Conference paper

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Synthesis of *p*-methoxyphenyl sulfated β-GalNAc derivatives with inhibitory activity against Japanese encephalitis virus

DOI 10.1515/pac-2016-0921

Abstract: The *N*-acetylgalactosamine (GalNAc) residue is one of the units of chondroitin sulfate E (CS-E) which has been reported to have inhibitory activity against Japanese encephalitis virus (JEV). Herein, we describe the synthesis of a series of *p*-methoxyphenyl β-GalNAc derivatives with a sulfate group at 3-, 4-, and/or 6-positions using an efficient route through a common synthetic intermediate. By measuring the inhibition activity of these compounds that bear different numbers and positions of sulfate groups, the effect of position specificity for interaction with the virus was determined. From these results, GalNAc6S and GalNAc4S6S derivatives inhibited JEV infections well; we suggest the 6-*O*-sulfate group is necessary for selective recognition by the virus.

Keywords: GalNAc; ICS-28; inhibitor; Japanese encephalitis virus; sulfated saccharide.

Introduction

Japanese encephalitis virus (JEV) is a member of the Flavivirus genus within the *Flaviviridae* family. Similarly to West Nile and dengue viruses that belong to the same genus, JEV infection is considered a major public health concern. JEV has caused a major outbreak of viral encephalitis in south, southeast, and east areas of Asia [1–5]. More than 67,000 cases, including approximately 20,000 deaths annually, occur from the viral infection [6]. In the northeastern part of Australia, JEV infection was first reported in 1995. Distribution areas of JEV are now growing inward in Australia [7, 8].

JEV, an arthropod-borne virus, is transmitted through swine to humans by *Culex tritaeniorhynchus* mosquitoes [9–11]. Pigs are considered to be important amplifying hosts because they develop an asymptomatic infection with a high level of viremia during for several days post-infection. Although JEV infection in humans and horses do not result in a level of viremia high enough for human-to-human transmission by mosquitoes, they develop clinical manifestations such as high fever, headache, diarrhea, and vomiting, followed by seizure, flaccid paralysis, meningitis, and encephalitis in severe cases. Therefore, humans and horses are considered to be dead-end hosts [3]. JEV almost results in asymptomatic infections in humans with

Article note: A collection of invited papers based on presentations at the XXVIII International Carbohydrate Symposium (ICS-28), New Orleans, July 17–21 2016.

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an incidence rate that varies from 0.1 to 4 % [12]; however, the mortality ratio in symptomatic cases is more than 30 % and a high population of survivors has severe and long-term neurological sequelae [12, 13].

Vaccination is the most effective way to prevent JEV infection. Inactivated vaccines have been developed using Vero cells and widely introduced with routine immunization programs, however, all healthy people who are immunized have some risk of adverse effects. In addition, JEV is not fully controlled in areas with relatively lower vaccine coverage [6, 11, 14]. Since there are no clinically-approved anti-JEV drugs available, patients are confined to symptomatic alleviation and supportive care. Therefore, therapeutic use of anti-JEV drugs need to be developed.

JEV is an envelope virus with an icosahedral lipid bilayer [15]. The virus has a positive-strand RNA genome that encodes a single polypeptide, including three structural (Capsid, Membrane, and Envelope) and seven non-structural proteins. The Envelope (E) protein, expressed in a heterodimer form in mature virus particles, consists of three functional domains, I, II, and III. The E protein is involved in early events of JEV infection, such as adsorption of the virus to host receptors and engagement of fusion between viral and host cell membranes [16–18]. This protein also induces production of neutralizing antibodies in humans [19]. Domain III of the E protein is critical for virus adsorption to receptors expressed on the host cell surface [20, 21].

In JEV-infected cells, low pH conditions in endosomes triggers conformational alterations of the E protein and receptor-mediated endocytosis of the virus particles, inducing fusion between viral and host cell membranes. After the viral RNA is released into the cytosol, viral genome replication occurs [22].

Glycosaminoglycans (GAGs), a family of complex glycoconjugates, consist of a linear polysaccharide chain composed of a repeating disaccharide unit. The repeating unit consists of uronic acid, glucuronic or iduronic acid, and amino sugar, *N*-acetylgalactosamine (GalNAc) or *N*-acetylglucosamine (GlcNAc). GAGs have many isomers and diverse structures formed from the combination of the constituent sugars and position and amount of sulfate groups; for example, heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate, and keratan sulfate.

The core structures of GAGs depend on their derived tissues that result from different sulfation positions on sugars. GAGs exist in the extracellular matrix (ECM) and on the cell surface. Interaction of GAGs with GAG-binding proteins induces cell-extracellular adhesion, cell growth, cell differentiation, and regulation of growth factor function.

A virus binding to a host cell surface is mediated through specific receptor molecules that play a significant role in viral infections. These specific interactions account for the definition of the tissue and host tropism [23]. Heparin and chondroitin sulfate E (CS-E), classified as sulfated GAGs, are involved in host cell recognition in the early stage of JEV infection as host co-receptors [24, 25].

In this study, we focused on the structure of GalNAc in CS-E because CS-E shows strong inhibitory activity against JEV infection. We designed, chemically synthesized, and characterized GalNAc derivatives with different numbers and positions of sulfate groups that showed effective inhibitory activity against JEV infection. The compounds that prevent JEV from attaching to cultured cells may contribute to elucidation of the molecular mechanisms for JEV-host recognition, as well as development of anti-JEV drugs.

Results and discussion

The position of the sulfate group, which effected inhibitory activity of JEV, is an important research area. To investigate the suitable position of a sulfate group for interaction with the virus, we synthesized compounds **1–7**. The compounds bear sulfate groups at the 3-, 4-, and/or 6-positions, and the 1- and 2-positions had *p*-methoxyphenyl (MP) and *N*-acetyl groups, respectively (Fig. 1). The MP group was chosen in this study to allow for easy detection of reaction progress through the aromatic ring that absorbs UV light. The MP group can be removed using diammonium cerium(IV) nitrate, an advantage for forming a variety of aglycons in the future syntheses, after which we will use a click reaction such as the copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction [26, 27]. After deprotection of the MP group, β-selective introduction of an

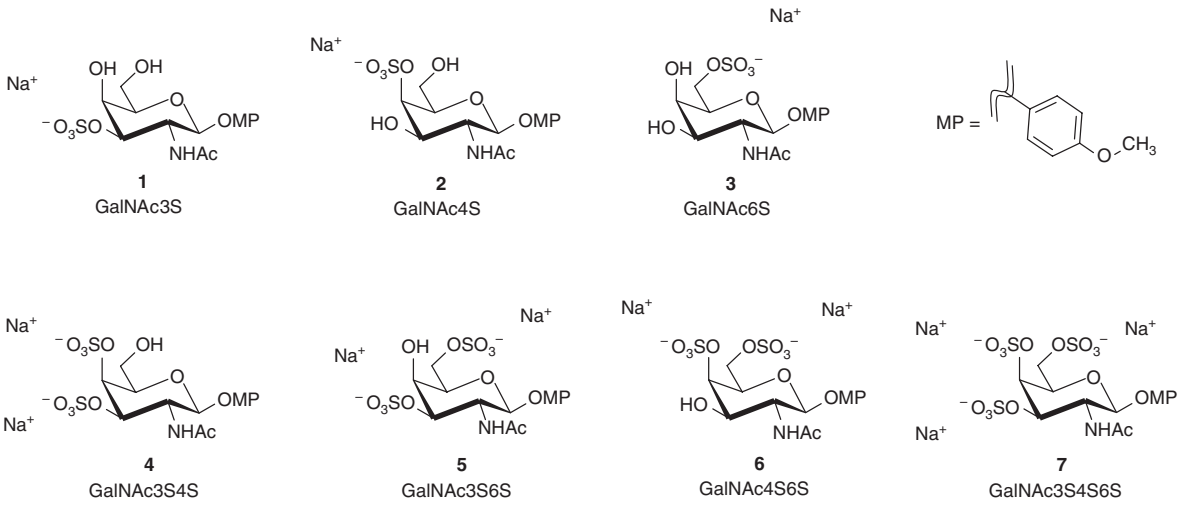


Fig. 1: Target sulfated GalNAc derivatives **1–7**.

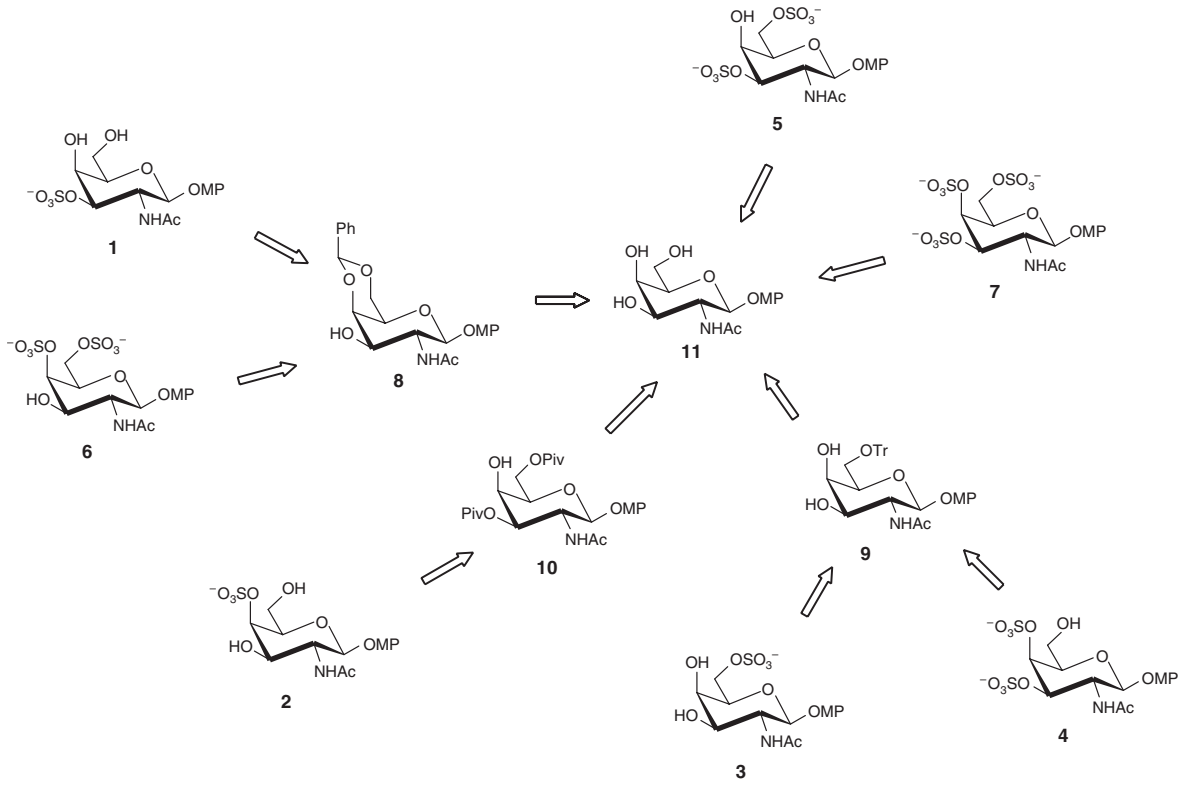
azide group at an anomeric position will be performed by reaction with sodium azide and 2-chloro-1,3-dimethylimidazolinium chloride (DMC), developed by Shoda and co-workers [28]. Then, the CuAAC reaction will be used to introduce various substituents in the final step without any protecting groups. We assumed the influence of the configuration of the glycon on the interaction with the virus was stronger than that of aglycons. So, we first sought to find a suitable glycon structure before optimizing their aglycons.

