Synthesis of Galabiose-chitosan Conjugate as Potent Inhibitor of *Streptococcus suis* Adhesion

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The aim of this work is to construct a safe and effective drug candidate against *Streptococcus suis* infection. A panel of chitosan-based polymer conjugates with branched galabiose (Galα1−4Gal) side chains was synthesized as inhibitors of *S. suis* adhesion. The synthesis was achieved by using an aldehyde-functionalized galabiose derivative to graft it onto chitosan amino groups. Structural compositions of the conjugates were verified by ^{1}H NMR spectroscopy and CHN elemental analyses. Potent inhibitory activities of the conjugates against *S. suis* adhesion to human erythrocytes were determined at low nanomolar concentration by HAI assay. An SPR study revealed a high affinity binding (K_d = 39.6 nM) of the conjugate with BSI-B_4 lectin. By using biocompatible chitosan as the scaffold for presenting *S. suis*-specific galabiose units, as well as the concise route tailored for the conjugate syntheses, the present study provides a practical way for explorations of new anti-*S. suis* therapies.

Introduction

Streptococcus suis is a serious zoonotic pathogen causing meningitis, septicemia, and pneumonia in both pigs and human. The repeated worldwide outbreaks of *S. suis* infection with high mortality, up to 17.8% in human cases, pinpoint the urgent need for safe and effective methods for microbial prevention and treatment.[1] Antibiotics such as penicillin G and gentamicin are powerful tools in the fight against this contagious pathogen. However, the effectiveness of traditional antibiotics is under threat from the alarming increase in bacterial resistance.[2] A recent alternative strategy termed antiadhesion therapy[3] is to use agents that interfere with microbial attachment to host tissues. Adhesion is the first key step in the infection process that in many cases is mediated by the specific binding of bacterial lectinlike proteins (adhesins) to complementary glycan receptors present on the host cell's surface. Creation of multivalent glyco-mimics[4] with high affinity for the bacterial surface proteins is by far one of the most promising approaches based on antiadhesion strategy. Since the first identification of carbohydrate receptor, galabiose (Galα1−4Gal), of *S. suis* in 1993,[5] several studies have been conducted to develop galabiose derivatives as *S. suis* blockers. In these reports,[6] enhanced inhibitory effect of the multivalent galabiosides as low as nanomolar concentrations was found by clustering[7] galabiose residue on the synthetic scaffolds such as aminoethoxyl benzoic acid and poly(amidoamine) dendrimer, although the preparations of such dendrimer blockers were complicated, involving multistep modifications of the galabiose unit as well as generations of the scaffolds, and therefore, could hardly meet the requirement of drug safety and practical applications to produce an abundant supply.

To meet the goal of developing safe and practical drug candidates against *S. suis* infection, here we report the facile synthesis of a new anti-*S. suis* agent, galabiose-chitosan (GC) conjugate **1** (Scheme 1), which has a chitosan backbone with branched galabiose side chains, and also demonstrate its ability to inhibit *S. suis* adhesion at low nanomolar concentration. It is advantageous to use chitosan as the scaffold for displaying multivalent galabiose residues because (i) chitosan is an abundant natural polysaccharide with amino groups that allow easy chemical modifications; (ii) chitosan has shown a broad-spectrum antibacterial activity toward various species;[8] and (iii) chitosan is biodegradable, biocompatible, and has been applied to medical areas such as wound healing.[9] The unique combination of *S. suis*-specific galabiose with diverse chitosan bioactivities prompts us to start the synthetic and bioevaluation research program.

Experimental Section

Materials. Five types of chitosans with the same degree of deacetylation (DDA, 95% GlcN, 5% GlcNAc) but different average molecular weight (M_w, 5500, 1000, 300, 100, and 50 kDa, respectively) were purchased from Zhejiang Golden-shell Biochemical Co., Ltd. The molecular weight distribution of chitosans was generally moderate, with a polydispersity index (PDI) at a range of 1.4−2.2. A galactose-specific virulent *S. suis* type 2 strain (HA9801, a vaccine strain in China),[10] isolated from diseased pigs in Jiangsu province of China, was used in this study. The strain was grown in Todd-Hewitt broth (THB) or agar medium and supplemented with 2% (*v*/*v*) newborn bovine serum at 37 °C. Human erythrocytes (blood group B) were obtained from General Hospital of China People's Liberation Army. BSI-B_4 lectin (*Bandeiraea simplicifolia*) was purchased from Sigma Co., Ltd. Compounds **3**, **5**, and **6** were synthesized as described in Supporting Information.

