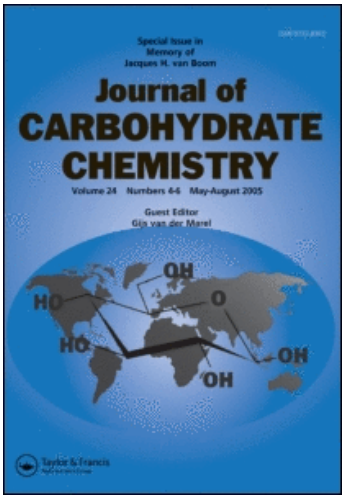

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Novel Glycosaminoglycan Glycotechnology: Method for Hybrid Synthesis of Glycosaminoglycan Chains Utilizing Chemo-enzymatic Procedures

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Novel Glycosaminoglycan Glycotechnology: Method for Hybrid Synthesis of Glycosaminoglycan Chains Utilizing Chemo-enzymatic Procedures

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A novel method for the synthesis of glycosaminoglycan chains is described. The 4,5-unsaturated hexuronic acid located at the nonreducing terminal, which derived from eliminase digestion of glycosaminoglycan, is chemically converted to glucuronic acid by employing the oxymercuration-demercuration reaction. Then, utilizing these oligosaccharides as acceptors for the transglycosylation reaction of bovine testicular hyaluronidase, glycosaminoglycan oligosaccharides were successfully constructed. It is thought that the present method will contribute to the development of glycosaminoglycan glycobiology and technology.

Keywords Glycosaminoglycan; Chondroitin sulfate; Glycotechnology; Proteoglycan; Transglycosylation; Oxymercuration-demercuration

INTRODUCTION

Several carbohydrate chains exist in the surface proteins of cells, each having specific functions. In eukaryotes, these proteins are usually glycoproteins and play important roles in biological phenomena.[1] Recent advances in gene

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engineering have now made it possible to mass produce useful proteins, but it is difficult to synthesize complete glycoproteins because DNA incorporated by gene recombination carries no direct information about the biosynthesis of these carbohydrate chains. Many reports have described that these glycoproteins have few biological activities because of the incomplete nature of their carbohydrate chains. [2,3] Therefore, to address this problem, glycotechnology techniques to construct individual carbohydrate chains have been developed (i.e., organic synthesis or enzymatic synthesis). [4,5] In the case of glycosaminoglycans, we have developed several original glycotechnology approaches utilizing the transglycosylation reaction of bovine testicular hyaluronidase (BTH), which is an endo-β-*N*-acetylhexsosaminidase, and endo-β-xylosidase. [6,7] These methods utilize the reverse hydrolysis reaction of glycosidase and have produced many new bioactive glycosaminoglycan oligosaccharides.[8,9] These endo-type glycosidases are useful tools for glycotechnology.