Retrosynthetic analysis gave strategies with simple synthetic steps for 7 types of targeted sulfated GalNAc derivatives from the non-sulfated GalNAc derivative **11**. Target compounds **1** and **6** were prepared from compound **8**, which was protected at the 4- and 6-positions using a benzylidene group. GalNAc4S6S derivative **6** was obtained by sulfation after protecting with an acyl group at the 3-position of **8** and removal of the benzylidene group. The 4-*O*-sulfated galactopyranoside **2** was synthesized from compound **10**, prepared by selective 3,6-di-*O*-pivaloylation [29] of **11**. Compounds **3** and **4** were synthesized from compound **9**, whose 6-position was selectively protected by a trityl group. Benzoylation at the 3- and 4-positions afforded fully a protected GalNAc derivative, then the 6-position was subjected to deprotection under acidic conditions, followed by sulfation to give GalNAc6S **3**. Targets **5** and **7** were obtained in one step from the synthetic intermediate **11** by controlled sulfation (Scheme 1).

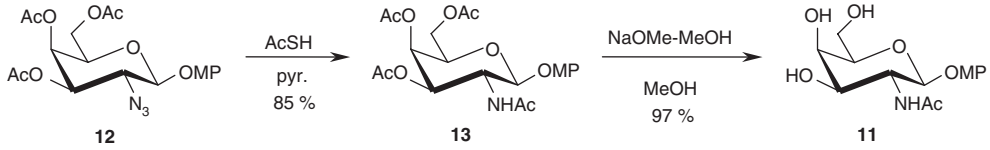
The synthetic intermediate GalNAc **11** was synthesized as depicted in Scheme 2. *p*-Methoxyphenyl 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyratnoside **12** [30] was used as the starting material. The azide group at the 2-position of **12** was converted to an acetamido group using thioacetic acid and pyridine [31, 32] to give compound **13** in 85 % yield. The GalNAc derivative **11**, with free hydroxy groups at the 3-, 4-, and 6-positions was obtained by treating **13** with sodium methoxide in methanol.

GalNAc3S **1** was prepared in 3 steps from **11** (Scheme 3). A solution of **11** was treated with benzaldehyde dimethyl acetal (BDA) and D-camphor-10-sulfonic acid (CSA) to give compound **8**. Target **1** was then obtained by sulfation of **8** with sulfur trioxide-pyridine complex in pyridine [33, 34], followed by deprotection of the benzylidene group under acidic reaction conditions. The low yield of synthetic intermediate **8** was due to competitive formation of methyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-galactopyranoside by glycosylation of **8** or **11** with the methanol byproduct under the acidic conditions. The formation of the methyl glycoside was confirmed by the ^{1}H NMR spectrum of the reaction mixture.

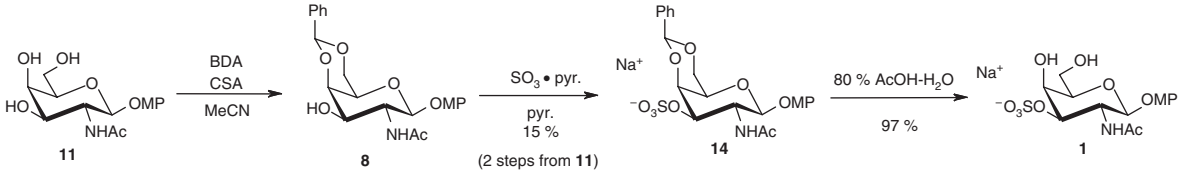
The synthetic route for GalNAc4S **2** is described in Scheme 4. Galactosamine **10** was synthesized by selective protection with a pivaloyl group at the 3- and 6-positions of **11** [29]. Then, compound **10** was subjected to sulfation by treating with sulfur trioxide-pyridine complex to afford **15**. De-*O*-pivaloylation with sodium methoxide in methanol gave compound **2** in 81 % yield.



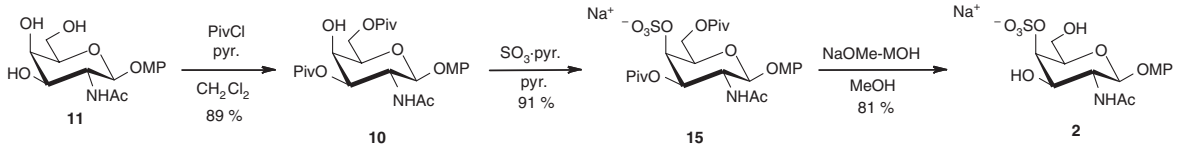
Scheme 1: Retrosynthetic analysis of target compounds **1–7**.



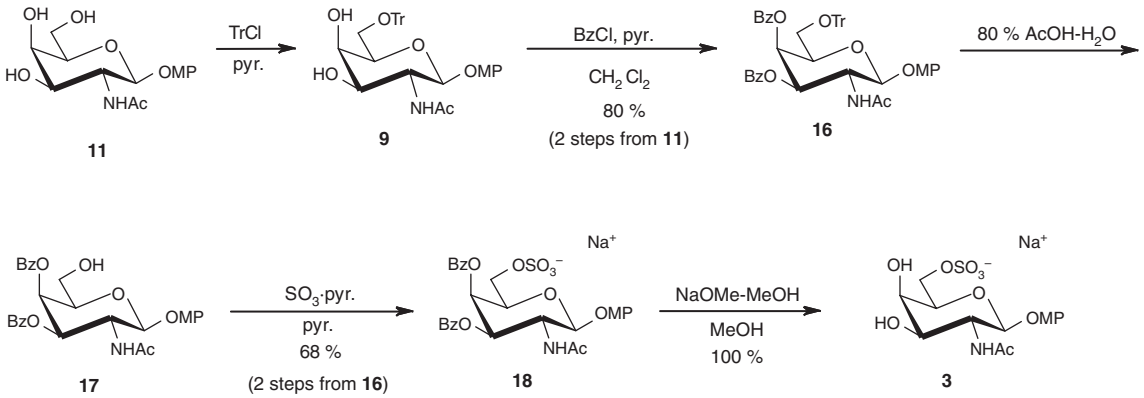
Scheme 2: Synthetic route to common synthetic intermediate **11**.



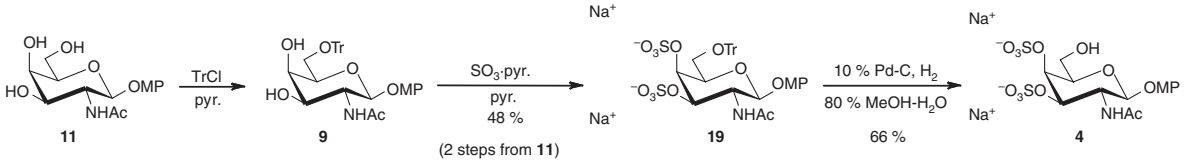
Scheme 3: Synthetic route of GalNAc3S derivative **1**.



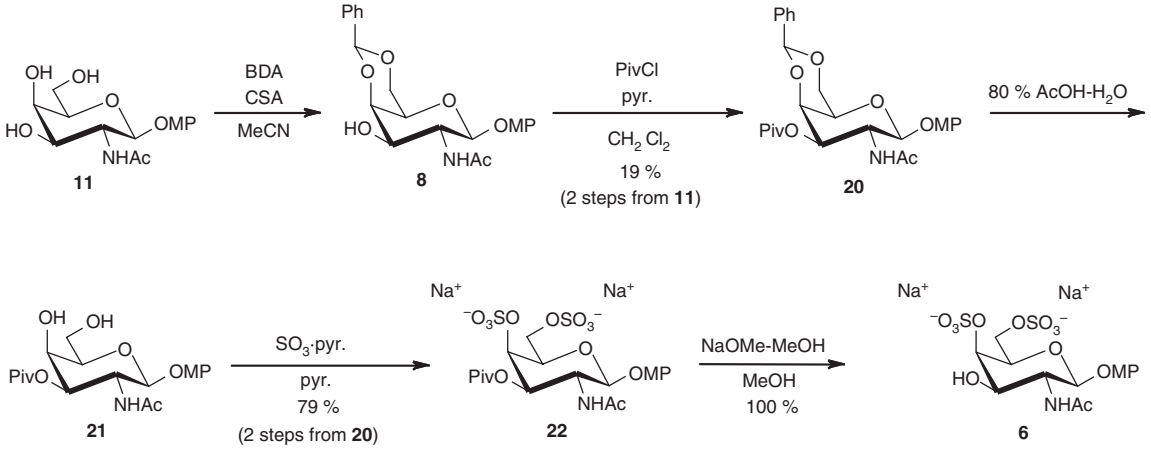
Scheme 4: Synthesis of GalNAc4S derivative **2**.



Scheme 5: Synthesis of GalNAc6S derivative **3**.



Scheme 6: Synthesis of GalNAc3S4S derivative **4**.



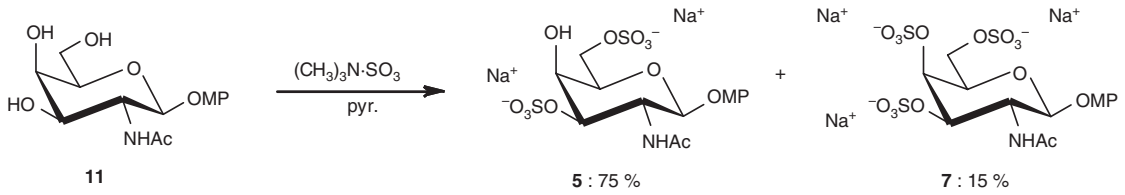
Scheme 7: Synthesis of GalNAc4S6S derivative **6**.