General Procedure for the Synthesis of GC Conjugate (1). Ozonolysis of compound **6** in methanol (0.1 mL/mg of **6**) at −78 °C quantitatively yielded aldehyde **7**. A reaction mixture for conjugate syntheses was composed of chitosan (2.0 mg/mL), aldehyde **7** (0.1−2.0 equiv of chitosan GlcN), and sodium cyanoborohydride (2 equiv of **7**) in an aqueous 5 wt % acetic acid solution. The mixture was stirred for

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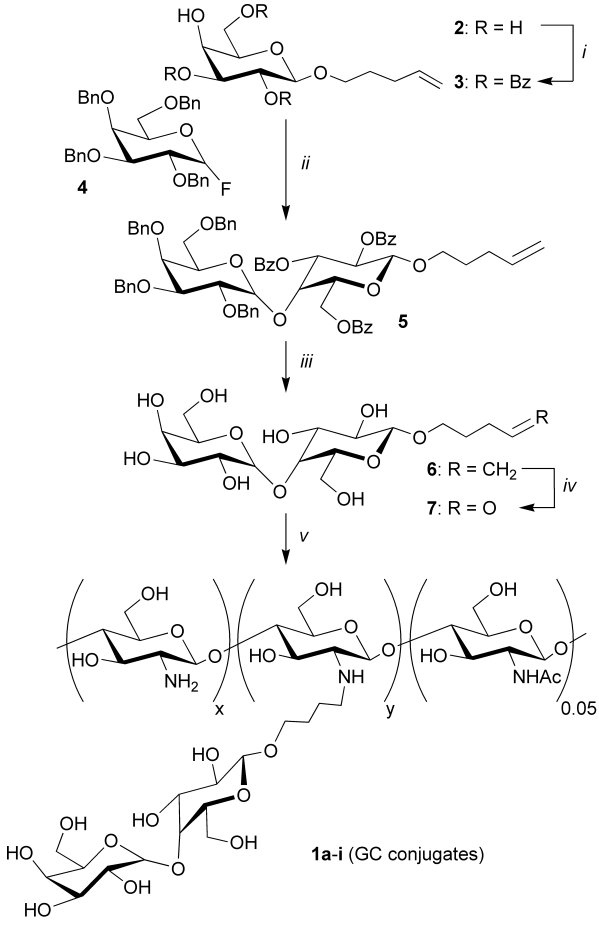
Scheme 1. Syntheses of GC Conjugates **1**[a]



Conjugate	1a	1b	1c	1d	1e	1f	1g	1h	1i
x	0.39	0.43	0.42	0.41	0.41	0.70	0.81	0.87	0.92
y (DS[b])	0.56	0.52	0.53	0.54	0.54	0.25	0.14	0.08	0.03
DP[c]	307	613	1839	6131	33722	1839	1839	1839	1839

[a] Reagents and conditions: (i) BzCl, pyridine, −20 °C, 65%; (ii) AgOTf, $SnCl_2$, CH_2Cl_2−Et_2O, 0 °C, 53%; (iii) MeONa, MeOH, and then Na/NH_3, MeOH, −78 °C, two steps, 56%; (iv) O_3, Me_2S, −78 °C, quantitatively; (v) chitosan (see Experimental Section), $NaCNBH_3$, AcOH (aq. 5 wt % solution), rt, 65−82%. [b]Degree of substitution (DS) was determined by the corresponding ^1H NMR spectrum. [c]Degree of polymerization (DP) was estimated from the M_w of starting chitosans.

24 h at room temperature and then dialyzed with a membrane tube (MWCO: 14 kDa). After filtration to remove the precipitate, lyophilization of the filtrate gave conjugate **1** as a white amorphous solid (65−82% yield). The quantity of reaction agents, yield, structural parameter of the products (GC conjugates **1a**−**i**), and the element analysis data were summarized in Supporting Information (Table S1 and Table S2). ^1H NMR of **1c** (400 MHz, D_2O): δ 4.99 (d, 1H, J = 3.6 Hz, Gal_α H-1), 4.67 (br, GlcNAc H-1), 4.50 (d, 1H, J = 7.6 Hz, Gal_β H-1), 4.41 (t, 1H, J = 6.4 Hz, Gal_α H-5), 4.16−3.54 (m, 21.6H), 3.22−2.74 (brm, 5.4H, GlcN H-2 and $NHCH_2$), 2.09 (br, 0.28H, $COCH_3$), 1.70 (br, 4H, linker CH_2). Other GC conjugates gave similar ^1H NMR data.

