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Inhibition of *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase by *ß*-D-4-*O*-sulfo-*N*-acetylgalactosaminides bearing various hydrophobic aglycons

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Abstract *N*-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) transfers sulfate to position 6 of GalNAc ($4SO_4$) residues of chondroitin sulfate to yield chondroitin sulfate E (CS-E). We have previously demonstrated that phenyl-*ß*-D-GalNAc($4SO_4$) could serve as an acceptor for GalNAc4S-6ST, thereby inhibiting GalNAc4S-6ST competitively. In this paper we compared the inhibitory effects of various glycosides in which various hydrophobic aglycons were attached to D-GalNAc($4SO_4$) via *ß* anomeric configuration. *p*-Nitrophenyl-*ß*-D-GalNAc($4SO_4$) and *p*-chlorophenyl-*ß*-D-GalNAc($4SO_4$) were stronger inhibitors than phenyl-*ß*-D-GalNAc($4SO_4$). Among inhibitors examined here, 3-estradiol-*ß*-D-GalNAc($4SO_4$) was the strongest inhibitor; the Ki of 3-estradiol-*ß*-D-GalNAc($4SO_4$) for the competitive inhibition was 0.008 mM, which was much lower than the Ki of phenyl-*ß*-D-GalNAc($4SO_4$), 0.98 mM. In contrast, 7-estradiol-*ß*-D-GalNAc($4SO_4$) showed only weak inhibition to GalNAc4S-6ST. 3-Estradiol- *ß*-D-GalNAc($4SO_4$) did not inhibit chondroitin 6-sulfotransferase and chondroitin 4-sulfotransferase under the concentration where GalNAc4S-6ST was inhibited by 90%. When 3-estradiol- *ß*-D-GalNAc($4SO_4$) was added to the culture medium of chondrosarcoma cells expressing human GalNAc4S-6ST, a significant, albeit small, reduction in the cellular synthesis of CS-E was observed. These results suggest that estradiol group of 3-estradiol-*ß*-D-GalNAc($4SO_4$) may enhance the inhibitory activity of the glycoside through increasing the affinity to the enzyme and may allow the glycosides to diffuse at a low efficiency into the cells to inhibit cellular synthesis of CS-E.

Keywords GalNAc4S-6ST · Sulfotransferase · Chondroitin sulfate E · Inhibitors

Introduction

Chondroitin sulfate E (CS-E) is an isomer of chondroitin sulfate containing unique repeating disaccharide units, GlcA*ß*1-3GalNAc(4,6-SO_4) *ß*1–4. CS-E has been reported to be involved in various physiological functions such as immunological response of mast cells [1–6], regulation of procoagulant activity [7], promotion of neurite outgrowth [8, 9], neural cell adhesion through binding to midkine [10], enhancement of plasminogen activation by plasminogen activator [11], binding of L-selectin, chemokines [12, 13] and various heparin binding growth factors [14], infection of herpes symplex virus [15, 16], metastasis of the Lewis lung carcinoma cells [17], migration of neuronal precursors during cortical development [18], and activation of matrilysin [19].

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N-acetylgalactosamine 4-sulfate 6-*O*-sulfotransferase (GalNAc4S-6ST) transfers sulfate to position 6 of GalNAc($4SO_4$) residues of chondroitin sulfate and catalyzes the final step of the synthesis of CS-E [20–22]. GalNAc(4,6-SO_4) residue was found not only in the repeating units of CS-E but also at the nonreducing terminal of CS-A [23–28]. GalNAc4S-6ST appears to be involved in the synthesis of GalNAc(4,6-SO_4) residues located both in the repeating disaccharide moiety and at the nonreducing end, because recombinant human and mouse GalNAc4S-6ST could sulfate both the GalNAc($4SO_4$) residues [21, 28, 29]. To obtain information about the functions of GalNAc (4,6-SO_4) residues contained in CS-E or located at the nonreducing terminal of chondroitin sulfate A (CS-A), it is important to regulate the activity of GalNAc4S-6ST.

We found previously that phenyl-*ß*-D-GalNAc($4SO_4$) could serve as the acceptor for GalNAc4S-6ST, and thereby inhibit sulfation of CS-A by GalNAc4S-6ST competitively [30]. Xylosides bearing various hydrophobic aglycons were reported to prime chondroitin sulfate or heparan sulfate in sliced tissues or various cultured cells [31–33]. Acetylated disaccharides bearing *ß*-*O*-naphthalenemethanol primed sialyl Le^X oligosaccharides and inhibited the expression of sialyl Le^X in cells [34–36]. The 4′-deoxy analog of the acetylated GlcNAc*ß*1-3Gal bearing *ß*-*O*-naphthalenemethanol did not act as a substrate but instead act as a competitive inhibitor [37]. GlcNAc and Gal-*ß*1-4GlcNAc bearing dodecyl group as the aglycon were found to prime neolacto-series oligosaccharides in HL60 cells [38]. From these observations, we expected that GalNAc($4SO_4$) bearing a certain hydrophobic aglycon might enter cells and affect the sulfation of chondroitin sulfate by GalNAc4S-6ST. To examine such possibility, we synthesized various glycosides in which various hydrophobic aglycons were attached to GalNAc($4SO_4$) via *ß* anomeric configuration, and determine their activity to inhibit GalNAc4S-6ST *in vitro* and CS-E synthesis in cells.

Materials and methods

Materials

The following commercial materials were used: $H_2{}^{35}SO_4$ was from Perkin-Elmer; chondroitinase ACII, CS-A (whale cartilage), ΔDi-0S, ΔDi-6S, ΔDi-4S, ΔDi-diS_D, ΔDi-diS_E, and ΔDi-triS were from Seikagaku Corporation, Tokyo; Partisil-10 SAX was from Whatman; unlabeled PAPS *N*-acetylgalactosamine 4-sulfate, *N*-acetylgalactosamine 6-sulfate were from Sigma; Hiload Superdex 30 HR 16/60 and Fast Desalting Column HR 10/10 were from Amersham Bioscience. [^{35}S]PAPS was prepared as described [39]. The FLAG-tagged human GalNAc4S-6ST, human chondroitin 4-sulfotransferase-1 (C4ST-1) and human chondroitin 6-sulfotransferase-1 (C6ST-1) were prepared as described previously [21, 40]. *N*-acetylgalactosamine 4, 6-bissulfate was prepared from 2-acetamide-2-deoxy-3-*O*-(*ß*-D-gluco-4-enepyranosyluronic acid)-4,6-bis-*O*-sulfo-D-galactose (ΔDi-diS_E), as described previously [41]. Phenyl-*ß*-D-GalNAc($4SO_4$) (**1**) was prepared as described previously [30]. Various glycosides containing GalNAc ($4SO_4$) or its derivatives were synthesized using glucosamine as a starting material. The detailed preparation procedures for 2-acetamido-2-deoxy-4-*O*-sulfonato-*β*-D-galactopyranosides will be reported elsewhere.

The structures of these glycosides were confirmed by ^{1}H NMR, ^{13}C NMR, and two-dimensional NMR (COSY, HMQC, HMBC). ^{1}H and ^{13}C NMR spectra were recorded with a JEOL LA-400 spectrometer operating at 399.65 MHz and 100.40 MHz. Mass spectra (FAB) were obtained on a LCQ (Thermoquest) or a LCT (Micromass) spectrometer.