Compound **16** was obtained by selective 6-*O*-tritylation of **11** followed by benzoylation at the 3- and 4-positions using benzoyl chloride and pyridine. After removal of the trityl group, sulfation at the 6-position gave **18**. Benzoyl groups were deprotected by treating with sodium methoxide to give GalNAc6S **3** in good yield (Scheme 5).

In order to get the di-*O*-sulfated saccharide **19**, the common intermediate **11** was subjected to a S_N1 reaction with trityl chloride in pyridine, followed by sulfation of the free hydroxy groups. Target compound **4** was obtained by removal of the trityl group using catalytic hydrogenation with a Pd-C catalyst (Scheme 6).

Protection of **11** at the 4- and 6-positions with benzylidene acetal, followed by pivaloylation afforded compound **20**. Deprotection of the benzylidene group with 80 % AcOH-H_2O followed by sulfation at the 4- and 6-positions gave **22**. Then, sulfated compound **22** was subjected to removal of the pivaloyl group with sodium methoxide to give target compound **6** quantitatively (Scheme 7).

GalNa3S4S6S **5** and GalNAc3S6S **7** were simultaneously obtained from compound **11** by sulfation without any protection (Scheme 8). This reaction afforded di-sulfated saccharide **5** in good yield because the highly



Scheme 8: Synthesis of GalNAc3S6S derivative **5** and GalNAc3S4S6S derivative **7**.

reactive 6-hydroxy group was sulfated at first, and then sulfation at the 3-position occurred due to the repulsion of their negative charge and steric hindrance.

Now, we have completed the synthesis of *N*-acetylgalactosamine derivatives with sulfate group at the 3- to 6-positions. The ^{1}H and ^{13}C NMR spectral data of compounds **1**–**7** and **11** are summarized in Table 1.

Comparing the signal in the ^{1}H NMR spectrum between the GalNAc derivatives bearing some sulfate groups and the corresponding precursor with no sulfate group reveals a downfield shift in the spectrum of sulfated compounds. Of note, the H-3 signal was slightly affected by the sulfate group located at the 4-position and the H-4 signal of GalNAc3S **1** and GalNAc3S6S **5** shifted downfield at least 0.3 ppm compared to that of compound **11**. In GalNAc4S6S **6** and GalNAc3S4S6S **7** derivatives, the signal from H-5 shifted downfield more than 0.4 ppm.

In the ^{13}C NMR spectrum of the GalNAc derivatives, the signal of the carbon atoms bearing a *O*-sulfate group was also shifted downfield relative to that of the corresponding carbon atoms without a sulfate group. The C-5 signal for GalNAc derivatives having a sulfate group at the 6-position showed a 2.5–2.9 ppm upfield shift compared to the corresponding signal from **11**.

The coupling constants in the ^{1}H NMR for the GalNAc derivatives showed values ($J_{1,2}$ = 8.5 Hz, $J_{3,4}$ = 3.1 Hz, and $J_{4,5}$ = 0.0 Hz) typical of a galactosamine configuration. A conformation change due to the position of the sulfate groups was not observed.

Compounds **1**–**6** were tested as inhibitors against JEV infection in Vero cells. In the presence of each compound in a final concentration of 0.1 mM, the inhibitory activity relative to a control treatment without the compound is shown in Fig. 2. GalNAc4S6S **6** and GalNAc6S **3** reduced JEV infection by approximately 35 %. Since the sugar structure of **6** constitutes the CS-E, the result is reasonable for strong inhibitory activity against JEV infection. Compound **3** also inhibited JEV infection in spite of bearing only one sulfate group,

Table 1: The chemical shifts in the ^{1}H and ^{13}C NMR spectra for synthetic intermediate **11** and target sulfated GalNAc derivatives **1**–**7**.

	GalNAc 11	GalNAc 3S 1	GalNAc 4S 2	GalNAc 6S 3	GalNAc 3S4S 4	GalNAc 3S6S 5	GalNAc 4S6S 6	GalNAc 3S4S6S 7
H-1	4.86	5.03	4.93	4.85	5.12	5.00	4.92	5.09
H-2	4.03	4.18	4.04	4.04	4.13	4.17	4.06	4.17
H-3	3.70[a]	4.40	3.87	3.72	4.49	4.40	3.89	4.51
H-4	3.88	4.21	4.64	3.93	4.88	4.24	4.69	4.91
H-5	3.69[a]	3.76	3.83	3.96	3.88	4.02	4.14	4.17
H-6a	3.69[a]	3.71[a]	3.71	4.11	3.72	4.12	4.15	4.18
H-6b		3.73[a]	3.76	4.15	3.76	4.17	4.27	4.27
C-1	101.12	100.66	100.90	101.06	100.32	100.54	100.87	100.27
C-2	52.40	50.62	52.72	52.28	51.08	50.48	52.60	50.96
C-3	70.73	77.51	69.72	70.56	74.99	77.21	69.70	74.91
C-4	67.60	66.14	75.44	67.33	73.99	65.92	75.27	73.81
C-5	75.36	75.04	74.64	72.89	74.67	72.66	72.44	72.49
C-6	60.74	60.67	60.75	66.97	60.76	67.02	67.66	67.69

[a]Chemical shift was determined by peak picking of HSQC spectra.

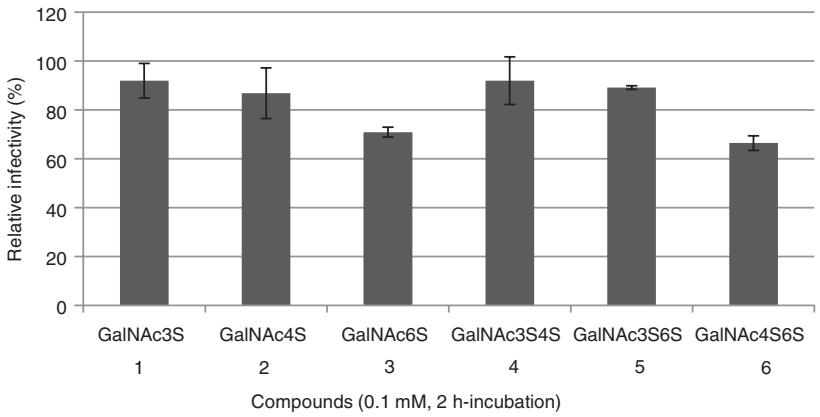


Fig. 2: Inhibitory effect of the compounds on JEV infection of Vero cells was evaluated using a real-time RT-PCR assay. The assay was performed as described in the Experimental section. Values indicate means ± SE of relative infectivity in the presence of compound at the indicated concentration to the virus alone as a control. Bars show standard deviation in the experiment.

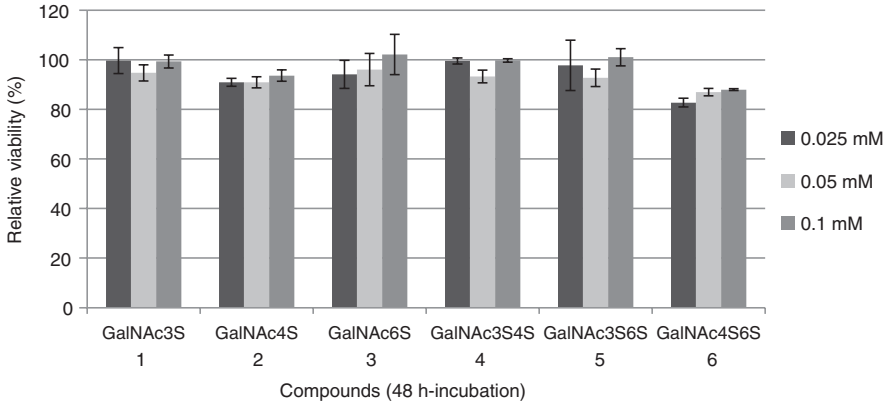


Fig. 3: Cytotoxicity of compounds **1–6**. Values were calculated after normalizing data to control cell viability based on vehicle (DMSO) treatment. Bars show standard deviation of triplicate measurements in each experiment.

suggesting that the sulfate group at the 6-position is necessary for JEV to interact with a host cell, however, GalNAc3S6S **5** showed inhibitory activity to a lower extent. This indicates that the 3-*O*-sufate group prevented the saccharide from binding to virus due to repulsion from the non-natural position of the negative charge. In contrast, a sulfate group at the 4-position might help produce an effective interaction by leading the 6-*O*-sulfate group to the appropriate binding site. In the presence of each compound up to 0.1 mM, a cytotoxic effect was not significantly observed (Fig. 3), meaning that the compounds did not affect the cell viability.

Conclusions

In the present study, an exhaustive synthisis of *p*-methoxyphenyl sulfated β-GalNAc derivatives was accomplished using a simple synthetic route from common synthetic intermediate **11**. Moreover, their inhibitory activities against JEV were evaluated, and we indentified GalNAc6S **3** and GalNAc4S6S **6** as novel monosaccharide JEV inhibitors. We found a key factor in JEV infection is that the virus may require a 6-*O*-sulfated saccharide on the host cell surface. These results contribute to the development of anti-JEV compounds with low molecular weights.

Experimental section

General

Compound **12** was prepared as previously reported [30]. All solvents were of reagent grade quality and purchased commercially. Structures of synthetic compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and two-dimensional NMR (COSY, HSQC, HMBC, and NOESY) spectroscopy. ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AVANCE III instrument operating at 400.13 and 100.62 MHz, respectively. Chemical shifts were referenced to TMS in $CDCl_3$ and δ values (ppm) of water in D_2O (^{1}H: $\delta = 4.70$) as internal standard. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectra were obtained on a Bruker Daltonics micrOTOF-QII. Thin layer chromatography (TLC) was performed on precoated Silica gel 60 F_{254} plates. Column chromatography was performed on silica gel 60 N (spherical neutral) purchased from Kanto Chemical Company, Japan.