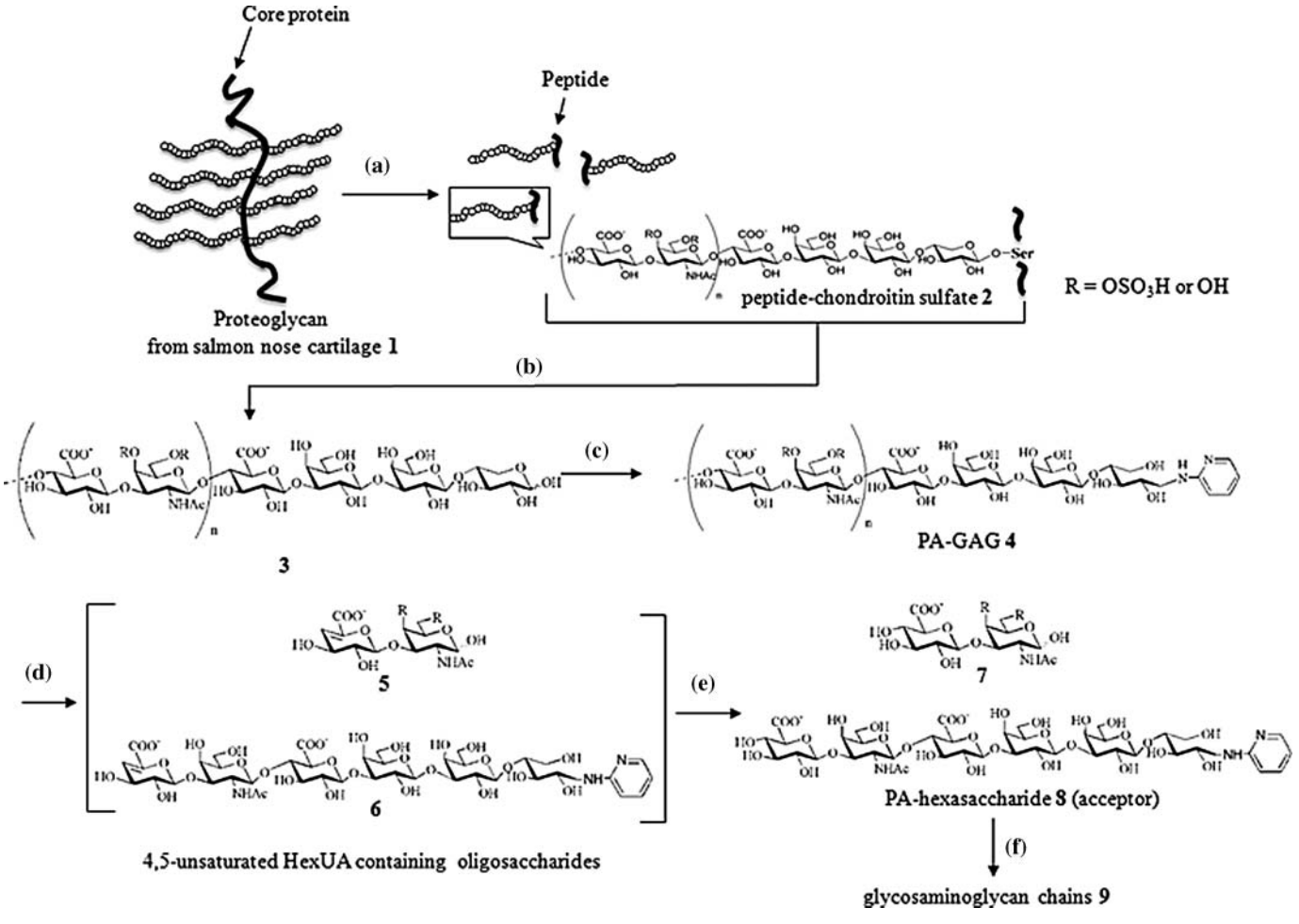
On the other hand, bacterial GAG lyases (i.e., chondroitinase ABC, ACI and ACII, etc.) are also known to be typical degradation enzymes for glycosaminoglycans. The eliminase digestion of glycosaminoglycans produces 4,5-unsaturated hexuronic acid (HexUA) at the nonreducing terminal of the resulting glycosaminoglycan oligosaccharides. These oligosaccharides provide important information for qualitative and quantitative analysis of glycosaminoglycans[10–13] but cannot be acceptors for the transglycosylation reaction because the nonreducing terminal saccharide is converted to 4,5-unsaturated HexUA.

In the present study, we focused on these 4,5-unsaturated HexUA-containing glycosaminoglycan oligosaccharides. Development of a convenient technique that could transform unsaturated HexUA into glucuronic acid (GlcA) would be of considerable significance in glycosaminoglycan glycotechnology, as it would allow the creation of novel bioactive glycosaminoglycan oligosaccharides by using the transformed oligosaccharide as an acceptor for the transglycosylation reaction. Here we describe for the first time the chemical conversion of 4,5-unsaturated HexUA into GlcA, and the construction of glycosaminoglycan chains by using the transformed oligosaccharide as an acceptor for the transglycosylation reaction.