*p-Nitrophenyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (p-Nitrophenyl-ß-D-GalNAc(4SO$_4$)) (**2**)^{1}H NMR (399.65 MHz, CD$_3$OD): δ 1.95 (s, 3 H, NHCOCH$_3$), 3.77 (dd, 1 H, J $_{6a,6b}$= 11.8 Hz, J $_{5,6a}$= 7.2 Hz, H-6a), 3.84 (dd, 1 H, J $_{5,6b}$= 5.4 Hz, H-6b), 3.90 (dd, 1 H, H-5), 3.95 (dd, 1 H, J $_{2,3}$= 11.0 Hz, J $_{3,4}$= 3.0 Hz, H-3), 4.18 (dd, 1 H, J $_{1,2}$= 8.4 Hz, H-2), 4.77 (d, 1 H, H-4), 5.23 (d, 1 H, H-1) 7.17–7.19 (m, 2 H, o-arom. H of nitrophenyl), 8.18-8.21 (m, 2 H, m-arom. H of nitrophenyl); ^{13}C NMR (100.40 MHz, CD$_3$OD): δ 23.00 (q, NHCOCH$_3$), 54.87 (d, C-2), 62.08 (t, C-6), 71.64 (d, C-3), 76.02 (d, C-4), 76.48 (d, C-5), 100.24 (d, C-1), 117.83 (d, o-arom. CH), 126.68 (d, m-arom. CH), 144.07 (s, arom. C-NO$_2$), 163.79 (s, arom. C-O), 174.09 (s, NHCOCH$_3$); HRMS (FAB$^-$): calcd for C$_{14}$H$_{17}$N$_2$O$_{11}$S [M]$^-$ 421.0553, found 421.0555.*

*p-Chlorophenyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (p-Chlorophenyl-ß-D-GalNAc(4SO$_4$)) (**3**)* ^{1}H NMR (399.65 MHz, D_2O): δ 1.89 (s, 3 H, NHCOCH_3), 3.68 (dd, 1 H, $J_{6a,6b}$=12.0 Hz, $J_{5,6a}$= 7.4 Hz, H-6a), 3.73 (dd, 1 H, $J_{5,6b}$=5.0 Hz, H-6b), 3.84 (dd, 1 H, H-5), 3.86 (dd, 1 H, $J_{2,3}$=10.9 Hz, $J_{3,4}$=3.0 Hz, H-3), 4.03 (dd, 1 H, $J_{1,2}$=8.4 Hz, H-2), 4.63 (d, 1 H, H-4), 4.99 (d, 1 H, H-1) 6.91–6.93 (m, 2 H, arom. H), 7.22–7.24 (m, 2 H, arom. H); ^{13}C NMR (100.40 MHz, D_2O): δ 22.97 (q, NHCO*C*H$_3$), 53.50 (d, C-2), 61.60 (t, C-6), 70.53 (d, C-3), 75.59 (d, C-5), 76.27 (d, C-4), 100.82 (d, C-1), 119.04 (d, arom. CH), 128.54 (s, arom. C), 130.42 (d, arom. CH), 156.21 (s, arom. C-O), 175.88 (s, NHCOCH$_3$); HRMS (FAB$^-$): calcd for $C_{14}H_{17}ClNO_9S$ [M]$^-$ 410.0312, found 410.0321.

p-Fluorophenyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (p-Fluorophenyl-ß-D-

GalNAc(4SO$_4$)) (**4**) ^{1}H NMR (399.65 MHz, CD_3OD): δ 1.99 (s, 3 H, NHCOC*H$_3$*), 3.75 (dd, 1 H, $J_{6a,6b}$= 8.3 Hz, $J_{5,6a}$=2.6 Hz, H-6a), 3.78–3.86 (m, 2 H, H-5, H-6b), 3.89 (dd, 1 H, $J_{2,3}$=11.0 Hz, $J_{3,4}$=3.2 Hz, H-3), 4.17 (dd, 1 H, $J_{1,2}$=8.5 Hz, H-2), 4.75 (d, 1 H, H-4), 4.99 (d, 1 H, H-1), 6.96–7.05 (m, 4 H, arom. CH of fluorophenyl); ^{13}C NMR (100.40 MHz, CD_3OD): δ 23.01 (q, NHCO*C*H$_3$), 54.70 (d, C-2), 61.88 (t, C-6), 71.98 (d, C-3), 76.01 (d, C-5), 76.14 (d, C-4), 101.76 (d, C-1), 116.67 (dd, $^2J_{C,F}$=23.2 Hz, *m*-arom. CH), 119.56 (dd, $^3J_{C,F}$= 8.3 Hz, *o*-arom. CH), 155.26 (sd, $^4J_{C,F}$=2.5 Hz, arom. C-O), 159.80 (sd, $^1J_{C,F}$=239.0 Hz, *p*-arom. C-F), 174.16 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{14}H_{17}FNO_9S$ [M]$^-$ 394.0608, found 394.0600.

Benzyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (Benzyl-β-D-GalNAc(4SO$_4$)) (**5**). ^{1}H NMR (399.65 MHz, D_2O): δ 1.76 (s, 3 H, NHCOC*H$_3$*), 3.60–3.74 (m, 5 H, H-5, H-3, H-6, H-2), 4.36 (d, 1 H, $J_{1,2}$=8.3 Hz, H-1), 4.51 (d, 1 H, $J_{3,4}$=2.9 Hz, H-4), 4.52 (d, 1 H, J_{gem}=12.2 Hz, OC*H*(H)Ph), 4.73 (d, 1 H, J_{gem}=12.2 Hz, OCH(*H*)Ph), 7.21–7.32 (m, 5 H, arom. H); ^{13}C NMR (100.40 MHz, D_2O): δ 23.06 (q, NHCO*C*H$_3$), 53.69 (d, C-2), 61.88 (t, C-6), 70.77 (d, C-3), 72.29 (t, O*C*H$_2$Ph), 75.25 (d, C-5), 76.55 (d, C-4), 101.02 (d, C-1), 129.33 (d, arom. CH), 129.51 (d, arom. CH), 129.61 (d, arom. CH), 137,65 (s, arom. C), 175.55 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{15}H_{20}NO_9S$ [M]$^-$ 390.0859, found 390.0861.

Octyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (Octyl-β-D-GalNAc(4SO$_4$)) (**6**) ^{1}H NMR (399.65 MHz, D_2O): δ 0.73 (t, *J*=6.3 Hz, 3 H, CH_3), 1.15 (br m, 10 H, CH_2 X 5), 1.38–1.43 (m, 2 H, CH_2), 1.90 (s, 3 H, NHCOC*H$_3$*), 3.47 (dt, 1 H, J_{gem}=10.3 Hz, *J*=6.2 Hz, OC*H*(H)), 3.64–3.79 (m, 6 H, H-5, H-3, H-6, H-2, OC*H*(H)), 4.34–4.40 (m, 1 H, H-1), 4.55 (br s, 1 H, H-4); ^{13}C NMR (100.40 MHz, D_2O): δ 14.23 (q, CH_3), 22.85 (t, CH_2), 23.09 (q, NHCO*C*H$_3$), 25.90 (t, CH_2), 29.19 (t, CH_2), 29.33 (t, CH_2), 29.36 (t, CH_2), 31.95 (t, CH_2), 53.74 (d, C-2), 61.80 (t, C-6), 70.86 (d, C-3), 71.31 (t, OCH_2), 75.08 (d, C-5), 76.55 (d, C-4), 102.18 (d, C-1), 175.48 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{16}H_{30}NO_9S$ [M]$^-$ 412.1641, found 412.1644.

1-Naphthyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (1-Naphthyl-β-D-GalNAc(4SO$_4$)) (**7**) ^{1}H NMR (399.65 MHz, D_2O): δ 1.83 (s, 3 H, NHCOC*H$_3$*), 3.72–3.81 (m, 2 H, H-6), 3.91 (dd, 1 H, $J_{2,3}$=11.0 Hz, $J_{3,4}$=3.1 Hz, H-3), 3.95 (dd, 1 H, $J_{5,6a}$= 7.3 Hz, $J_{5,6b}$=5.1 Hz, H-5), 4.26 (dd, 1 H, $J_{1,2}$=8.5 Hz, H-2), 4.68 (d, 1 H, H-4), 5.14 (d, 1 H, H-1), 7.12 (d, 1 H, *J*=8.1 Hz, arom. H-2), 7.35 (t, 1 H, *J*=8.1 Hz, arom. H-3), 7.44–7.48 (m, 2 H, arom. H-6, H-7), 7.53 (d, 1 H, *J*= 8.1 Hz, arom. H-4), 7.78–7.82 (m, 1 H, arom. H-5), 7.96–8.00 (m, 1 H, arom. H-8); ^{13}C NMR (100.40 MHz, D_2O): δ 23.01 (q, NHCO*C*H$_3$), 53.47 (d, C-2), 61.69 (t, C-6), 70.55 (d, C-3), 75.70 (d, C-5), 76.34 (d, C-4), 101.44 (d, C-1), 110.11 (d, arom. CH-2), 121.89 (d, arom. CH-8), 123.56 (d, arom. CH-4), 125.80 (s, arom. C-8a), 127.06 (d, arom. CH), 127.13 (d, arom. CH), 127.80 (d, arom. CH), 128.58 (d, arom. CH-5), 135.02 (s, arom. C-4a), 153.51 (s, arom. C-1), 175.88 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{18}H_{20}NO_9S$ [M]$^-$ 426.0859, found 426.0869.

Cyclohexyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (Cyclohexyl-β-D-GalNAc(4SO$_4$)) (**8**) ^{1}H NMR (399.65 MHz, D_2O): δ 1.06-1.33 (br m, 6 H, CH_2 X 3), 1.45–1.53 (br m, 2 H, CH_2), 1.64–1.71 (br m, 2 H, OCHC*H$_2$*), 1.89 (s, 3 H, NHCOC*H$_3$*), 3.59–3.76 (m, 6 H, H-2, H-3, H-5, H-6a, H-6b, OCH), 4.48 (d, 1H, $J_{1,2}$= 7.8 Hz, H-1), 4.54 (d, 1H, $J_{3,4}$=2.9 Hz, H-4); ^{13}C NMR (100.40 MHz, D_2O): δ 23.00, 23.79, 24.02, 25.80, 31.74, 33.37, 53.89 (C-2), 61.69 (C-6), 70.83, 75.00, 76.48, 79.04, 100.37 (C-1), 175.52 (NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{14}H_{24}NO_9S$ [M]$^-$ 382.1172, found 382.1175.

(1S,3S,4R)-Menthyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (l-Menthyl-β-D-GalNAc (4SO$_4$)) (**9**) ^{1}H NMR (399.65 MHz, D_2O): δ 0.61–0.90 (m, 12 H, menthyl), 1.03–1.10 (m, 1 H, menthyl), 1.22 (br m, 1 H, menthyl), 1.50 (br m, 2 H, menthyl), 1.84–1.89 (m, 1 H, menthyl), 1.89 (s, 3 H, NHCOC*H$_3$*), 1.97–2.01 (m, 1 H, H-8 of menthyl), 3.45 (td, *J*=10.9 Hz, *J*=4.3 Hz, H-3 of menthyl), 3.59–3.70 (m, 4 H, H-2, H-5, H-6a, H-6b), 3.75 (dd, 1 H, $J_{2,3}$=10.9 Hz, $J_{3,4}$=3.0 Hz, H-3), 4.46 (d, 1 H, $J_{1,2}$=8.3 Hz, H-1), 4.55 (d, 1 H, H-4); ^{13}C NMR (100.40 MHz, D_2O): δ 17.56 (q, C-10 of menthyl), 23.27 (q, C-9 of menthyl), 24.46 (q, C-7 of menthyl), 25.14 (q + t, NHCOC*H$_3$*, C-5 of menthyl), 27.36 (d, C-8 of menthyl), 33.93 (d, C-1 of menthyl), 36.55 (t, C-6 of menthyl), 43.83 (t, C-2 of menthyl), 49.94 (d, C-4 of menthyl), 56.11 (d, C-2), 63.53 (t, C-6), 73.12 (d, C-3), 76.98 (d, C-5), 78.58 (d, C-4), 83.88 (d, C-3 of menthyl), 102.85 (d, C-1), 177.50 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{18}H_{32}NO_9S$ [M]$^-$ 438.1798, found 438.1795.

(1R,3R,4S)-Menthyl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (d-Menthyl-β-D-GalNAc (4SO$_4$)) (**10**) ^{1}H NMR (399.65 MHz, D_2O): δ 0.60–0.89 (m, 12 H, menthyl), 1.02–1.12 (m, 1 H, menthyl), 1.23 (br m, 1 H, menthyl), 1.47–1.50 (m, 2 H, menthyl), 1.84–1.88 (m, 1 H, menthyl), 1.88 (s, 3 H, NHCOC*H$_3$*), 2.05–2.08 (m, 1 H, H-8 of menthyl), 3.35 (td, *J*=10.6 Hz, *J*=4.1 Hz, H-3 of menthyl), 3.63–3.72 (m, 5 H, H-2, H-3, H-5, H-6a, H-6b), 4.44 (m, 1 H, H-1), 4.53 (s, 1 H, H-4); ^{13}C NMR

(100.40 MHz, D_2O): δ 15.88 (q, C-10 of menthyl), 21.16 (q, C-9 of menthyl), 22.42 (q, C-7 of menthyl), 23.10 (q + t, NHCO*C*H_3, C-5 of menthyl), 25.45 (d, C-8 of menthyl), 31.84 (d, C-1 of menthyl), 34.54 (t, C-6 of menthyl), 43.57 (t, C-2 of menthyl), 48.77 (d, C-4 of menthyl), 54.05 (d, C-2), 61.49 (t, C-6), 70.90 (d, C-3), 74.76 (d, C-5), 76.37 (d, C-4), 84.60 (d, C-3 of menthyl), 103.53 (d, C-1), 175.40 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{18}H_{32}NO_9S$ [M]$^-$ 438.1798, found 438.1794.

Cyclohexyl 2-acetamido-2,6-dideoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (Cyclohexyl-ß-D-6-deoxy-GalNAc(4SO$_4$)) (**11**) ^{1}H NMR (500.16 MHz, D_2O): δ 1.07–1.25 (br m, 8 H), 1.35 (br s, 1 H), 1.51 (br s, 2 H), 1.90 (s, 3 H, NHCOCH_3), 3.58–3.74 (m, 6 H), 4.23 (br s, 1 H), 4.47 (br m, 1 H).

17β-Hydroxyestra-1,3,5(10)-trien-3-yl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (3-Estradiol-ß-D-GalNAc(4SO$_4$)) (**12**) ^{1}H NMR (399.65 MHz, D_2O): δ 0.62 (s, 3 H, CH_3-18), 1.06–1.41 (m, 8 H), 1.55–1.63 (m, 1 H), 1.73–1.81 (m, 2 H), 1.91 (s, 3 H, NHCOCH_3), 2.03–2.10 (m, 1 H), 2.17–2.21 (m, 1 H), 2.69–2.71 (m, 2 H), 3.61 (t, 1 H, J=8.5 Hz, CH-17), 3.70 (dd, 1 H, $J_{6a,6b}$= 11.8 Hz, $J_{5,6a}$=7.2 Hz, H-6a), 3.74 (dd, 1 H, H-6b), 3.82–3.86 (m, 1 H, H-5), 3.87 (dd, 1 H, $J_{2,3}$=10.8 Hz, H-3), 4.03 (dd, 1 H, $J_{1,2}$=8.7 Hz, H-2), 4.57–4.65 (m, 1 H, H-4), 4.97 (d, 1 H, H-1), 6.73 (s, 1 H, arom. CH-4), 6.76 (d, 1 H, J= 8.4 Hz, arom. CH-2), 7.21 (d, 1 H, J=8.4 Hz, arom. CH-1); ^{13}C NMR (100.40 MHz, D_2O): δ 11.47 (q, CH_3-18 of estratrienyl), 23.02 (q, NHCO*C*H_3), 23.37 (t, CH_2-15 of estratrienyl), 26.68 (t, CH_2-11 of estratrienyl), 27.36 (t, CH_2-7 of estratrienyl), 29.76 (t, CH_2-16 of estratrienyl), 29.96 (t, CH_2-6 of estratrienyl), 37.03 (t, CH_2-12 of estratrienyl), 39.14 (d, CH-8 of estratrienyl), 43.57 (s, C-13 of estratrienyl), 44.24 (d, CH-9 of estratrienyl), 50.12 (d, CH-14 of estratrienyl), 53.66 (d, C-2), 61.67 (t, C-6), 70.63 (d, C-3), 75.56 (d, C-5), 76.35 (d, C-4), 82.29 (d, CH-17 of estratrienyl), 101.07 (d, C-1), 115.11 (d, arom. CH-2), 117.68 (d, arom. CH-4), 127.71 (d, arom. CH-1), 136.85 (s, arom. C-10), 140.04 (s, arom. C-5), 155.55 (s, arom. C-3), 175.85 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{26}H_{36}NO_{10}S$ [M]$^-$ 554.2060, found 554.2064.