Synthesis of *p*-methoxyphenyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranoside (13)

A solution of **12** (1.283 g, 2.93 mmol, 1.0 equiv) in dry pyridine (14.67 mL, 0.20 M) was cooled by ice-water bath, and then thioacetic acid (6.30 mL, 6.71 g, 88.1 mmol, 30 equiv) was added to the solution dropwisely. The mixture was stirred at room temperature for 4 h and then at 40 °C for 20 h. The reaction mixture was diluted with chloroform (100 mL). The organic layer was washed with 5 % aq HCl, saturated aq $NaHCO_3$, and saturated aq NaCl successively, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was separated by silica gel column chromatography eluting with 1 % methanol-chloroform to give **13** (1.126 g, 2.48 mmol, 85 %) as a colorless solid. $R_f = 0.50$ (10 % methanol-chloroform); ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ 1.98 (s, 3 H, 2-NHCOCH_3), 2.03 (s, 3 H, 3-OCOCH_3), 2.05 (s, 3 H, 6-OCOCH_3), 2.17 (s, 3 H, 4-OCOCH_3), 3.77 (s, 3 H, *p*-OCH_3), 4.03 (t, 1 H, $J_{5,6b} = 6.8$ Hz, $J_{5,6a} = 6.6$ Hz, H-5), 4.15 (dd, 1 H, $J_{6a,6b} = 11.3$ Hz, $J_{5,6a} = 6.6$ Hz, H-6a), 4.13–4.24 (m, 1 H, H-2), 4.21 (dd, 1 H, $J_{6a,6b} = 11.3$ Hz, $J_{5,6b} = 6.8$ Hz, H-6b), 5.18 (d, 1 H, $J_{1,2} = 8.4$ Hz, H-1), 5.40 (dd, 1 H, $J_{2,3} = 12.8$ Hz, $J_{3,4} = 3.3$ Hz, H-3), 5.41 (d, 1 H, $J_{3,4} = 3.3$ Hz, H-4), 5.56 (d, 1 H, $J_{2,NH} = 8.6$ Hz, 2-N*H*COCH_3), 6.78–6.83 (m, 2 H, *m*-arom. H), 6.94–6.98 (m, 2 H, *o*-arom. H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ 20.63 (q, OCO*C*H_3X2), 20.66 (q, OCO*C*H_3), 23.44 (q, 2-NHCO*C*H_3), 51.79 (d, C-2), 55.62 (q, *p*-OCH_3), 61.46 (t, C-6), 66.63 (d, C-4), 69.61 (d, C-3), 70.83 (d, C-5), 100.34 (d, C-1), 114.50 (d, *m*-arom. CH), 118.51 (d, *o*-arom. CH), 151.14 (s, arom. C), 155.58 (s, *p*-arom. C), 170.24 (s, 4-O*C*OCH_3), 170.36 (s, 6-O*C*OCH_3), 170.41 (s, 3-O*C*OCH_3), 170.44 (s, 2-NH*C*OCH_3); HRMS (APCI) m/z $[M+H]^+$ calcd for $C_{21}H_{28}NO_{10}^+$ 454.1708, found 454.1704.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-β-D-galactopyranoside (11)

A suspension of compound **13** (1.126 g, 2.48 mmol, 1.0 equiv) in dry methanol (24.83 mL, 0.10 M) was treated with 1.0 M sodium methoxide in methanol (0.37 mL, 0.37 mmol, 0.15 equiv) and stirred at room temperature for 5 h. The mixture was neutralized with Amberlite IRC-50 H^+ resin and concentrated under diminished pressure to give **11** (789 mg, 2.41 mmol, 97 %) as a colorless solid. $R_f = 0.56$ (30 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.92 (s, 3 H, 2-NHCOCH_3), 3.66–3.72 (m, 4 H, H-5, H-6, H-3), 3.69 (s, 3 H, *p*-OCH_3), 3.88 (d, 1 H, $J_{3,4} = 3.3$ Hz, H-4), 4.03 (dd, 1 H, $J_{2,3} = 10.9$ Hz, $J_{1,2} = 8.5$ Hz, H-2), 4.86 (d, 1 H, $J_{1,2} = 8.5$ Hz, H-1), 6.83–6.87 (m, 2 H, *m*-arom. H), 6.92–6.96 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.11 (q, 2-NHCO*C*H_3), 52.40 (d, C-2), 55.73 (q, *p*-OCH_3), 60.74 (t, C-6), 67.60 (d, C-4), 70.73 (d, C-3), 75.36 (d, C-5), 101.12 (d, C-1), 115.00 (d, *m*-arom. CH), 118.21 (d, *o*-arom. CH), 151.21 (s, arom. C), 154.71 (s, *p*-arom. C), 175.01 (s, 2-NH*C*OCH_3); HRMS (APCI) m/z $[M+H]^+$ calcd for $C_{15}H_{22}NO_7^+$ 328.1391, found 328.1386.

Synthesis of *p*-methoxyphenyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-sulfonato-β-D-galactopyranoside sodium salt (14)

Benzaldehyde dimethyl acetal (0.13 mL, 0.132 g, 0.866 mmol, 3.2 equiv) and D-camphor-10-sulfonic acid (6.8 mg, 29.2 μmol, 0.11 equiv) were added to a suspension of **11** (87.5 mg, 0.267 mmol, 1.0 equiv) in dry acetonitrile (3.8 mL, 70 mM), the mixture was stirred at 40 °C for 15.5 h. After cooling, the reaction was quenched with triethyl amine (1.0 mL), and the mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluting with 2 % methanol-chloroform to give **8** as colorless solid. Sulfur trioxide-pyridine complex (130.2 mg, 0.818 mmol, 3.1 equiv) was added to a solution of **8** in dry pyridine (1.66 mL, 0.16 M), and the mixture was stirred for 36.5 h at 40 °C. Methanol (2.0 mL) was added to the reaction mixture, and the resulting solution was passed through the column of Dowex 50WX8 Na^+ resin. The effluent was concentrated under diminished pressure. The residue was separated by reversed phase column chromatography eluting with 50 % water-methanol to give **14** (21.0 mg, 40.6 μmol, 15 %) as colorless solid. R_f = 0.45 (30 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.93 (s, 3 H, 2-NHCOCH_3), 3.72 (s, 3 H, *p*-OCH_3), 3.87 (s, 1 H, H-5), 4.19 (d, 1 H, $J_{6a,6b}$ = 14.6 Hz, H-6a), 4.22 (d, 1 H, $J_{6a,6b}$ = 14.6 Hz, H-6b), 4.29 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{1,2}$ = 8.7 Hz, H-2), 4.58 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{3,4}$ = 3.0 Hz, H-3), 4.63 (d, 1 H, $J_{3,4}$ = 3.0 Hz, H-4), 5.19 (d, 1 H, $J_{1,2}$ = 8.7 Hz, H-1), 5.73 (s, 1 H, 4,6-OCHC$_6$H$_5$), 6.88–6.90 (m, 2 H, *m*-arom. H of 1-OMP), 7.02–7.04 (m, 2 H, *o*-arom. H of 1-OMP), 7.39–7.41 (m, 3 H, *p*-arom. H and *m*-arom. H of benzylidene), 7.51–7.54 (m, 2 H, *o*-arom. H of benzylidene); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.18 (q, 2-NHCOCH$_3$), 50.28 (d, C-2), 55.81 (q, *p*-OCH_3), 66.54 (d, C-5), 68.66 (t, C-6), 73.38 (d, C-4), 75.43 (d, C-3), 100.37 (d, C-1), 101.28 (d, 4,6-OCHC$_6$H$_5$), 115.05 (d, *m*-arom. CH of 1-OMP), 118.70 (d, *o*-arom. CH of 1-OMP), 126.53 (d, *o*-arom. CH of benzylidene), 128.70 (d, *m*-arom. CH of benzylidene), 129.94 (d, *p*-arom. CH of benzylidene), 136.68 (s, arom. C of benzylidene), 150.88 (s, arom. C of 1-OMP), 154.96 (s, *p*-arom. C of 1-OMP), 174.95 (s, 2-NHCOCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{22}H_{24}NO_{10}S^-$ 494.1126, found 494.1121.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-3-*O*-sulfonato-β-D-galactopyranoside sodium salt (1)

A solution of **14** (17.8 mg, 34.4 μmol, 1.0 equiv) in 80 % acetic acid-water (0.40 mL) was stirred at room temperature for 3 h. 80 % acetic acid-water (0.20 mL) was added to the mixture, and then it was stirred at 40 °C for 3 h. The mixture was concentrated under reduced pressure. The residue was diluted with methanol and passed through the column of Dowex 50WX8 Na^+ resin. The effluent was evaporated under reduced pressure. The residue was separated by reversed phase column chromatography eluting with 85 % water-methanol to give **1** (9.9 mg, 23.1 μmol, 67 %) as a colorless solid. R_f = 0.13 (30 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.92 (s, 3 H, 2-NHCOCH_3), 3.71 (s, 3 H, *p*-OCH_3), 3.67–3.76 (m, 2 H, H-6), 3.76 (dd, 1 H, $J_{5,6b}$ = 9.9 Hz, $J_{5,6a}$ = 5.9 Hz, H-5), 4.18 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{1,2}$ = 8.6 Hz, H-2), 4.21 (d, 1 H, $J_{3,4}$ = 3.1 Hz, H-4), 4.40 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.1 Hz, H-3), 5.03 (d, 1 H, $J_{1,2}$ = 8.6 Hz, H-1), 6.85–6.89 (m, 2 H, *m*-arom. H), 6.95–6.99 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.18 (q, 2-NHCOCH$_3$), 50.62 (d, C-2), 55.79 (q, *p*-OCH_3), 60.67 (t, C-6), 66.14 (d, C-4), 75.04 (d, C-5), 77.51 (d, C-3), 100.66 (d, C-1), 115.05 (d, *m*-arom. CH), 118.43 (d, *o*-arom. CH), 151.06 (s, arom. C), 154.85 (s, *p*-arom. C), 174.93 (s, 2-NHCOCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{15}H_{20}NO_{10}S^-$ 406.0813, found 406.0830.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-3,6-di-*O*-pivaloyl-β-D-galactopyranoside (10)