RESULTS

Strategy for Synthesis of Neo-Glycosaminoglycan Chains

Our strategy in the present study consisted of four steps (Sch. 1). The first step was the preparation of pyridylaminated glycosaminoglycan (PA-GAG) **4** from proteoglycan **1** derived from salmon nose cartilage. The second step was enzymatic digestion of **4** using chondroitinase ABC to obtain disaccharide **5** and PA-hexasaccharide **6**, which each contain 4,5-unsaturated HexUA at the



Scheme 1: Strategy for synthesis of glycosaminoglycan chains. (a) Actinase E, 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$, 40°C, 48 h; (b) endo-β-xylosidase, 100 mM sodium acetate buffer (pH 4.0), 37°C, 24 h; (c) 1) 2-aminopyridine 90°C, 20 min, 2) $NaBH_3CN$, 90°C, 3 h; (d) chondroitinase ABC (0.3U), 400 mM Tris-HCl buffer (pH 8.0), 400 mM sodium acetate buffer (pH 5.0), 37°C, 48 h; (e) 1) $(CH_3COO)_2Hg$, 0°C, 10 min, 2) $NaBH_4$, 0°C, 1 h; (f) hyaluronic acid, bovine testicular hyaluronidase (1.0 NFU), 150 mM Tris-HCl buffer (pH 7.0), 37°C, 1 h.

nonreducing terminal. The third step was the chemical conversion of unsaturated HexUA into GlcA utilizing the oxymercuration-demercuration reaction.[14] Optimization of the reaction conditions and instrumental analysis of the reaction products were performed using disaccharide **5**.

Next, to develop a general procedure for preparation of the transglycosylation reaction acceptor from 4,5-unsaturated HexUA-containing oligosaccharide, we employed PA-hexasaccharide **6**, which was chemically converted into the terminal GlcA-containing PA-hexasaccharide **8**. The final step was the enzymatic elongation of **8** as the transglycosylation reaction acceptor, producing glycosaminoglycan chains **9**.

Optimization of the Conditions for the Oxymercuration-Demercuration Reaction

For converting 4,5-unsaturated HexUA to GlcA, we employed the oxymercuration-demercuration reaction, which is a useful procedure for synthesizing alcohols from alkenes employing a two-step method.[14] The 4,5-unsaturated HexUAβ(1→3)GalNAc 4-*O*-sulfate **5** was used as the model compound for the optimization process. The reaction should proceed below 0°C to prevent decomposition of the HexUA and sulfate group, and the most appropriate amounts of mercury (II) acetate ($(CH_3COO)_2Hg$) and sodium borohydride ($NaBH_4$) were 2 and 1.5 molar equivalents, respectively, relative to unsaturated oligosaccharide. The addition of excess $(CH_3COO)_2Hg$ led to release of 4,5-unsaturated HexUA.[15] The optimal oxymercuration and demercuration times were 10 min and 1 h, respectively (Table 1). The present procedure was also available for 4,5-unsaturated HexUAβ(1→3)GalNAc, 4,5-unsaturated HexUAβ(1→3)GalNAc 6-*O*-sulfate, and 4,5-unsaturated HexUAβ(1→3)GlcNAc.

Structural Analysis of Synthesized Saturated Disaccharide 7

The 4,5-unsaturated HexUA-containing disaccharide mixture [ratio of 4,5-unsaturated HexUAβ(1→3)GalNAc 4-*O*-sulfate to 4,5-unsaturated

Table 1: Conditions for the oxymercuration-demercuration reaction Entry 1, decomposition of 4,5-unsatuated HexUA; entry 2, remaining starting material **5**

Entry	Oxymercuration time/temp.	Demercuration time/temp.	Yield (%)
1	1 h/r.t.	1 h/r.t.	0
2	5 min/0°C	1 h/0°C	59
3	10 min/0°C	1 h/0°C	72
4	10 min/0°C	2 h/0°C	68

HexUAβ(1→3)GalNAc 6-*O*-sulfate was 3 to 1; prepared by exhaustive digestion of PA-GAG **4**] was subjected to the oxymercuration-demercuration reaction, and the resulting products were analyzed by HPLC, ESI-MS, NMR, and β-glucuronidase digestion.