Synthesis of Lactose-Chitosan Conjugate (10). Conjugate **10** was prepared with a similar procedure as described for GC conjugate **1**. Ozonolysis of compound **8**[11] (20 mg, 48.8 μmol) yielded aldehyde **9**, which reacted with chitosan (4.2 mg, 25.7 μmol, M_w 300 kDa, DDA 95%), affording 7 mg (22.1 μmol) of conjugate **10** (42% DS, 83% yield). ^1H NMR (400 MHz, D_2O): δ 4.90 (br, GlcN H-1), 4.49 (d, 1H, J = 8.0 Hz, Gal_β H-1), 4.44 (d, 1H, J = 7.6 Hz, Glc H-1), 3.98−3.47 (m, 18.5H), 3.28−2.91 (brm, 6.8H, GlcN H-2 and $NHCH_2$), 2.06 (br, 0.35H, $COCH_3$), 1.69 (br, 4H, linker CH_2). Anal. Calcd for $(C_6H_{11}NO_4)_{0.53}(C_{22}H_{39}NO_{15})_{0.42}(C_8H_{13}NO_5)_{0.05}$: C, 46.69; H, 6.94; N, 4.26. Found: C, 46.53; H, 7.05; N, 4.18.

Hemagglutination Inhibition (HAI) Assay. Hemagglutination assays were performed according to a previously reported method.[5,6] *S. suis* was grown overnight at 37 °C in 5% CO_2 incubator. The bacteria were harvested by centrifugation (5000 × g, 15 min, 4 °C) and washed twice with phosphate-buffered saline (PBS, pH 7.2). The hemagglutination activity was titrated and the lowest bacterial density causing agglutination was used for the inhibition studies. Conjugates **1a**−**f**, conjugate **10**, and compound **6** were dissolved in PBS at the concentration of 1.0 mg/mL. Conjugates **1g**−**i**, chitosan (M_w 300 kDa) were dissolved in acidic PBS (pH 4.5, adjusted with 1 N HCl) at the same concentrations. The above solutions were serially diluted (1:10 for the first time, and then 1:2). PBS (pH 7.2 and 4.5, respectively) was used as the blank. For inhibition assays, a volume of 25 mL of each dilution was mixed with an equal volume of bacteria suspension. After incubation at room temperature for 5 min, a suspension of 4% human erythrocytes in PBS (50 mL) was added. The hemagglutinations and minimum inhibitory concentrations (MIC) were recorded.

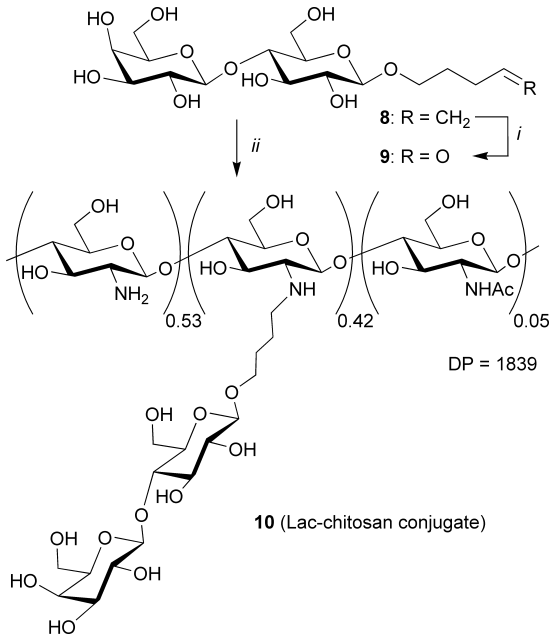
Surface Plasmon Resonance (SPR) Analysis. The binding of conjugate **1c** with BSI-B_4 lectin was analyzed by a BIAcore biosensor system based on the SPR technique. Conjugate **1c** was covalently immobilized by a standard amine-coupling method on a sensor chip (CM-5, research grade) that was coated with carboxymethyl dextran. A solution of BSI-B_4 lectin at the concentration of 0−0.114 mg/mL (0−1000 nM) in running buffer (PBS, pH 7.2) was injected over the immobilized conjugate **1c** at a flow rate of 20 μL/min for 4 min (association phase). During the dissociation phase, the sensor surface was exposed to running buffer at the flow rate of 20 μL/min. Conjugate **10** and chitosan (M_w 300 kDa, DDA 95%) were used as the negative controls. The affinity constant (K_d) of the interactions were calculated by a standard BIA evaluation software (version 3.1).