Pivaloyl chloride (0.12 mL, 118 mg, 0.975 mmol, 3.9 equiv) was added to a solution of **11** (82.7 mg, 0.253 mmol, 1.0 equiv) in dry pyridine (3.53 mL, 72 mM), and the mixture was stirred at – 10 °C for 2.5 h. After addition of methanol (1.0 mL), the mixture was concentrated under diminished pressure. The residue was diluted

with chloroform (20 mL) and washed with 2.5 % aq HCl (20 mL). The aqueous phase was extracted with chloroform (5 mL × 3). The combined organic phase was washed with 2.5 % aq HCl (15 mL × 2), saturated aq $NaHCO_3$ (15 mL × 2), and saturated aq NaCl (15 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was separated by silica gel chromatography eluting with chloroform to afford **10** (111.6 mg, 0.225 mmol, 89 %) as a colorless amorphous. R_f = 0.26 (50 % ethyl acetate-hexane); ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ 1.16 (s, 9 H, 6-OCOC(CH_3)$_3$), 1.21 (s, 9 H, 3-OCOC(CH_3)$_3$), 1.93 (s, 3 H, 2-NHCOCH_3), 2.99 (d, 1 H, $J_{4,OH}$ = 6.0 Hz, 4-OH), 3.73 (s, 3 H, *p*-OCH$_3$), 3.91 (dd, 1 H, $J_{5,6b}$ = 7.3 Hz, $J_{5,6a}$ = 4.8 Hz, H-5), 4.02 (dd, 1 H, $J_{4,OH}$ = 6.0 Hz, $J_{3,4}$ = 3.1 Hz, H-4), 4.28 (dd, 1 H, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6a}$ = 4.8 Hz, H-6a), 4.31 (dd, 1 H, $J_{6a,6b}$ = 11.6 Hz, $J_{5,6b}$ = 7.3 Hz, H-6b), 4.51 (dt, 1 H, $J_{2,3}$ = 11.1 Hz, $J_{2,NH}$ = 9.0 Hz, $J_{1,2}$ = 8.8 Hz, H-2), 4.97 (d, 1 H, $J_{1,2}$ = 8.8 Hz, H-1), 5.19 (dd, 1 H, $J_{2,3}$ = 11.1 Hz, $J_{3,4}$ = 3.1 Hz, H-3), 6.42 (d, 1 H, $J_{2,NH}$ = 9.0 Hz, 2-N*H*COCH$_3$), 6.66–6.69 (m, 2 H, *m*-arom. H), 6.87–6.89 (m, 2 H, *o*-arom. H); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ 23.14 (q, 2-NHCO*C*H$_3$), 26.97 (q, 3-OCOC(*C*H$_3$)$_3$), 27.06 (q, 6-OCOC(*C*H$_3$)$_3$), 38.66 (s, 6-OCO*C*(CH$_3$)$_3$), 39.02 (s, 3-OCO*C*(CH$_3$)$_3$), 50.59 (d, C-2), 55.52 (q, *p*-OCH$_3$), 63.13 (t, C-6), 66.97 (d, C-4), 72.40 (d, C-3), 72.58 (d, C-5), 100.93 (d, C-1), 114.33 (d, *m*-arom. CH), 118.55 (d, *o*-arom. CH), 151.39 (s, arom. C), 155.32 (s, *p*-arom. C), 170.45 (s, 2-NH*C*OCH$_3$), 178.21 (s, 6-O*C*OC(CH$_3$)$_3$), 178.50 (s, 3-O*C*OC(CH$_3$)$_3$); HRMS (FAB) m/z [M + H]$^+$ calcd for $C_{25}H_{38}NO_9^+$ 496.2541, found 496.2547.

Synthesis *p*-methoxyphenyl 2-acetamido-2-deoxy-3,6-di-*O*-pivaloyl-4-*O*-sulfonato-β-D-galactopyranoside sodium salt (15)

A mixture of **10** (63 mg, 0.127 mmol, 1.0 equiv) and sulfur trioxide-pyridine complex (101 mg, 0.635 mmol, 5.0 equiv) in dry pyridine (3.5 mL, 36 mM) was stirred for 49 h at room temperature. Methanol (5.0 mL) was then added, and the mixture was passed through the column of Dowex 50WX8 Na$^+$ resin. The effluent was concentrated under reduced pressure. The residue was separated by silica gel column chromatography eluting with 15 % methanol-chloroform to give **15** (69 mg, 0.115 mmol, 91 %) as colorless crystals. R_f = 0.50 (20 % methanol-chloroform); ^{1}H NMR (CD_3OD, 400.13 MHz): δ 1.20 (s, 9 H, 6-OCOC(CH_3)$_3$), 1.24 (s, 9 H, 3-OCOC(CH_3)$_3$), 1.95 (s, 3 H, 2-NHCOCH_3), 3.75 (s, 3 H, *p*-OCH$_3$), 4.04 (dd, 1 H, $J_{5,6a}$ = 8.2 Hz, $J_{5,6b}$ = 3.7 Hz, H-5), 4.39 (dd, 1 H, $J_{6a,6b}$ = 11.9 Hz, $J_{5,6a}$ = 8.2 Hz, H-6a), 4.36–4.41 (m, 1 H, H-2), 4.44 (dd, 1 H, $J_{6a,6b}$ = 11.9 Hz, $J_{5,6b}$ = 3.7 Hz, H-6b), 4.85 (d, 1 H, $J_{3,4}$ = 3.3 Hz, H-4), 5.07 (d, 1 H, $J_{1,2}$ = 8.7 Hz, H-1), 5.06–5.09 (m, 1 H, H-3), 6.79–6.84 (m, 2 H, *m*-arom. H), 6.98–7.02 (m, 2 H, *o*-arom. H); ^{13}C NMR (CD_3OD, 100.62 MHz): δ 21.47 (q, 2-NHCO*C*H$_3$), 26.18 (q, 6-OCOC(*C*H$_3$)$_3$), 26.23 (q, 3-OCOC(*C*H$_3$)$_3$), 38.36 (s, 6-OCO*C*(CH$_3$)$_3$), 38.56 (s, 3-OCO*C*(CH$_3$)$_3$), 50.48 (d, C-2), 54.68 (q, *p*-OCH$_3$), 64.25 (t, C-6), 70.95 (d, C-3), 71.79 (d, C-4), 72.49 (d, C-5), 100.41 (d, C-1), 114.10 (d, *m*-arom. CH), 118.12 (d, *o*-arom. CH), 151.56 (s, arom. C), 155.53 (s, *p*-arom. C), 171.97 (s, 2-NH*C*OCH$_3$), 178.36 (s, 6-O*C*OC(CH$_3$)$_3$), 178.41 (s, 3-O*C*OC(CH$_3$)$_3$); HRMS (FAB) m/z [M + Na]$^+$ calcd for $C_{25}H_{36}NNa_2O_{12}S^+$ 620.1748, found 620.1755.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-4-*O*-sulfonato-β-D-galactopyranoside sodium salt (2)

A solution of **15** (50 mg, 83.7 μmol, 1.0 equiv) in dry methanol (1.0 mL, 84 mM) was treated with 1.0 M sodium methoxide-methanol solution (0.17 mL, 0.170 mmol, 2.0 equiv) for 20 h at room temperature and then for 6 h at 40 °C. The mixture was neutralized with Amberlite IRC-50 H$^+$ resin and concentrated under diminished pressure. The residue was purified by reversed phase column chromatography eluting with 90 % and 85 % water-methanol to give **2** (29.0 mg, 67.5 μmol, 81 %) as a colorless powder. R_f = 0.49 (30 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.93 (s, 3 H, 2-NHCOCH_3), 3.70 (s, 3 H, *p*-OCH$_3$), 3.71 (dd, 1 H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6a}$ = 7.7 Hz, H-6a), 3.76 (dd, 1 H, $J_{6a,6b}$ = 12.0 Hz, $J_{5,6b}$ = 4.6 Hz, H-6b), 3.83 (dd, 1 H, $J_{5,6a}$ = 7.7 Hz, $J_{5,6b}$ = 4.6 Hz, H-5), 3.87 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 4.04 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{1,2}$ = 8.4 Hz, H-2), 4.64 (d, 1 H, $J_{3,4}$ = 3.2 Hz,

H-4), 4.93 (d, 1 H, $J_{1,2}$ = 8.4 Hz, H-1), 6.84–6.88 (m, 2 H, *m*-arom. H), 6.93–6.97 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.11 (q, 2-NHCO*C*H$_3$), 52.72 (d, C-2), 55.73 (q, *p*-O*C*H$_3$), 60.75 (t, C-6), 69.72 (d, C-3), 74.64 (d, C-5), 75.44 (d, C-4), 100.90 (d, C-1), 115.00 (d, *m*-arom. CH), 118.33 (d, *o*-arom. CH), 151.05 (s, arom. C), 154.80 (s, *p*-arom. C), 174.99 (s, 2-NH*C*OCH$_3$); HRMS (FAB) m/z [M + Na]$^+$ calcd for $C_{15}H_{20}NNa_2O_{10}S^+$ 452.0598, found 452.0614.

Synthesis of *p*-methoxyphenyl 2-acetamido-3,4-di-*O*-benzoyl-2-deoxy-6-*O*-trityl-β-D-galactopyranoside (16)