3-Hydroxyestra-1,3,5(10)-trien-17β-yl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galactopyranoside sodium salt (17-Estradiol-ß-D-GalNAc(4SO$_4$)) (**13**) ^{1}H NMR (399.65 MHz, D_2O): δ 0.76 (s, 3 H, CH_3-18), 1.14–1.69 (m, 8 H), 1.82–2.29 (m, 5 H), 1.97 (s, 3 H, NHCOCH_3), 2.74–2.78 (m, 2 H, CH_2-6), 3.62 (t, 1 H, $J_{5,6}$=6.6 Hz, H-5), 3.66–3.73 (m, 2 H, H-6a, CH-17), 3.80–3.85 (m, 3 H, H-6b, H-3, H-2), 4.49–4.51 (m, 1 H, H-1), 4.70 (br s, 1 H, H-4), 6.46 (d, 1 H, J=2.7 Hz, arom. CH-4), 6.52 (dd, 1 H, J=8.5 Hz, J=2.7 Hz, arom. CH-2), 7.05 (d, 1 H, J=8.5 Hz, arom. CH-1); ^{13}C NMR (100.40 MHz, D_2O): δ 12.09 (q, CH_3-18 of estratrienyl), 23.16 (q, NHCO*C*H_3), 24.02 (t, CH_2-15 of estratrienyl), 27.60 (t, CH_2-11 of estratrienyl), 28.43 (t, CH_2-7 of estratrienyl), 29.84 (t, CH_2-16 of estratrienyl), 30.67 (t, CH_2-6 of estratrienyl), 38.68 (t, CH_2-12 of estratrienyl), 40.29 (d, CH-8 of estratrienyl), 44.42 (s, C-13 of estratrienyl), 45.31 (d, CH-9 of estratrienyl), 51.03 (d, CH-14 of estratrienyl), 55.59 (d, C-2), 61.91 (t, C-6), 71.67 (d, C-3), 75.47 (d, C-5), 76.14 (d, C-4), 90.64 (d, CH-17 of estratrienyl), 103.61 (d, C-1), 113.74 (d, arom. CH-2), 116.06 (d, arom. CH-4), 127.17 (d, arom. CH-1), 132.59 (s, arom. C-10), 138.82 (s, arom. C-5), 155.86 (s, arom. C-3), 173.58 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{26}H_{36}NO_{10}S$ [M]$^-$ 554.2060, found 554.2064.

Cholesteryl 2-acetamido-2-deoxy-4-O-sulfonato-β-D-galacto-pyranoside sodium salt (Cholesteryl-ß-D-GalNAc(4SO$_4$)) (**14**) ^{1}H NMR (399.65 MHz, CD_3OD): δ 0.71 (s, 3 H, CH_3 of cholesteryl), 0.86–1.66 (m, 33 H, cholesteryl), 1.82–2.15 (m, 6 H, cholesteryl), 1.96 (s, 3 H, NHCOCH_3), 2.28–2.33 (m, 1 H, cholesteryl), 3.59–3.86 (m, 5 H, H-5, H-6, H-2, cholesteryl), 4.57 (d, 1 H, $J_{1,2}$=8.5 Hz, H-1), 4.68 (d, 1 H, $J_{3,4}$=2.7 Hz, H-4), 4.79–4.90 (m, 1 H, H-3), 5.33–5.35 (m, 1 H, cholesteryl); ^{13}C NMR (100.40 MHz, DMSO-d_6): δ 11.73 (q, CH_3-18 of cholesteryl), 18.61 (q, CH_3-21 of cholesteryl), 19.15 (q, CH_3-19 of cholesteryl), 20.64 (t, CH_2-11 of cholesteryl), 22.45 (q), 22.73 (q), 23.10 (q, NHCO*C*H_3), 23.21 (t, CH_2 of cholesteryl), 23.92 (t), 27.44 (d), 27.84 (t), 29.32 (t), 31.43 (t), 31.48 (d), 35.23 (d), 35.70 (d), 36.23 (s), 36.85 (t), 38.57 (t), 39.50 (t), 39.71 (t), 41.91 (s), 49.64 (d), 53.46 (d, C-2), 55.63 (d, CH-17 of cholesteryl), 56.23 (d, CH-14 of cholesteryl), 60.41 (t, C-6), 70.05 (d), 73.50 (d), 74.05 (d), 77.62 (d, CH-3 of cholesteryl), 99.69 (d, C-1), 121.32 (d, CH-6 of cholesteryl), 140.50 (s, C-5 of cholesteryl), 169.13 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{35}H_{58}NO_9S$ [M]$^-$ 668.3833, found 668.3809.

Phenyl 2-acetamido-2-deoxy-4-O-sulfonato-1-thio-β-D-galacto-pyranoside sodium salt (Phenyl-S-ß-D-GalNAc(4SO$_4$)) (**15**) ^{1}H NMR (399.65 MHz, D_2O): δ 1.86 (s, 3 H, NHCOCH_3), 3.58–3.63 (m, 2 H, H-6a, H-3), 3.67–3.71 (m, 2 H, H-5, H-2), 3.77 (d, 1 H, $J_{5,6}$=12.4 Hz, H-6b), 4.65 (d, 1 H, H-4 overlap with H_2O), 4.95 (d, 1 H, $J_{1,2}$= 10.7 Hz, H-1) 7.25–7.28 (m, 3 H, arom. H), 7.40–7.42 (m, 2 H, arom. H); ^{13}C NMR (100.40 MHz, D_2O): δ 22.69 (q, NHCO*C*H_3), 51.61 (d, C-2), 61.77 (t, C-6), 66.64 (d, C-3), 77.66 (d, C-5), 78.99 (d, C-4), 82.79 (d, C-1), 129.45 (d, arom. CH), 130.04 (d, arom. CH), 131.14 (d, arom. CH), 133.79 (s, arom. C), 174.15 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{14}H_{18}NO_8S_2$ [M]$^-$ 392.0474, found 392.0466.

*Phenyl 2-acetamido-2-deoxy-1-seleno-4-O-sulfonato-β-D-galactopyranoside sodium salt (Phenyl-Se-β-D-GalNAc (4SO$_4$)) (***16***)* ^{1}H NMR (399.65 MHz, D_2O): δ 1.85 (s, 3 H, NHCOC*H$_3$*), 3.60–3.69 (m, 3 H, H-6, H-5), 3.72 (dd, 1 H, $J_{2,3}$=10.5 Hz, $J_{3,4}$=3.0 Hz, H-3), 3.94 (t, 1 H, $J_{1,2}$= 10.5 Hz, H-2), 4.65 (d, 1 H, H-4 overlap with H_2O), 4.95 (d, 1 H, H-1) 7.21–7.26 (m, 3 H, arom. H), 7.50–7.53 (m, 2 H, arom. H); ^{13}C NMR (100.40 MHz, D_2O): δ 22.97 (NHCO*C*H$_3$), 53.10 (C-2), 61.87 (C-6), 71.89, 76.70, 80.35, 84.04 (C-1), 128.72, 129.11, 130.25, 134.54, 175.25 (s, NH*C*OCH$_3$); HRMS (FAB$^-$): calcd for $C_{14}H_{18}NO_8SSe$ [M]$^-$ 439.9919, found 439.9916.

The final products free from the protecting groups were purified with Superdex 30 chromatography except for 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**), 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**), and cholesteryl-*β*-D-GalNAc(4SO$_4$) (**14**). These compounds were purified by recrystallization from methanol/chloroform. The galactosamine contents of the glycosides except for phenyl-*S*-*β*-D-GalNAc(4SO$_4$) (**15**) and phenyl-*Se*-*β*-D-GalNAc(4SO$_4$) (**16**) were determined by the Morgan-Elson method as modified by Strominger *et al.* [42] after hydrolysis with 6 M HCl at 100°C for 4 h. Concentration of phenyl-*S*-*β*-D-GalNAc(4SO$_4$) (**15**) and phenyl-*Se*-*β*-D-GalNAc(4SO$_4$) (**16**) were determined by weight.

The Swarm rat chondrosarcoma cell line, RCS-LTC [43], was a gift from Dr. James H. Kimura, Department of Surgery, John A. Burns School of Medicine, University of Hawaii.

Assay of Sulfotransferase Activity

GalNAc4S-6ST activity was assayed by the method described previously [21] with a slight modification. The standard reaction mixture contained, in a final volume of 50 µl, 2.5 µmol imidazole-HCl, pH 6.8, 0.5 µmol $CaCl_2$, 1 µmol reduced glutathione, 25 nmol (as galactosamine) of CS-A, 50 pmol [^{35}S] 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (about 5.0×10^5 cpm), and enzyme. When 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**), 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) or cholesteryl-*β*-D-GalNAc(4SO$_4$) (**14**) were added to the reaction mixtures, 1% Triton X-100 was included in the reaction mixture. The reaction mixtures were incubated at 37 ˚C for 20 min and the reaction was stopped by immersing the reaction tubes in a boiling water bath for 1 min. After the reaction was stopped, ^{35}S-labeled glycosaminoglycans were isolated by the precipitation with ethanol followed by gel chromatography with a Fast Desalting Column as described previously [44] and radioactivity was determined. To obtain the kinetics parameters, the sulfotransferase activity was determined at the concentration of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 mM CS-A in the absence or presence of 0.05 or 0.1 mM of 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**). The kinetics parameters were determined by a set of the Lineweaver-Burk's plot.