Measurements. The M_w and PDI (M_w/M_n) of starting chitosans and all conjugates were measured by GPC (CTO-20A, Shimadzu Co., Ltd.) equipped with a multiangle laser light scattering detector (DAWN HELEOS-II, Wyatt Co., Ltd.) in NaOAc buffer (pH 4.5). The ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX 400 spectrometer at 400 and 100 MHz, respectively. The DDA of starting chitosans was determined by the corresponding ^1H NMR spectrum. ESIMS and HRESIMS data were recorded on a Mariner ESI-TOF spectrometer (ABI Co., Ltd.). Elemental analysis was performed with a Flash EA 1112 instrument (ThermoQuest Co., Ltd.). Surface plasmon resonance (SPR) analysis was performed by a BIAcore biosensor system (Biacore 3000, Biacore Co., Ltd.).

Results and Discussion

Syntheses commenced with the assembly of galabiose derivative **7** having an alkyl linker terminated by an aldehyde group (Scheme 1), which was designed for attaching galabiose unit onto the amino groups of chitosan. Selective benzoylation of galactoside **2**[12] according to a published procedure[13] afforded **3** in a 65% yield. Glycosidation of galactosyl fluoride **4**[14] with acceptor **3** provided the exclusively α-linked disaccharide **6** after two steps of deprotection reactions. TLC indicated ozonolysis of the C−C double bond of **6** proceeded quantitatively to provide aldehyde **7**, which was directly employed for the next coupling reaction with chitosan without further purification. Reductive *N*-alkylation of chitosan with **7** was conducted smoothly in an aqueous AcOH solution in the presence of $NaCNBH_3$ to afford the GC conjugates **1a**−**i** in 65−82% yield. The degree of substitution (DS) of the galabiose branch in GC conjugate was adjusted by the molar ratio of aldehyde **7** to the amino groups of chitosan (see Supporting Information). The maximum DS was obtained at 56% by using 2 equiv **7**. A large excess of **7**, up to 4 equiv, has been tested and found to have no effect on further increasing the DS value, likely due to

Scheme 2. Syntheses of Lac-Chitosan Conjugate **10**[a]



[a] Reagents and conditions: (i) O_3, Me_2S, −78 °C, quantitatively; (ii) chitosan (DDA 95%, M_w 300 kDa), $NaCNBH_3$, AcOH (aq. 5 wt %), rt.

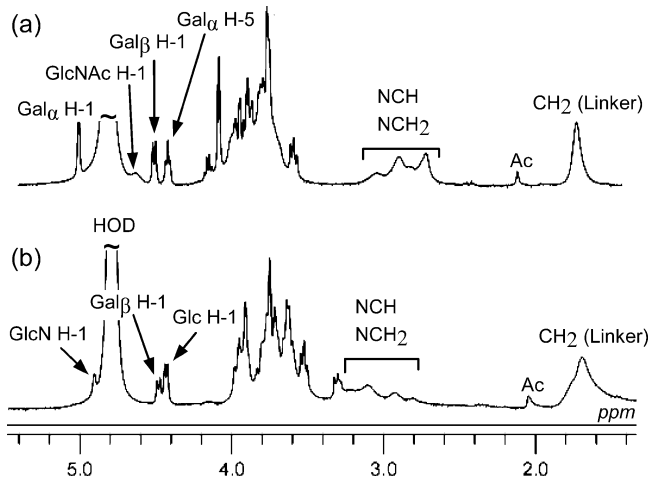


Figure 1. ^{1}H NMR spectra of (a) GC conjugate **1c** and (b) Lac-chitosan conjugate **10** in D_2O at 300 K. Abbreviations: Gal_α, α-linked Gal in **1c**; Gal_β, β-linked Gal in **1c** or in **10**.