A mixture of **11** (355.4 mg, 1.09 mmol, 1.0 equiv) and trityl chloride (956 mg, 3.43 mmol, 3.2 equiv) in dry pyridine (10.88 mL, 0.10 M) was stirred for 24.5 h at 40 °C. Methanol (4.0 mL) was added, and the reaction mixture was evaporated under reduced pressure. The residue was dissolved in dry pyridine (5.5 mL) and CH_2Cl_2 (2.8 mL, 0.13 M), benzoyl chloride (0.277 mL, 0.336 g, 2.39 mmol, 2.2 equiv) was added to the solution. The mixture was stirred at 0 °C for 3 h and then 5 °C for 14 h. Methanol (1.0 mL) was then added, the reaction mixture was diluted with chloroform (50 mL). The organic phase was washed with 2.5 % aq HCl (30 mL × 2), saturated aq $NaHCO_3$ (30 mL × 2), and saturated aq NaCl (30 mL × 1), dried over Na_2SO_4, and concentrated under diminished pressure. The residue was separated by silica gel column chromatography eluting with 40 % ethyl acetate-hexane to give **16** (678.8 mg, 0.873 mmol, 80 %) as a colorless amorphous. R_f = 0.43 (50 % ethyl acetate-hexane); ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ 1.88 (s, 3 H, 2-NHCOC*H*$_3$), 3.26 (dd, 1 H, $J_{6a,\,6b}$ = 9.5 Hz, $J_{5,6a}$ = 6.8 Hz, H-6a), 3.51 (dd, 1 H, $J_{6a,6b}$ = 9.5 Hz, $J_{5,6b}$ = 6.5 Hz, H-6b), 3.76 (s, 3 H, *p*-OCH$_3$), 4.01 (t, 1 H, $J_{5,6a}$ = 6.8 Hz, $J_{5,6b}$ = 6.5 Hz, H-5), 4.45 (dt, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{2,NH}$ = 8.7 Hz, $J_{1,2}$ = 8.6 Hz, H-2), 5.21 (d, 1 H, $J_{1,2}$ = 8.6 Hz, H-1), 5.54–5.61 (m, 1 H, 2-N*H*COCH$_3$), 5.66 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 3.1 Hz, H-3), 5.86 (d, 1 H, $J_{3,4}$ = 3.1 Hz, H-4), 6.77–6.82 (m, 2 H, *m*-arom. H of 1-OMP), 7.02–7.06 (m, 2 H, *o*-arom. H of 1-OMP), 7.12–7.16 (m, 3 H, *p*-arom. H of 6-OTr), 7.16–7.20 (m, 6 H, *m*-arom. H of 6-OTr), 7.30–7.33 (m, 2 H, *m*-arom. H of 3-OBz), 7.36–7.38 (m, 6 H, *o*-arom. H of 6-OTr), 7.40–7.44 (m, 2 H, *m*-arom. H of 4-OBz), 7.48–7.52 (m, 1 H, *p*-arom. H of 3-OBz), 7.57–7.61 (m, 1 H, *p*-arom. H of 4-OBz), 7.83–7.85 (m, 2 H, *o*-arom. H of 3-OBz), 7.93–7.95 (m, 2 H, *o*-arom. H of 4-OBz); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ 23.46 (q, 2-NHCO*C*H$_3$), 52.21 (d, C-2), 55.65 (q, *p*-O*C*H$_3$), 61.57 (t, C-6), 67.80 (d, C-4), 70.89 (d, C-3), 72.88 (d, C-5), 87.03 (s, 6-O*C*Ph$_3$), 100.81 (d, C-1), 114.56 (d, *m*-arom. CH of 1-OMP), 118.47 (d, *o*-arom. CH of 1-OMP), 127.04 (d, *p*-arom. CH of 6-OTr), 127.83 (d, *m*-arom. CH of 6-OTr), 128.38 (d, *m*-arom. CH of 3-OBz), 128.43 (d, *m*-arom. CH of 4-OBz), 128.58 (d, *o*-arom. CH of 6-OTr), 129.03 (s, arom. C of 3-OBz), 129.42 (s, arom. C of 4-OBz), 129.92 (d, *o*-arom. CH of 3-OBz), 130.00 (d, *o*-arom. CH of 4-OBz), 133.25 (d, *p*-arom. CH of 4-OBz), 133.37 (d, *p*-arom. CH of 3-OBz), 143.43 (s, arom. C of 6-OTr), 151.44 (s, arom. C of 1-OMP), 155.47 (s, *p*-arom. C of 1-OMP), 165.41 (s, 4-O*C*OC$_6$H$_5$), 166.17 (s, 3-O*C*OC$_6$H$_5$), 170.36 (s, 2-NH*C*OCH$_3$); HRMS (APCI) m/z [M + H]$^+$ calcd for $C_{48}H_{44}NO_9^+$ 778.3011, found 778.3009.

Synthesis of *p*-methoxyphenyl 2-acetamido-3,4-di-*O*-benzoyl-2-deoxy-6-*O*-sulfonato-β-D-galactopyranoside sodium salt (18)

A solution of **16** (182.1 mg, 0.234 mmol, 1.0 equiv) in 80 % acetic acid-water (1.17 mL) was stirred at 80 °C for 1 h. After cooling, triethylamine (2.6 mL) was added to the mixture. The product was extracted by chloroform (5 mL × 3). The organic phase was washed with saturated aq $NaHCO_3$ (30 mL × 3) and saturated aq NaCl (30 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The residue was dissolved in dry pyridine (2.34 mL, 0.10 M) and sulfur trioxide-pyridine complex (180 mg, 1.13 mmol, 4.8 equiv) was added to the mixture. The mixture was stirred for 15.5 h at room temperature and then for 25.5 h at 40 °C. Methanol (1 mL) was added to the reaction mixture, and the resulting solution was passed through the column of Dowex 50WX8 Na^+ resin. The effluent was concentrated under reduced pressure. The residue was separated by reversed phase column chromatography eluting with 40 % water-methanol to afford **18** (101.4 mg,

0.159 mmol, 68 %) as a colorless solid. R_f = 0.40 (20 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.79 (s, 3 H, 2-NHCOCH_3), 3.72 (s, 3 H, *p*-OCH_3), 4.15 (d, 1 H, $J_{5,6}$ = 6.3 Hz, H-6), 4.48 (t, 1 H, $J_{5,6}$ = 6.3 Hz, H-5), 4.60 (dd, 1 H, $J_{2,3}$ = 10.8 Hz, $J_{1,2}$ = 9.0 Hz, H-2), 5.28 (d, 1 H, $J_{1,2}$ = 9.0 Hz, H-1), 5.46 (dd, 1 H, $J_{2,3}$ = 10.8 Hz, $J_{3,4}$ = 3.0 Hz, H-3), 5.79 (d, 1 H, $J_{3,4}$ = 3.0 Hz, H-4), 6.89–6.91 (m, 2 H, *m*-arom. H of 1-OMP), 7.05–7.07 (m, 2 H, *o*-arom. H of 1-OMP), 7.27–7.31 (m, 2 H, *m*-arom. H of 3-OBz), 7.44–7.48 (m, 2 H, *m*-arom. H of 4-OBz), 7.50–7.53 (m, 1 H, *p*-arom. H of 3-OBz), 7.61–7.64 (m, 1 H, *p*-arom. H of 4-OBz), 7.68–7.70 (m, 2 H, *o*-arom. H of 3-OBz), 7.95–7.97 (m, 2 H, *o*-arom. H of 4-OBz); ^{13}C NMR (D_2O, 100.62 MHz): δ 21.86 (q, 2-NHCOCH_3), 50.41 (d, C-2), 55.76 (q, *p*-OCH_3), 65.54 (t, C-6), 67.55 (d, C-4), 71.21 (d, C-5), 71.48 (d, C-3), 100.41 (d, C-1), 115.06 (d, *m*-arom. CH of 1-OMP), 118.61 (d, *o*-arom. CH of 1-OMP), 128.06 (s, arom. C of 3-OBz), 128.33 (s, arom. C of 4-OBz), 128.73 (d, *m*-arom. CH of 3-OBz), 128.84 (d, *m*-arom. CH of 4-OBz), 129.40 (d, *o*-arom. CH of 3-OBz), 129.75 (d, *o*-arom. CH of 4-OBz), 134.21 (d, *p*-arom. CH of 3-OBz), 134.34 (d, *p*-arom. CH of 4-OBz), 150.73 (s, arom. C of 1-OMP), 155.04 (s, *p*-arom. C of 1-OMP), 167.00 (s, 3-O*C*OC$_6H_5$), 167.43 (s, 4-O*C*OC$_6H_5$), 174.68 (s, 2-NH*C*OCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{29}H_{28}NO_{12}S^-$ 614.1338, found 614.1325.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-6-*O*-sulfonato-β-D-galactopyranoside sodium salt (3)

Compound **18** (49.7 mg, 77.9 μmol, 1.0 equiv) was dissolved in dry methanol (1.0 mL, 78 mM) and treated with 1 M sodium methoxide in methanol (0.16 mL, 0.160 mmol, 2.1 equiv) for 7.5 h at room temperature. The mixture was neutralized with Amberlite IRC-50 H$^+$ resin and concentrated under reduced pressure. The residue was purified by reversed phase column chromatography eluting with 90 % and 85 % water-methanol to afforded **3** (33.4 mg, 77.8 μmol, 100 %) as colorless solid. R_f = 0.56 (50 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.92 (s, 3 H, 2-NHCOCH_3), 3.68 (s, 3 H, *p*-OCH_3), 3.72 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{3,4}$ = 3.3 Hz, H-3), 3.93 (d, 1 H, $J_{3,4}$ = 3.3 Hz, H-4), 3.96 (dd, 1 H, $J_{5,6a}$ = 7.1 Hz, $J_{5,6b}$ = 5.3 Hz, H-5), 4.04 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{1,2}$ = 8.5 Hz, H-2), 4.11 (dd, 1 H, $J_{6a,6b}$ = 10.8 Hz, $J_{5,6a}$ = 7.1 Hz, H-6a), 4.15 (dd, 1 H, $J_{6a,6b}$ = 10.8 Hz, $J_{5,6b}$ = 5.3 Hz, H-6b), 4.85 (d, 1 H, $J_{1,2}$ = 8.5 Hz, H-1), 6.83–6.87 (m, 2 H, *m*-arom. H), 6.93–6.97 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.11 (q, 2-NHCOCH_3), 52.28 (d, C-2), 55.73 (q, *p*-OCH_3), 66.97 (t, C-6), 67.33 (d, C-4), 70.56 (d, C-3), 72.89 (d, C-5), 101.06 (d, C-1), 115.00 (d, *m*-arom. CH), 118.17 (d, *o*-arom. CH), 151.25 (s, arom. C), 154.70 (s, *p*-arom. C), 175.00 (s, 2-NH*C*OCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{15}H_{20}NO_{10}S^-$ 406.0813, found 406.0835.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-3,4-di-*O*-sulfonato-6-*O*-trityl-β-D-galactopyranoside disodium salts (19)

A mixture of **11** (21.8 mg, 66.6 μmol, 1.0 equiv) and trityl chloride (55.7 mg, 0.200 mmol, 3.0 equiv) in dry pyridine (0.61 mL, 0.11 M) was stirred for 19 h at 40 °C. Methanol (2.0 mL) was then added, and the mixture was concentrated under reduced pressure. The residue was dissolved in chloroform and purified by silica gel column chromatography eluting with 5 % methanol-chloroform to give **9** as colorless amorphous. Sulfur trioxide-pyridine complex (68.1 mg, 0.428 mmol, 6.4 equiv) was added to a solution of **9** in dry pyridine (1.00 mL, 67 mM), and the mixture was stirred for 14.5 h at room temperature. Methanol (2 mL) was added to the reaction mixture, and the resulting solution was passed through the column of Dowex 50WX8 Na$^+$ resin. The effluent was concentrated under reduced pressure. The residue was separated by reversed phase column chromatography eluting with 50 %, 40 %, 30 %, and 20 % water-methanol to give **19** (24.6 mg, 31.8 μmol, 48 %) as a colorless solid. R_f = 0.35 (50 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.95 (s, 3 H, 2-NHCOCH_3), 3.08 (d, 1 H, $J_{6a,6b}$ = 10.9 Hz, H-6a), 3.57 (dd, 1 H, $J_{6a,6b}$ = 10.9 Hz, $J_{5,6b}$ = 9.5 Hz, H-6b), 3.62 (s, 3 H, *p*-OCH_3), 3.70 (d, 1 H, $J_{5,6b}$ = 9.5 Hz, H-5), 4.09 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{1,2}$ = 8.6 Hz, H-2), 4.41 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{3,4}$ = 3.1 Hz, H-3), 4.58 (d, 1 H, $J_{3,4}$ = 3.1 Hz, H-4), 5.27 (d, 1 H, $J_{1,2}$ = 8.6 Hz, H-1), 6.86–6.88 (m, 2 H, *m*-arom. H of 1-OMP), 7.16–7.19 (m, 2 H, *o*-arom. H of 1-OMP), 7.20–7.26 (m, 9 H, *p*- and *m*-arom. H of 6-OTr),