Preparation of chondrosarcoma cells overexpressing human GalNAc4S-6ST

Cells that synthesize CS-E were established from rat chondrosarcoma LTC cells by transfection of human GalNAc4S-6ST cDNA. A DNA fragment which codes for full open reading frame of the human GalNAc4S-6ST was subcloned into EcoRI site of pCXN2 plasmid [45]. The resulting plasmid was transfected in the rat chondrosarcoma cells using Trans Fast reagent (Promega) according to the methods recommended by the manufacturer. Chondrosarcoma cells overexpressing human GalNAc4S-6ST were selected in the presence of 500 µg/ml G418. GalNAc4S-6ST activity of the extracts prepared from the G418-resistant clones were determined. Cells that synthesize CS-E were selected from the clone showing high GalNAc4S-6ST activity.

Metabolic labeling of chondroitin sulfate synthesized by chondrosarcoma cells expressing human GalNAc4S-6ST

Rat chondrosarcoma cells expressing human GalNAc4S-6ST obtained as above were plated in 6-well plates at a density of 1.5×10^5 cells/well. The medium in which the cells were plated consisted of Dulbecco's modified Eagle's medium containing penicillin (100 units/ml), streptomycin (50 mg/ml), 5% fetal bovine serum and 5 µg/ml insulin, and cells were grown at 37 ˚C in 5% CO_2, 95% air. After 2 days, the cells were grown to 3.6×10^5 cells/well. 3-Estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) dissolved in dimethyl sulfoxide (17 µl/dish) was then added to the culture medium (500 µl) of the chondrosarcoma cells expressing human GalNAc4S-6ST. After 30 min, $^{35}SO_4$ (40 µCi/ml) was added to the culture medium, and the cells were cultured for further 6 h. Under the culture conditions, viability of the cells was more than 99% in the absence or presence of 2.5 mM 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) when examined by Trypan Blue exclusion assay. The cell layer and medium were separated and treated with 0.5 M NaOH for 24 h at 4°C and digested with Actinase (0.3 mg/ml) after neutralization. To the digests, trichloroacetic acid was added to a final concentration of 5%, and the precipitates formed were removed by centrifugation at 10,000×g for 10 min. The resulting supernatant fractions were mixed with a 3-vol of ethanol containing 1.3% potassium acetate, and ^{35}S-labeled glycosaminoglycans were precipitated by centrifugation at 10,000×g for 10 min. The precipitates were dissolved in water and precipitation with 3-vol of ethanol was repeated twice. The final precipitates were dissolved in water and the radioactivity was determined. For determining

the disaccharide composition of the ^{35}S-labeled glycosaminoglycans, the ^{35}S-labeled glycosaminoglycans were digested with chondroitinase ACII, and the disaccharides formed were separated with HPLC using a Whatman Partisil-10 SAX column as described below, and ^{35}S-radioactivity was determined. Digestion with chondroitinase ACII was carried out for 4 h at 37°C in the reaction mixture containing, in a final volume of 25 µl, 1.25 µmol of Tris-acetate buffer, pH 7.5, 2.5 µg of bovine serum albumin and 30 milliunits of chondroitinase ACII.

Chromatography on Superdex 30 and HPLC

A Superdex 30 16/60 column was equilibrated with 0.2 M NH_4HCO_3, and run at a flow rate of 2 ml/min. One-ml fractions were collected. Separation of the degradation products formed from the ^{35}S-labeled glycosaminoglycans were carried out by HPLC using a Whatman Partisil-10 SAX column (4.6 mm×25 cm) equilibrated with 8 mM or 5 mM KH_2PO_4. The column was developed with 8 mM KH_2PO_4 for 10 min followed by a linear gradient from 10 to 720 mM KH_2PO_4, or developed with 5 mM KH_2PO_4 for 10 min followed by a linear gradient from 5 to 500 mM KH_2PO_4. Fractions (0.5 ml) were collected at a flow rate of 1 ml/min and a column temperature of 40°C.

Results

Inhibition of GalNAc4S-6ST activity by glycosides bearing various hydrophobic aglycons attached to GalNAc(4SO$_4$)

We have shown previously that phenyl-*β*-D-GalNAc(4SO$_4$) (**1**) inhibited GalNAc4S-6ST, which transfers sulfate to position 6 of GalNAc(4SO$_4$) residues of chondroitin sulfate to yield chondroitin sulfate E (CS-E), through competing with the acceptor substrate. To obtain more efficient inhibitors than phenyl-*β*-D-GalNAc(4SO$_4$) (**1**), we synthesized glycosides bearing various aglycons attached to D-GalNAc(4SO$_4$) via *β* anomeric configuration and compared their activity to inhibit GalNAc4S-6ST. Structures of these compounds are shown in Fig. 1. In Fig. 2, inhibition of GalNAc4S-6ST activity by these compounds are shown. *p*-Nitrophenyl-*β*-D-GalNAc(4SO$_4$) (**2**) and *p*-chlorophenyl-*β*-D-GalNAc(4SO$_4$) (**3**) inhibited GalNAc4S-6ST more strongly than phenyl-*β*-D-GalNAc(4SO$_4$) (**1**), but the inhibitory activity of *p*-fluorophenyl-*β*-D-GalNAc(4SO$_4$) (**4**) was nearly the same as that of phenyl-*β*-D-GalNAc(4SO$_4$) (**1**) (Fig. 2a). Concentration at which the enzyme activity was decreased to 50% of the control was 0.25 mM for *p*-chlorophenyl-*β*-D-GalNAc(4SO$_4$) (**3**) compared to 2.5 mM for phenyl-*β*-D-GalNAc(4SO$_4$) (**1**). As shown in Fig. 2b, benzyl-*β*-D-GalNAc(4SO$_4$) (**5**) showed nearly the same inhibitory activity as *p*-nitrophenyl-*β*-D-GalNAc(4SO$_4$) (**2**) and *p*-chlorophenyl-*β*-D-GalNAc(4SO$_4$) (**3**), suggesting that the aglycon moiety of benzyl-*β*-D-GalNAc(4SO$_4$) (**5**) may be accessed by GalNAc4S-6ST more easily than phenyl-*β*-D-GalNAc(4SO$_4$) (**1**), because the methylene bridge of benzyl group may function as the spacer. Octyl-*β*-D-GalNAc(4SO$_4$) (**6**) and 1-naphthyl-*β*-D-GalNAc(4SO$_4$) (**7**) showed intermediate activity between phenyl-*β*-D-GalNAc(4SO$_4$) (**1**) and benzyl-*β*-D-GalNAc(4SO$_4$) (**5**). The inhibitory activity of cyclohexyl-*β*-D-GalNAc(4SO$_4$) (**8**) was as high as those of phenyl-*β*-D-GalNAc(4SO$_4$) (**1**). Of the two optical isomers, *l*-menthyl-*β*-D-GalNAc(4SO$_4$) (**9**) and *d*-menthyl-*β*-D-GalNAc(4SO$_4$) (**10**), only *l*-isomer (**9**) inhibited GalNAc4S-6ST weakly (Fig. 2c). Cyclohexyl-*β*-D-6-deoxy-GalNAc(4SO$_4$) (**11**) showed no detectable inhibitory activity at 2.5 mM (data not shown), indicating that 6-OH of GalNAc(4SO$_4$) is indispensable for the recognition by GalNAc4S-6ST. 3-Estradiol-*β*-D-xyloside was reported to prime heparan sulfate in the cultured cells. Because cholesterol is a common component of plasma membranes, glycosides containing cholesterol as the aglycon were expected to show membrane permeability as well as glycosides containing estradiol. We synthesized glycosides containing these relatively large hydrophobic aglycons and determined their inhibitory activity. The inhibition by these compounds were determined in the presence of 1% Triton X-100. 3-Estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) inhibited GalNAc4S-6ST most strongly among all compounds examined; 40% inhibition was observed at 0.05 mM. On the other hand, 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) showed much weaker inhibitory activity than 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**); 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) inhibited GalNAc4S-6ST activity nearly completely at 0.25 mM, whereas 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) showed only 10% inhibition at the same concentration. Cholesteryl-*β*-D-GalNAc(4SO$_4$) (**14**) showed nearly the same inhibitory activity as that of 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) (Fig. 2d).