the increased steric hindrance of the conjugate. For negative control of the bioevaluation of GC conjugates, a lactose (Lac, Galβ1−4Glc) branched chitosan derivative, Lac-chitosan conjugate **10**, was also synthesized from compound **8**[11] in a similar procedure (Scheme 2). In contrast to water-insoluble chitosan, conjugates **1a**−**f** and **10** showed good solubility in neutral water (solubility ≥ 1.0 mg/mL, PBS, pH 7.2) owing to the highly incorporated hydrophilic sugar branches, but conjugates **1g**−**i** with the lower DS value were less soluble.

Characterizations of the conjugates were carried out by ^{1}H NMR spectroscopy, CHN elemental, and GPC analyses. The structural compositions were verified by ^{1}H NMR assignments of several baseline-separated signals of the galabiose (or lactose) moiety, alkyl linker, and acetyl group of chitosan backbone, as typically illustrated by the spectra of conjugate **1c** and **10** in Figure 1. The DS values determined on the basis of integral ratio of the branch-derived peaks to chitosan backbone acetyl peaks were in good agreement with the CHN elemental analyses (see Supporting Information). The M_w of all conjugates measured by a GPC system was generally at the same level with that estimated from the formula weight (F_w) and DP of chitosans (F_w × DP), and the PDIs had approximately the same range as those of starting chitosans as well (data not shown), suggesting that little change had occurred in the molecular weight of chitosan backbones during the synthetic process. However, attempts for the further characterization by ^{13}C NMR spectroscopy has failed because of the rigid chitosan backbone[15] and increased macromolecule size of the conjugates.

Table 1. Inhibitory Effect of GC Conjugates on the Hemagglutination of Human Erythrocytes Caused by *S. suis* HA9801 Strain

sample	DP	DS (%)	MIC[a] (ng/mL)
1a	307	56	5 (7.1 nM)
1b	613	52	3 (4.1 nM)
1c	1839	53	1.25 (1.7 nM)
1d	6131	54	2.5 (3.4 nM)
1e	33722	54	2.5 (3.5 nM)
1f	1839	25	2.0 (1.8 nM)
1g[b]	1839	14	25 (15.3 nM)
1h[b]	1839	8	5000 (1970 nM)
1i[b]	1839	3	NA[c]
6			5500 (13410 nM)
10	1839	42	NA[c]
chitosan[b,d]	1839		NA[c]

[a] MIC (minimum inhibitory concentration): ng/mL of sample (nM of galabiose moiety). [b] Sample was dissolved in acidic PBS (pH 4.5) that was tested no influence on the assay (blank). [c] No activity at the concentration of 1.0 mg/mL or less. [d] The chitosan with DDA of 95% and M_w of 300 kDa was used.

Inhibitory effect of GC conjugates on the adhesion of *S. suis* to human erythrocytes (expressing galabiose-terminated glycolipids on the surface[5]) was evaluated by HAI assay. As the results in Table 1 show, potent inhibition of the conjugates **1a**−**g** was observed at low nanomolar concentrations. However, the MIC values were strongly dependent on two structural variants of the conjugates, that is, the DP of chitosan backbone and the DS of galabiose branch. Conjugates **1a**−**e** with the similar DS (52−56%) but different DP showed the highest inhibitory effect at the DP of 1839 (**1c**, MIC = 1.7 nM), whereas the smaller (**1a**, **1b**) and larger (**1d**, **1e**) conjugates were relatively less effective. Umemura et al. have demonstrated that the scaffold length of the multivalent inhibitor is a significant factor in control of their antiadhesion activity through a molecular simulation study.[4d] Here the HIA assay results also supported the assumption that polymer size was of particular importance for the access and binding of bacterial cells to the conjugates, and **1c**, with a moderate molecular length, would be the most favorable one for the *S. suis* bindings among the conjugates tested. The result that larger conjugates (**1d**, **1e**) showed a same activity might indicate a critical point of polymer size existing for the bacterial preference; otherwise, the conformation of conjugates such as the distribution and availability of galabiose branches would also affect the inhibitions as demonstrated by Umemura et al. In addition, no clear correlation between the molecular weight distributions and inhibitions has been found by a comparison of PDI and MIC values of the conjugate (data not shown). On the other hand, the inhibitory activity decreased in accordance with the DS value, as illustrated by conjugates **1c** and **1f**−**h**, due to the multivalency effect.[7] Monovalent galabioside **6** showed a very weak activity at the MIC of 13410 nM. Conjugate **1i** had no activity, even at the concentration of 1.0 mg/mL, probably due to the fact that the large number of chitosan backbone (extremely low DS) hampered the access of the galabiose moiety to *S. suis* cells. In addition, no inhibition of hemagglutination was observed for conjugate **10** and chitosan,