7.33–7.35 (m, 6 H, *o*-arom. H of 6-OTr); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.29 (q, 2-NHCO*C*H$_3$), 51.18 (d, C-2), 55.78 (q, *p*-OCH$_3$), 64.08 (t, C-6), 73.74 (d, C-5), 74.26 (d, C-4), 75.18 (d, C-3), 87.11 (s, 6-O*C*Ph$_3$), 99.17 (d, C-1), 115.01 (d, *m*-arom. CH of 1-OMP), 118.80 (d, *o*-arom. CH of 1-OMP), 127.53 (d, *p*-arom. CH of 6-OTr), 128.19 (d, *m*-arom. CH of 6-OTr), 128.42 (d, *o*-arom. CH of 6-OTr), 143.41 (s, arom. C of 6-OTr), 150.34 (s, arom. C of 1-OMP), 154.89 (s, *p*-arom. C of 1-OMP), 175.02 (s, 2-NH*C*OCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{34}H_{33}NNaO_{13}S_2^-$ 750.1296 found 750.1282.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-3,4-di-*O*-sulfonato-β-D-galactopyranoside disodium salts (4)

To a solution of **19** (10 mg, 12.9 μmol) in 80 % methanol-water (1.7 mL, 7.6 mM) was added 10 % Pd-C (11 mg) and the mixture was stirred for 23 h at room temperature under hydrogen. The reaction mixture was filtered through Celite and concentrated under reduced pressure. The residue was purified by reversed phase column chromatography eluting with 80 % water-methanol and then HPLC (XSelect CSH C_{18}, 4.6 × 250 mm, 5 μm) eluting with water to give **4** (4.5 mg, 8.47 μmol, 66 %) as a colorless solid. R_f = 0.45 (30 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.92 (s, 3 H, 2-NHCOC*H*$_3$), 3.70 (s, 3 H, *p*-OCH$_3$), 3.72 (dd, 1 H, $J_{6a,6b}$ = 12.1 Hz, $J_{5,6a}$ = 7.5 Hz, H-6a), 3.76 (dd, 1 H, $J_{6a,6b}$ = 12.1 Hz, $J_{5,6b}$ = 4.7 Hz, H-6b), 3.88 (dd, 1 H, $J_{5,6a}$ = 7.5 Hz, $J_{5,6b}$ = 4.7 Hz, H-5), 4.13 (dd, 1 H, $J_{2,3}$ = 11.1 Hz, $J_{1,2}$ = 8.5 Hz, H-2), 4.49 (dd, 1 H, $J_{2,3}$ = 11.1 Hz, $J_{3,4}$ = 3.1 Hz, H-3), 4.88 (d, 1 H, $J_{3,4}$ = 3.1 Hz, H-4), 5.12 (d, 1 H, $J_{1,2}$ = 8.5 Hz, H-1), 6.85–6.89 (m, 2 H, *m*-arom. H), 6.96–7.00 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.20 (q, 2-NHCO*C*H$_3$), 51.08 (d, C-2), 55.77 (q, *p*-OCH$_3$), 60.76 (t, C-6), 73.99 (d, C-4), 74.67 (d, C-5), 74.99 (d, C-3), 100.32 (d, C-1), 115.03 (d, *m*-arom. CH), 118.49 (d, *o*-arom. CH), 150.90 (s, arom. C), 154.91 (s, *p*-arom. C), 174.95 (s, 2-NH*C*OCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{15}H_{19}NNaO_{13}S_2^-$ 508.0201, found 508.0234.

Synthesis of *p*-methoxyphenyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-pivaloyl-β-D-galactopyranoside (20)

A mixture of **11** (130 mg, 0.397 mmol, 1.0 equiv), benzaldehyde dimethyl acetal (0.34 mL, 0.345 g, 2.27 mmol, 5.7 equiv), and D-camphor-10-sulfonic acid (9.1 mg, 41.7 μmol, 0.10 equiv) in dry acetonitrile (10.2 mL, 39 mM) was stirred at room temperature for 14 h and then at 40 °C for 23.5 h. After cooling, the reaction was quenched with triethylamine (10 μL). The mixture was concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluting with 5 % methanol-chloroform to give **8** as a colorless solid. Pivaloyl chloride (0.21 mL, 206 mg, 1.71 mmol, 4.3 equiv) and dry pyridine (0.14 mL, 137 mg, 1.73 mmol, 4.4 equiv) were added to a solution of **8** in dry dichloromethane (3.80 mL, 0.10 M). The mixture was stirred for 11 h at room temperature. Methanol (0.2 mL) was added to the mixture. The resulting solution was diluted with chloroform (50 mL), and washed with 5 % aq HCl (30 mL × 3), saturated aq $NaHCO_3$ (30 mL × 2), and saturated aq NaCl (30 mL). The organic layer was dried over Na_2SO_4, and concentrated under reduced pressure. Purification was carried out by silica gel column chromatography eluting with chloroform to give **20** (37 mg, 74.1 μmol, 19 %) as a colorless solid. R_f = 0.54 (10 % methanol-chloroform); ^{1}H NMR ($CDCl_3$, 400.13 MHz): δ 1.21 (s, 9 H, 3-OCOC(C*H*$_3$)$_3$), 1.96 (s, 3 H, 2-NHCOC*H*$_3$), 3.65 (d, 1 H, $J_{5,6b}$ = 1.1 Hz, H-5), 3.76 (s, 3 H, *p*-OCH$_3$), 4.09 (dd, 1 H, $J_{6a,6b}$ = 12.4 Hz, $J_{5,6a}$ = 1.4 Hz, H-6a), 4.18 (dt, 1 H, $J_{2,3}$ = 11.3 Hz, $J_{1,2}$ = 8.3 Hz, $J_{2,NH}$ = 8.1 Hz, H-2), 4.36 (dd, 1 H, $J_{6a,6b}$ = 12.4 Hz, $J_{5,6b}$ = 1.1 Hz, H-6b), 4.43 (d, 1 H, $J_{3,4}$ = 3.5 Hz, H-4), 5.40 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1), 5.47 (dd, 1 H, $J_{2,3}$ = 11.3 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 5.53 (d, 1 H, $J_{2,NH}$ = 8.1 Hz, 2-N*H*COCH$_3$), 5.56 (s, 1 H, 4,6-OC*H*C$_6$H$_5$), 6.77–6.81 (m, 2 H, *m*-arom. H of 1-OMP), 6.98–7.02 (m, 2 H, *o*-arom. H of 1-OMP), 7.34–7.39 (m, 3 H, *p*- and *m*-arom. H of benzylidene), 7.50–7.52 (m, 2 H, *o*-arom. H of benzylidene); ^{13}C NMR ($CDCl_3$, 100.62 MHz): δ 23.48 (q, 2-NHCO*C*H$_3$), 27.02 (q, 3-OCOC(*C*H$_3$)$_3$), 39.01 (s, 3-OCO*C*(CH$_3$)$_3$), 52.04 (d, C-2), 55.63 (q, *p*-OCH$_3$), 66.51 (d, C-5), 69.12 (t, C-6), 69.89 (d, C-3), 72.97 (d, C-4), 99.88 (d, C-1), 100.49 (d, 4,6-O*C*HC$_6$H$_5$), 114.43 (d, *m*-arom. CH of 1-OMP), 119.12 (d, *o*-arom. CH of 1-OMP), 126.06 (d, *o*-arom. CH of benzylidene), 128.09 (d, *m*-arom. CH of benzylidene), 128.83 (d, *p*-arom. CH of benzylidene), 137.67 (s, arom. C of benzylidene), 151.29 (s, arom. C of 1-OMP), 155.48

(s, *p*-arom. C of 1-OMP), 170.25 (s, 2-NH*C*OCH$_3$), 178.24 (s, 3-O*C*OC(CH$_3$)$_3$); HRMS (APCI) m/z [M+H]$^+$ calcd for $C_{27}H_{34}NO_8^+$ 500.2279, found 500.2270.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-3-*O*-pivaloyl-4,6-di-*O*-sulfonato-β-D-galactopyranoside disodium salts (22)

Galactosamine **20** (37 mg, 74.1 μmol, 1.0 equiv) was dissolved in 80 % acetic acid-water (5.0 mL) and stirred at 80 °C for 2 h. The mixture was concentrated under reduced pressure. Then, the residue was diluted with chloroform (50 mL) and washed with saturated aq $NaHCO_3$ (20 mL). The aqueous phase was extracted by chloroform (5 mL × 3). The combined organic layer was dried over Na_2SO_4 and evaporated under diminished pressure. The residue was dissolved in dry pyridine (1.0 mL, 74 mM), sulfur trioxide–pyridine complex (142 mg, 0.892 mmol, 12 equiv) was added to the solution, and the mixture was stirred for 64.5 h at 30 °C. Methanol (1.0 mL) was added to the reaction mixture, and the resulting solution was passed through the column of Dowex 50WX8 Na^+ resin. The effluent was concentrated under diminished pressure. The residue was separated by reversed phase column chromatography eluting with 95 % and 90 % water-methanol to give **22** (38.7 mg, 62.9 μmol, 85 %) as a colorless solid. R_f = 0.15 (30 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.08 (s, 9 H, 3-OCOC(C*H*$_3$)$_3$), 1.90 (s, 3 H, 2-NHCOC*H*$_3$), 3.71 (s, 3 H, *p*-OCH$_3$), 4.13–4.28 (m, 3 H, H-6, H-5), 4.28 (dd, 1 H, $J_{2,3}$ = 11.3 Hz, $J_{1,2}$ = 8.5 Hz, H-2), 4.80 (d, 1 H, $J_{3,4}$ = 3.2 Hz, H-4), 4.98 (dd, 1 H, $J_{2,3}$ = 11.3 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 5.06 (d, 1 H, $J_{1,2}$ = 8.5 Hz, H-1), 6.86–6.90 (m, 2 H, *m*-arom. H), 6.99–7.03 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 21.96 (q, 2-NHCO*C*H$_3$), 26.22 (q, 3-OCOC(*C*H$_3$)$_3$), 38.70 (s, 3-OCO*C*(CH$_3$)$_3$), 50.18 (d, C-2), 55.78 (q, *p*-OCH$_3$), 67.50 (t, C-6), 70.96 (d, C-3), 72.41 (d, C-5), 72.49 (d, C-4), 100.23 (d, C-1), 115.07 (d, *m*-arom. CH), 118.42 (d, *o*-arom. CH), 150.96 (s, arom. C), 154.92 (s, *p*-arom. C), 174.54 (s, 2-NH*C*OCH$_3$), 180.64 (s, 3-O*C*OC(CH$_3$)$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{20}H_{27}NNaO_{14}S_2^-$ 592.0776, found 592.0736.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-4,6-di-*O*-sulfonato-β-D-galactopyranoside disodium salts (6)