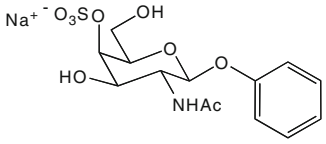
Fig. 1 Structures of various glycosides in which various hydrophobic aglycons were attached to D-GalNAc(4SO$_4$) via *β* anomeric configuration ▶

Effects of glycoside bonds on the inhibitory effects

Effects of phenyl-*S*-*β*-D-GalNAc(4SO$_4$) (**15**) and phenyl-*Se*-*β*-D-GalNAc(4SO$_4$) (**16**) were compared with the effects of phenyl-*O*-*β*-D-GalNAc(4SO$_4$) (**1**) (Fig. 2e). S- and Se-glycosides hardly inhibited GalNAc4S-6ST, indicating that O-glycosidic bond is required for recognition by the enzyme.

Inhibition mechanism by 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**)

As described above, 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) was found to inhibit GalNAc4S-6ST at the lowest concen-



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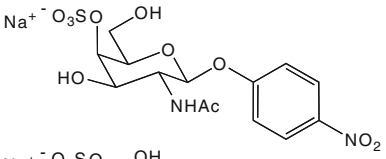

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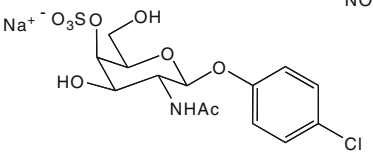

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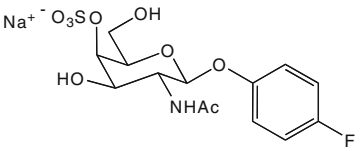

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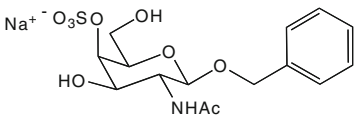

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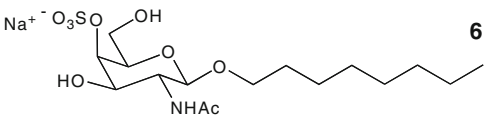

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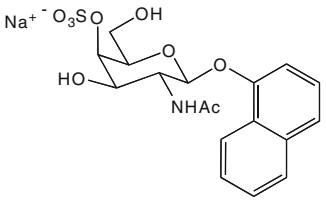

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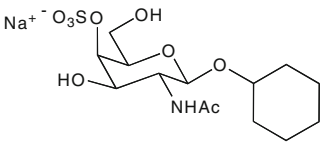

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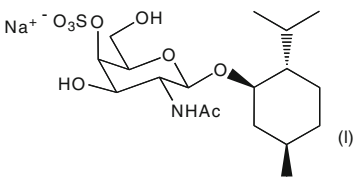

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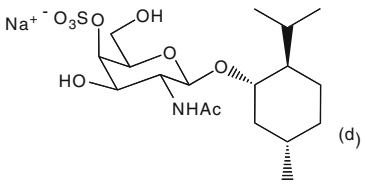

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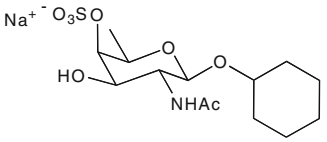

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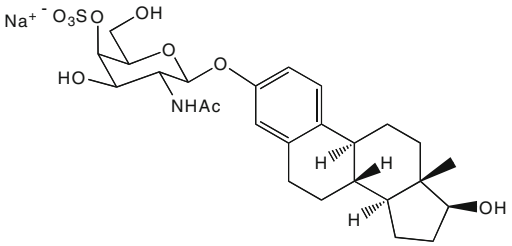

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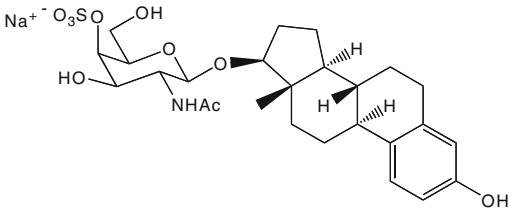

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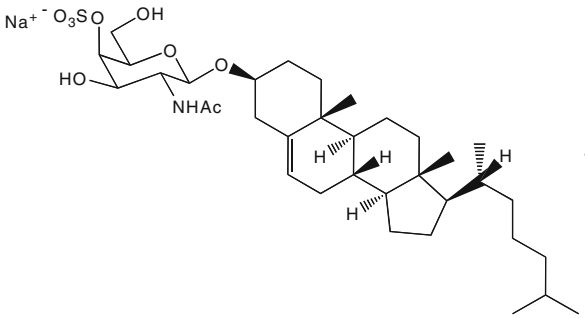

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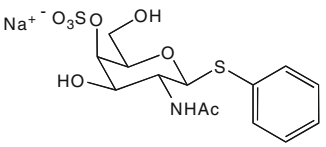

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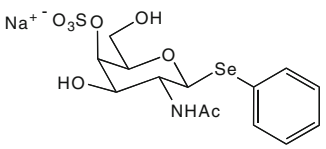

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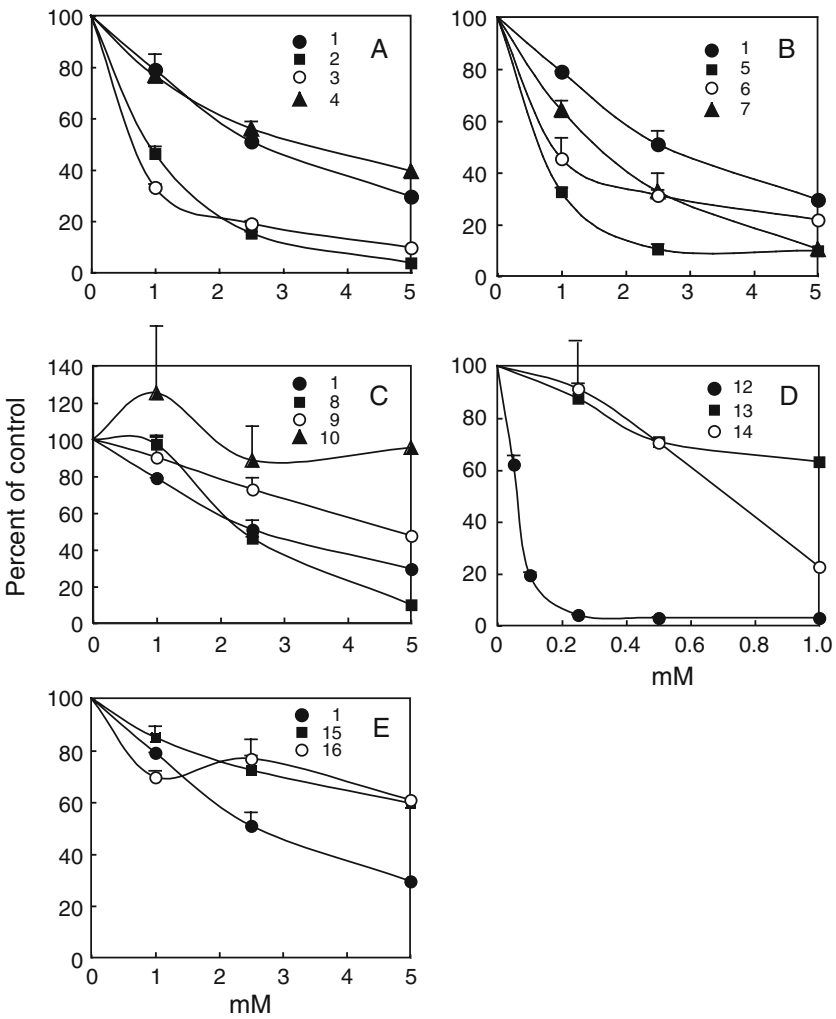

