % protein) for 2 weeks to achieve PCM and then challenged intragastrically with 2×10^6 cfu of *E. coli* O157:H7 strain N-9 (day 0). The infection was confirmed by detection of the fecal Stx by ELISA in the early morning of day 2. Each group of 10 mice received intragastrically 1.0 mg of the samples (chitosan was suspended but others were dissolved in 0.1 mL of saline) twice a day from day 2 to day 4. The control group was treated with 0.1 mL of saline alone. Fecal pellets of each group were collected on day 4 after treatment with the samples and then subjected to ELISA for detection of Stx2. The mice were continuously fed with the low-protein diet throughout the experiment until the end of the monitoring on day 22. The survival analysis of the mice was performed using Kaplan−Meier estimator or Fisher's exact test.

Quantification of Stx2 in Mouse Fecal Samples. ELISA was carried out using a commercially available kit (Bio-Rad Laboratories) according to the known procedures.[59] The fecal samples were homogenized in PBS at 50 mg/mL and diluted 5-fold with the dilution buffer obtained from the supplier. The homogenate solutions (100 mL) were assayed with the ELISA kit. The limit of detection for Stx2 in the stool was tested to be 12 pg/mL.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Details of preparations, structural parameters, and element analysis data of GCs **1a−d** (Tables S1 and Table S2) and spectral data (^{1}H NMR, ^{13}C NMR, mass) of the synthetic

compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

CAN, ceric ammonium nitrate; DAST, (diethylamino)sulfur trifluoride; DMAP, 4-dimethylaminopyridine; DMF, *N*,*N*-dimethylformamide; DS, degree of substitution; ELISA, enzyme-linked immunosorbent assay; GPC, gel permeation chromatography; HUS, hemolytic uremic syndrome; LLS, laser light scattering; MPLC, medium pressure liquid chromatography; NBS, *N*-bromosuccinimide; PCM, protein calorie malnutrition; PDI, polydispersity index; PMB, *p*-methoxybenzyl; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPR, surface plasmon resonance; Stx, Shiga toxin; StxB, Shiga toxin B-subunits; STEC, Shiga toxin-producing *Escherichia coli*; TFA, trifluoroacetic acid; THF, tetrahydrofuran

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