Sodium methoxide in methanol (1.0 M) (6.43 mL, 6.43 mmol, 14 equiv) was added to the suspension of **22** (273 mg, 0.444 mmol, 1.0 equiv) in methanol (9.0 mL, 49 mM), and stirred at room temperature for 67.5 h. The reaction mixture was neutralized with Amberlite IRC-50 H^+ resin, and concentrated under reduced pressure to afford **5** (234.9 mg, 0.442 mmol, 100 %) as colorless solid. R_f = 0.53 (50 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.94 (s, 3 H, 2-NHCOC*H*$_3$), 3.71 (s, 3 H, *p*-OCH$_3$), 3.89 (dd, 1 H, $J_{2,3}$ = 10.3 Hz, $J_{3,4}$ = 2.7 Hz, H-3), 4.06 (dd, 1 H, $J_{2,3}$ = 10.3 Hz, $J_{1,2}$ = 8.8 Hz, H-2), 4.14 (dd, 1 H, $J_{5,6a}$ = 8.2 Hz, $J_{5,6b}$ = 7.6 Hz, H-5), 4.15 (dd, 1 H, $J_{6a,6b}$ = 15.5 Hz, $J_{5,6a}$ = 8.2 Hz, H-6a), 4.27 (dd, 1 H, $J_{6a,6b}$ = 15.5 Hz, $J_{5,6b}$ = 7.6 Hz, H-6b), 4.69 (d, 1 H, $J_{3,4}$ = 2.7 Hz, H-4), 4.92 (d, 1 H, $J_{1,2}$ = 8.8 Hz, H-1), 6.86–6.88 (m, 2 H, *m*-arom. H), 6.98–6.99 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.14 (q, 2-NHCO*C*H$_3$), 52.60 (d, C-2), 55.78 (q, *p*-OCH$_3$), 67.66 (t, C-6), 69.70 (d, C-3), 72.44 (d, C-5), 75.27 (d, C-4), 100.87 (d, C-1), 115.05 (d, *m*-arom. CH), 118.27 (d, *o*-arom. CH), 151.20 (s, arom. C), 154.80 (s, *p*-arom. C), 175.03 (s, 2-NH*C*OCH$_3$); HRMS (ESI) m/z [M-Na]$^-$ calcd for $C_{15}H_{19}NNaO_{13}S_2^-$ 508.0201, found 508.0200.

Synthesis of *p*-methoxyphenyl 2-acetamido-2-deoxy-3,6-di-*O*-sulfonato-β-D-galactopyranoside disodium salts (5) and *p*-methoxyphenyl 2-acetamido-2-deoxy-3,4,6-tri-*O*-sulfonato-β-D-galactopyranoside trisodium salts (7)

Sulfur trioxide-trimethylamine complex (331 mg, 2.38 mmol, 15.0 equiv) was added to a solution of **11** (51.9 mg, 0.159 mmol, 1.0 equiv) in dry pyridine (1.59 mL, 0.10 M), and the mixture was stirred for 13.5 h at

room temperature. After addition of methanol (3.0 mL), the mixture was subjected to Dowex 50WX8 Na^+ resin. The effluent was concentrated under diminished pressure. The residue was separated by reversed phase column chromatography eluting with 100 % water-methanol to give **7** (15.4 mg, 23.2 μmol, 15 %) as a colorless solid, then with 97.5 % water-methanol to give **6** (62.9 mg, 0.118 mmol, 75 %) as a colorless solid. **6**: R_f = 0.18 (50 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.91 (s, 3 H, 2-NHCOCH_3), 3.69 (s, 3 H, *p*-OCH_3), 4.02 (dd, 1 H, $J_{5,6a}$ = 7.2 Hz $J_{5,6b}$ = 5.2 Hz, H-5), 4.12 (dd, 1 H, $J_{6a,6b}$ = 10.9 Hz, $J_{5,6a}$ = 7.2 Hz, H-6a), 4.17 (dd, 1 H, $J_{6a,6b}$ = 10.9 Hz, $J_{5,6b}$ = 5.2 Hz, H-6b), 4.17 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{1,2}$ = 8.3 Hz, H-2), 4.24 (d, 1 H, $J_{3,4}$ = 3.2 Hz, H-4), 4.40 (dd, 1 H, $J_{2,3}$ = 10.9 Hz, $J_{3,4}$ = 3.2 Hz, H-3), 5.00 (d, 1 H, $J_{1,2}$ = 8.3 Hz, H-1), 6.83–6.87 (m, 2 H, *m*-arom. H), 6.95–6.99 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.14 (q, 2-NHCOCH_3), 50.48 (d, C-2), 55.72 (q, *p*-OCH_3), 65.92 (d, C-4), 67.02 (t, C-6), 72.66 (d, C-5), 77.21 (d, C-3), 100.54 (d, C-1), 115.00 (d, *m*-arom. CH), 118.32 (d, *o*-arom. CH), 151.09 (s, arom. C), 154.80 (s, *p*-arom. C), 174.90 (s, 2-NH*C*OCH_3); HRMS (ESI) m/z $[M\text{-}Na]^-$ calcd for $C_{15}H_{19}NNaO_{13}S_2^-$ 508.0201, found 508.0196. **7**: R_f = 0.10 (50 % methanol-chloroform); ^{1}H NMR (D_2O, 400.13 MHz): δ 1.93 (s, 3 H, 2-NHCOCH_3), 3.71 (s, 3 H, *p*-OCH_3), 4.14–4.20 (m, 3 H, H-2, H-6a, H-5), 4.27 (dd, 1 H, $J_{6a,6b}$ = 16.6 Hz, $J_{5,6b}$ = 8.9 Hz, H-6b), 4.51 (dd, 1 H, $J_{2,3}$ = 11.0 Hz, $J_{3,4}$ = 2.9 Hz, H-3), 4.91 (d, 1 H, $J_{3,4}$ = 2.9 Hz, H-4), 5.09 (d, 1 H, $J_{1,2}$ = 8.5 Hz, H-1), 6.87–6.89 (m, 2 H, *m*-arom. H), 7.00–7.02 (m, 2 H, *o*-arom. H); ^{13}C NMR (D_2O, 100.62 MHz): δ 22.21 (q, 2-NHCOCH_3), 50.96 (d, C-2), 55.79 (q, *p*-OCH_3), 67.69 (t, C-6), 72.49 (d, C-5), 73.81 (d, C-4), 74.91 (d, C-3), 100.27 (d, C-1), 115.07 (d, *m*-arom. CH), 118.42 (d, *o*-arom. CH), 151.05 (s, arom. C), 154.88 (s, *p*-arom. C), 174.96 (s, 2-NH*C*OCH_3); HRMS (ESI) m/z $[M\text{-}Na]^-$ calcd for $C_{15}H_{18}NNa_2O_{16}S_3^-$ 609.9589 found 609.9579.

Viral infection

A Japanese encephalitis virus (JEV) strain, Beijing-1, was used in this study. Compounds were dissolved in DMSO at a concentration of 10 mM for a stock solution. Inhibition of JEV infection with compounds was determined by real-time reverse transcription (RT)-PCR targeting the gene encoding viral envelope (E) protein. Vero cells were seeded onto 96-well plastic plates and cultured overnight at 37 °C. The virus was premixed in a serum-free medium, Virus Production Serum Free medium (VPSFM) (GIBCO Life Technologies, Carlsbad, CA, USA) on ice with compounds at the indicated concentration. The virus-compound premixtures were immediately inoculated on the cells for 2 h at 37 °C. After removal of the premixtures, VPSFM was added, and plates were incubated at 37 °C for 20–24 h.

Quantitative real-time RT-PCR

Total RNA was extracted from the infected cells using NucleoSpin RNA (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. mRNA was reverse-transcribed using Random 6 mers with a PrimeScript RT reagent kit (Takara Bio Inc., Kyoto). The cellular expression of the genes encoding the E protein and β-actin were quantitatively measured by a real-time RT-PCR method using primers as follows: forward primer (5′-CATAGGGAAAGCTGTTCACCA-3′) and reverse primer (5′-ATTGACCGGTCTCGTGCGTT-3′) for JEV E protein gene amplification, and forward primer (5′-AGAAAATCTGGCACCACACC-3′) and reverse primer (5′-TGCTATCCCTGTACGCCTCT-3′) for β-actin gene amplification. Real-Time PCR was performed using an ABI Prism7000 Sequence Detection System and a *Power* SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Reaction cycles was performed for 45 cycles of denaturation (95 °C, 30 s), anneal-ing (55 °C, 30 s), and extension (60 °C, 30 s). The PCR efficiency was examined by serially diluting template cDNA and the melting curve data were collected to check the PCR specificity. Values were calculated using the delta Ct method normalizing to β-actin expression for each sample. Relative infectivity (%) was shown in the presence of compound at the indicated concentration to virus treated with DMSO as control.

Cellular cytotoxicity of compounds

The cytotoxicity of compounds was determined by a MTT assay according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plastic plates and cultured overnight at 37 °C. After removal of the media, the compounds were diluted to the indicated concentrations with VPSFM and added onto the cells. Plates were incubated at 37 °C for 48 h. Cell viability was then measured by the MTT assay.

Acknowledgments: This work was supported by a Cooperative Research Grant (to K.I.P.J.H.) of the Institute of Tropical Medicine, Nagasaki University, 2016.

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