Fig. 2 Comparison of the inhibition of GalNAc4S-6ST activity by various glycosides. The sulfotransferase reaction was carried out as described under "Materials and Methods". The results indicated in panel d were obtained in the presence of 1% Triton X-100 in the reaction mixtures. Numbers indicated in the respective panels indicate the number of the compounds shown in Fig. 1. In each panel except for panel d, effect of phenyl-*β*-D-GalNAc(4SO_4) (**1**) was indicated for comparison. (a) *p*-Nitrophenyl-*β*-D-GalNAc (4SO_4) (**2**), *p*-chlorophenyl-*β*-D-GalNAc(4SO_4) (**3**) and *p*-fluorophenyl-*β*-D-GalNAc (4SO_4) (**4**); (b) benzyl-*β*-D-GalNAc(4SO_4) (**5**), octyl-*β*-D-GalNAc(4SO_4) (**6**) and 1-naphthyl-*β*-D-GalNAc(4SO_4) (**7**); (c) cyclohexyl-*β*-D-GalNAc (4SO_4) (**8**), l-Menthyl-*β*-D-GalNAc(4SO_4) (**9**), and d-menthyl-*β*-D-GalNAc(4SO_4) (**10**); (d) 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**), 17-estradiol-*β*-D-GalNAc(4SO_4) (**13**) and cholesteryl-*β*-D-GalNAc(4SO_4) (**14**); (e), phenyl-S-*β*-D-GalNAc(4SO_4) (**15**) and phenyl-*Se*-*β*-D-GalNAc(4SO_4) (**16**). Values are averages of three determinations and S.D. is indicated by perpendicular lines

tration among the glycosides examined here. To determine the specificity of inhibition by this compound, we compared the effects of 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) on the sulfotransferase activity among GalNAc4S-6ST, C4ST-1 and C6ST-1. Under the conditions where GalNAc4S-6ST was inhibited by 90%, both C4ST-1 and chondroitin 6-sulfotransferase-1 (C6ST-1) were not inhibited at all (Fig. 3), suggesting that GalNAc(4SO_4) moiety of 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) might compete with GalNAc(4SO_4) residue of the acceptor CS-A for the acceptor binding site of the enzyme. To confirm this assumption, we examined the kinetics of GalNAc4S-6ST. As shown in Fig. 4, 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) exhibited competitive inhibition at 0.05 mM. The Ki value calculated from these data indicated in Fig. 4 was 0.008 mM; this value was much lower than the value for phenyl-*β*-D-GalNAc(4SO_4) (**1**) (0.98 mM) previously reported [30]. Aglycon moiety of 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) thus appears to contribute to the high affinity of 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) with the enzyme. The mode of inhibition by 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**), however, could not be simply attributed to competitive inhibition at 0.1 mM as shown in Fig. 4; at this concentration the aglycon moiety of 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) might also contribute to the interaction with the enzyme.

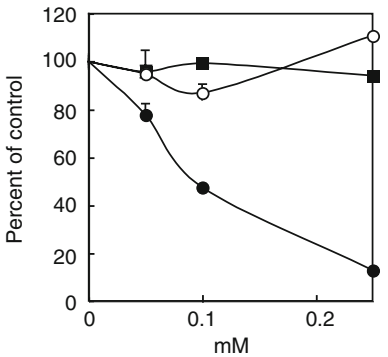

Fig. 3 Effects of 3-estradiol-*β*-D-GalNAc(4SO_4) (**12**) on the activities of GalNAc4S-6ST (closed circle), C4ST-1 (closed rectangle) and C6ST-1 (open circle). The sulfotransferase reaction was carried out as described under "Materials and Methods" in the presence of 1% Triton X-100. Values are averages of three determinations and S.D. is indicated by perpendicular lines

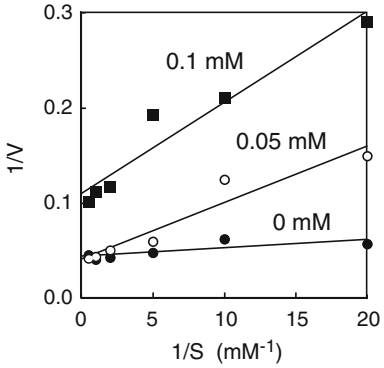

Fig. 4 The kinetics of inhibition of GalNAc4S-6ST by 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**). The GalNAc4S-6ST activity was determined at the varying concentration of CS-A and 3-estradiol-*β*-D-GalNAc ($4SO_4$). Data were expressed by a Lineweaver-Burk plot

In our previous paper, we reported that 6-sulfation of the nonreducing terminal GalNAc($4SO_4$) residue and internal GalNAc($4SO_4$) residue was inhibited equally by phenyl-β-D-GalNAc($4SO_4$) (**1**). To examine whether 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) also inhibits the sulfation of both the nonreducing terminal and internal GalNAc($4SO_4$) residues, we analyzed the ^{35}S-labeled glycosaminoglycans formed by the enzyme reaction with GalNAc4S-6ST in the absence or presence of 0.05 mM 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**). As shown in Fig. 5, the radioactivity was detected at the position of GalNAc(4,6-SO_4), ΔDi-diS_E and a oligosaccharide, GalNAc(4,6-SO_4)-GlcA($2SO_4$)- GalNAc($6SO_4$) [28]. The ratio of the radioactivity recovered in GalNAc(4,6-SO_4) and GalNAc(4,6-SO_4)-GlcA($2SO_4$)- GalNAc($6SO_4$), which were derived from the nonreducing terminal, to the total radioactivity was hardly altered in the presence of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**), indicating that this compound is able to inhibit 6-sulfation of both the nonreducing terminal and internal GalNAc($4SO_4$) residues.

Effects of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) on the intracellular synthesis of CS-E

To determine if 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) could influence the intracellular synthesis of CS-E, we examined the effects of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) on the synthesis of CS-E in the cells, which were capable of synthesizing CS-E. The cells were established from rat chondrosarcoma LTC cells by introducing human GalNAc4S-6ST cDNA as described under "Materials and Methods". The chondrosarcoma cells expressing human GalNAc4S-6ST were labeled by $^{35}SO_4$ in the absence or presence of 0.5 to 2.5 mM 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) and the ^{35}S-labeled glycosaminoglycans were isolated. When the ^{35}S-labeled glycosaminoglycans were digested with chondroitinase ACII and subjected to SAX-HPLC, major part of ^{35}S-radioactivity was detected at two peaks corresponding to 2-acetamide-2-deoxy-3-*O*-(*β*-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S) and ΔDi-diS_E. The ratio, ΔDi-diS_E /(ΔDi-4S + ΔDi-diS_E), was decreased slightly as the concentration of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) was increased (Fig. 6b, d), whereas total incorporation of $^{35}SO_4$ into glycosaminoglycans was not significantly affected (Fig. 6a, c). Incorporation into GalNAc(4,6-SO_4) was below the detection limits in these experiments. These results suggest that a small portion of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) could diffuse into the cells and affect the intracellular sulfation by GalNAc4S-6ST. However, a possibility that 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) might affect the expression of GalNAc4S-6ST protein by a yet unknown manner cannot be excluded.

Discussion

In this study, we found that the degree of inhibition of GalNAc4S-6ST by glycosides in which *β*-D-GalNAc ($4SO_4$) was linked to various hydrophobic aglycons was

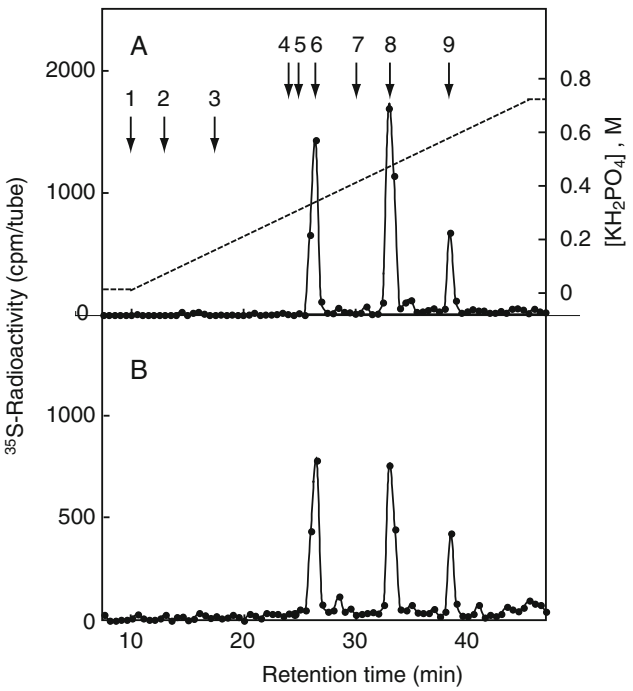

Fig. 5 Effects of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) on the sulfation of internal and nonreducing terminal GalNAc($4SO_4$) residues. The sulfotransferase reaction with GalNAc4S-6ST was carried out as described under "Materials and Methods" in the absence **a** or presence **b** of 0.05 mM 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**). The ^{35}S-labeled glycosaminoglycans were digested with chondroitinase ACII, and subjected to SAX-HPLC. The *arrows* indicate the elution position of ΔDi-0S (*arrow 1*); GalNAc($6SO_4$) (*arrow 2*); GalNAc($4SO_4$) (*arrow 3*); ΔDi-6S (*arrow 4*); ΔDi-4S (*arrow 5*); GalNAc(4,6-SO_4) (*arrow 6*); ΔDi-diS_D (*arrow 7*); and ΔDi-diS_E (*arrow 8*); and GalNAc(4,6-SO_4)-GlcA($2SO_4$)-GalNAc($6SO_4$) (*arrow 9*)