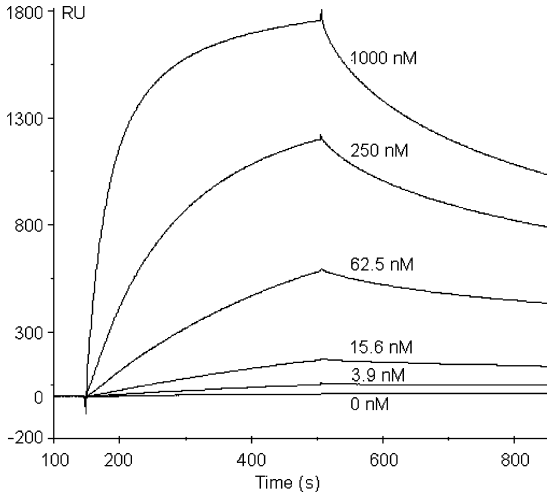


Figure 2. Sensorgram for the binding of GC conjugate **1c** with BSI-B_4 lectin. Immobilized ligand: **1c** on the SPR sensor chip; injected analyte: BSI-B_4 lectin at various concentrations in PBS buffer (pH 7.2).

proving that the inhibitions were derived from interactions of *S. suis* with the galabiose moieties of the conjugates.

The proposed molecular mechanism of inhibition of multivalent galabiose derivatives is the interactions with adhesins present on *S. suis* surface.[6] Because galabiose-specific adhesins have not been exactly identified from the *S. suis*, a plant lectin (BSI-B_4: *Bandeiraea simplicifolia*, specific to α-galactoside) was employed to further characterize the binding property of GC conjugates at the molecular level. The binding assay was carried out on the basis of SPR technique. Conjugate **1c** was covalently immobilized on the carboxymethylated dextran-coated sensor chip by the general amine-coupling method. The solutions containing various concentrations (0−1 μM) of BSI-B_4 lectin were injected over the sensor chip surface, and the binding affinity was determined. The SPR sensorgram (in resonance units, RU) is shown in Figure 2. The significant bindings of BSI-B_4 lectin with conjugate **1c** was detected in a dose-dependent manner with the maximum RU value at about 1800 (concentration = 1000 nM). The dissociation constant (K_d) estimated by the standard BIAcore software was 39.6 nM that represented the high binding affinity of **1c** with BSI-B_4 lectin. In contrast, no interaction of the conjugate **10** or chitosan with BSI-B_4 lectin was observed by the SPR analysis (data not shown). These results indirectly suggested a high affinity binding of **1c** with the proposed galabiose-specific adhesins of *S. suis*, and therefore indirectly elucidated the potent inhibitory effect of **1c** at the molecular level.

Conclusions

An efficient approach for construction of a novel galabiose-branched chitosan derivative, GC conjugate, has been presented to explore safe and practical anti-*S. suis* therapies. The key advantages compared to previously reported dendrimer inhibitors are the concise synthetic procedure as well as the use of biocompatible and cheaply available chitosan as the scaffold that enables not only the large-scale preparation but also the improved drug safety. The preliminary bioevaluation of GC conjugates revealed that **1c** completely inhibited the *S. suis*-induced hemagglutination at a concentration of 1.7 nM, which was comparable with the most potent inhibitor[6b] known by far [octavalent galabioside with poly(amidoamine) dendrimer scaffold, MIC = 2.4 nM]. SPR study also revealed that **1c** was of high affinity with the BSI-B_4 lectin (K_d = 39.6 nM). Currently, more extensive and in-depth bioevaluations of the GC conjugate is being pursued, and efforts are ongoing to develop these promising antiadhesion agents into the drug for *S. suis* infection.

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Supporting Information Available. Experimental details; NMR and elemental analysis data; and NMR and mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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