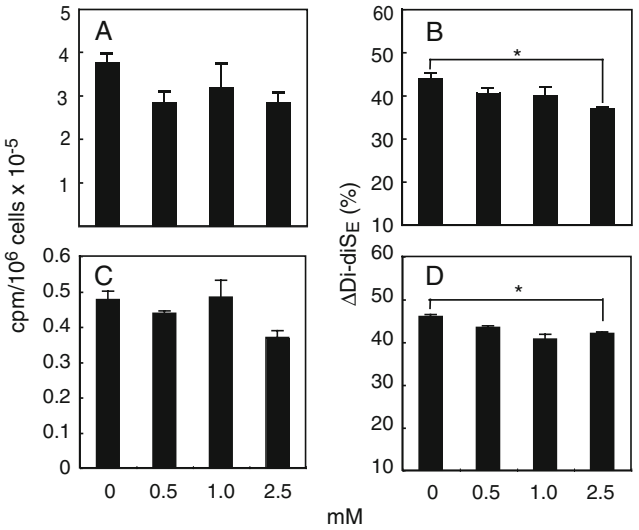

Fig. 6 Inhibition of synthesis of chondroitin sulfate E in chondrosarcoma cells overexpressing human GalNAc4S-6ST. Chondrosarcoma cells overexpressing human GalNAc4S-6ST were established from rat chondrosarcoma LTC cells and cultured as described under "Materials and Methods". 3-Estradiol-*β*-D-GalNAc(4SO$_4$) was dissolved in dimethyl sulfoxide and 17 µl of the solution was added to the culture medium at the final concentration up to 2.5 mM. To the control dishes, equal volume of dimethyl sulfoxide was added. Conditions for metabolic labeling and extraction of the ^{35}S-labeled glycosaminoglycan were as described under "Materials and Methods". Incorporation of ^{35}S-radioactivity into the glycosaminoglycan of the cell layer **a** and the medium fraction **c** was determined. Values are averages of three dishes and S.D. is indicated by perpendicular lines. The ^{35}S-labeled glycosaminoglycans obtained from the cell layer **b** and the medium fraction **d** were digested with chondroitinase ACII and radioactivity of ΔDi-4S and ΔDi-diS$_E$ were determined. Proportions of the radioactivity of ΔDi-diS$_E$ to the sum of radioactivity of ΔDi-4S and ΔDi-diS$_E$ are shown. Values are averages of three dishes and S.D. is indicated by perpendicular lines. *P<0.02 (*Student's t-test*)

dependent on the structure of the aglycons. *p*-Nitrophenyl-*β*-D-GalNAc(4SO$_4$) (**2**) and *p*-chlorophenyl-*β*-D-GalNAc (4SO$_4$) (**3**) were better inhibitors than phenyl-*β*-D-GalNAc (4SO$_4$) (**1**), suggesting that electron withdrawing group of these aglycons may strengthen the inhibitory activity. The observation that *p*-fluorophenyl-*β*-D-GalNAc(4SO$_4$) (**4**) inhibited more weakly than *p*-Nitrophenyl-*β*-D-GalNAc (4SO$_4$) (**2**) and *p*-chlorophenyl-*β*-D-GalNAc(4SO$_4$) (**3**) may be relevant to the fact that the para substituent constant for fluorine in the Hammett equation is much smaller than those for chlorine and nitro substituents [46]. Benzyl-*β*-D-GalNAc(4SO$_4$) (**5**) was a better inhibitor than phenyl-*β*-D-GalNAc(4SO$_4$) (**1**), suggesting that the methylene bridge of benzyl group may function as the spacer and GalNAc (4SO$_4$) moiety of the glycoside may be accessed by GalNAc4S-6ST more easily than phenyl-*β*-D-GalNAc (4SO$_4$) (**1**). *l*-Menthyl-*β*-D-GalNAc(4SO$_4$) (**9**) but not *d*-menthyl-*β*-D-GalNAc(4SO$_4$) (**10**) showed inhibitory activity. Configuration of *l*-menthyl group may be required for binding to the surface of GalNAc4S-6ST. Cyclohexyl-*β*-D-6-deoxy-GalNAc(4SO$_4$) (**11**), which is devoid of 6-OH showed no inhibitory activity, supporting the idea that glycosides containing GalNAc(4SO$_4$) could serve as the acceptor for GalNAc4S-6ST, and thereby inhibit sulfation of CS-A by GalNAc4S-6ST competitively as previously observed in phenyl-*β*-D-GalNAc(4SO$_4$) (**1**) [30]. 3-Estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) was the best inhibitor for GalNAc4S-6ST among glycosides examined here, whereas 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) was a much weaker inhibitor than 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**). Because 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) and 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) are different from each other only in the position of hydroxyl group through which these compounds are linked to GalNAc(4SO$_4$) by glycosidic bonds, GalNAc4S-6ST appears to recognize the structure of aglycons strictly. At present it is not clear what structure of compound **12** contributed its high inhibitory activity. A six-membered aromatic ring linked to GalNAc (4SO$_4$) through the *β*-O-glycosidic bond may play an important role in the higher inhibitory activity of 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**), because 17-estradiol-*β*-D-GalNAc(4SO$_4$) (**13**) and cholesteryl-*β*-D-GalNAc(4SO$_4$) (**14**) with less inhibitory activity contain five-membered and six-membered cycloalkane, respectively, instead of the six-membered aromatic ring.

Xylosides bearing hydrophobic aglycons have been thought to pass through plasma membrane and prime chondroitin sulfate or heparan sulfate at the Golgi. 2-Naphthyl-*β*-D-xyloside was actually shown to be incorporated into the CHO cells [34]. GalNAc(4SO$_4$) bearing hydrophobic aglycons was also expected to enter cells and affect the synthesis of CS-E. However, *p*-nitrophenyl-*β*-D-GalNAc(4SO$_4$) (**2**) with relatively high inhibitory activity did not affect the synthesis of CS-E in the chondrosarcoma cells overexpressing human GalNAc4S-6ST (data not shown). In contrast, 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) reduced the synthesis of CS-E significantly at 2.5 mM. Although 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) inhibited GalNAc4S-6ST nearly completely at 0.25 mM, this compound reduced the synthesis of CS-E in the cultured cells only 7% when this compound was added to the culture medium at 2.5 mM. One of reasons for the low cellular effects of 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) may be attributable to the low permeability of this glycoside due to the presence of negatively charged sugar residue, GalNAc(4SO$_4$). We examined cellular effects of *p*-chlorophenyl-*β*-D-GalNAc (4SO$_4$) (**3**) and octyl-*β*-D-GalNAc(4SO$_4$) (**6**) at 2.5 mM but no significant effects were obtained (data not shown). We also examined the effect of 3-estradiol-*β*-D-GalNAc(4SO$_4$) (**12**) on the cellular synthesis of CS-E using bone marrow derived mast cells [28] and CHO cells, which stably express human GalNAc4S-6ST; however, no significant inhibition of CS-E synthesis was observed in these cells (data not shown).

To achieve efficient introduction of the glycoside into the cultured cells, studies on the efficient delivery methods will be required. Further studies on the modification of both aglycon and glycon moieties will also be necessary. Acetylated disaccharides bearing *β*-O-naphthalenemethanol primed sialyl Le^X oligosaccharides and inhibited the expression of sialyl Le^X in cells [34–36]. It remains to be clarified whether acetylation of GalNAc($4SO_4$) residue of 3-estradiol-*β*-D-GalNAc($4SO_4$) (**12**) may enhance its permeability and cause more efficient reduction in the synthesis of CS-E.

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