First analysis

The HPLC (Polyamine II column) profiles of the saccharide before and after the reaction are shown in Figure 1. The data in charts A and B indicate the profiles before the reaction (i.e., 4,5-unsaturated HexUA-containing disaccharide), and these eluates were monitored for UV absorbance at 215 nm and 232 nm, respectively. Two peaks (4-*O*-sulfate and 6-*O*-sulfate) were observed in both charts. Next, charts C and D indicate the profiles after the reaction, and these eluates were also monitored for UV absorbance at 215 nm and 232 nm, respectively. There are no peaks in chart D, because the 4,5-unsaturated HexUA was converted to a saturated form.

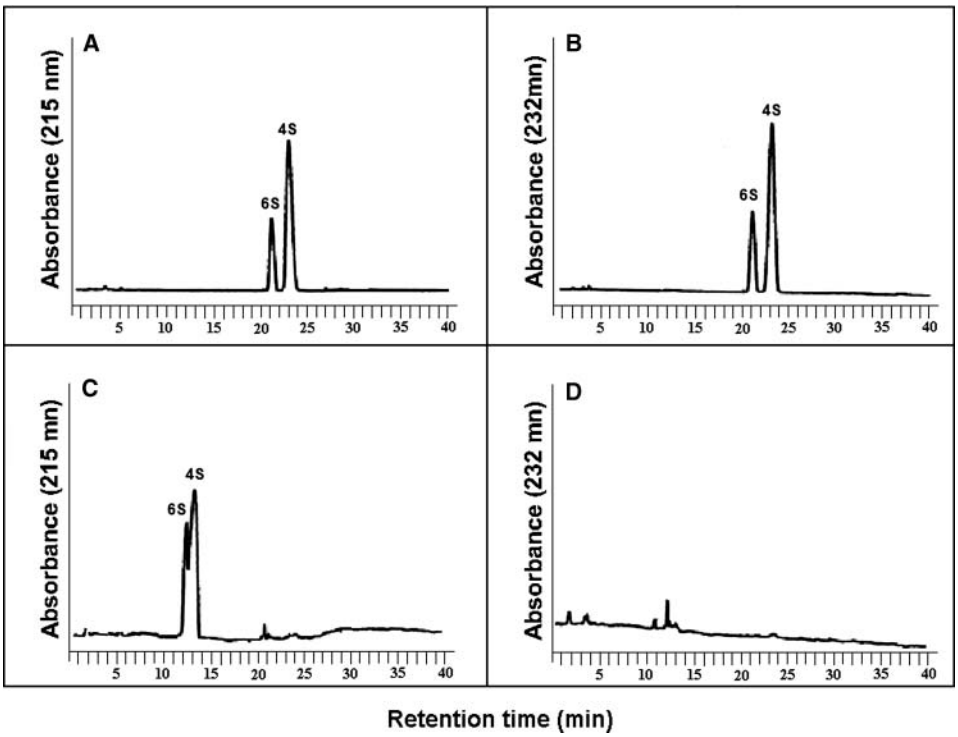


Figure 1: HPLC chromatograms (Polyamine II column) of disaccharides **5** (charts A and B) and **7** (charts C and D). The eluates were monitored by UV absorbance at 215 nm (charts A and C) and 232 nm (charts B and D). 6S and 4S shown in the chromatograms indicate 6-*O*-sulfate disaccharide and 4-*O*-sulfate disaccharide, respectively.

Second analysis

The reaction products were analyzed using ESI-MS spectrometry in negative ion mode (Fig. 2A, B). The unsaturated disaccharide was demonstrated at m/z 458.2 $[M\text{-}H]^-$ and 480.3 $[M\text{-}2H+Na]^-$ (Fig. 2A) and the reacted disaccharide at m/z 476.3 $[M\text{-}H]^-$, 498.3 $[M\text{-}2H+Na]^-$, and 521.3 $[M\text{-}3H+2Na]^{-,}$ which coincided with the saturated disaccharide (Fig. 2B). The molecular weight alteration indicated completion of the oxymercuration-demercuration reaction on the unsaturated disaccharide. Moreover, no structural degradation (i.e., desulfation and excess reduction) occurred during the reaction process.

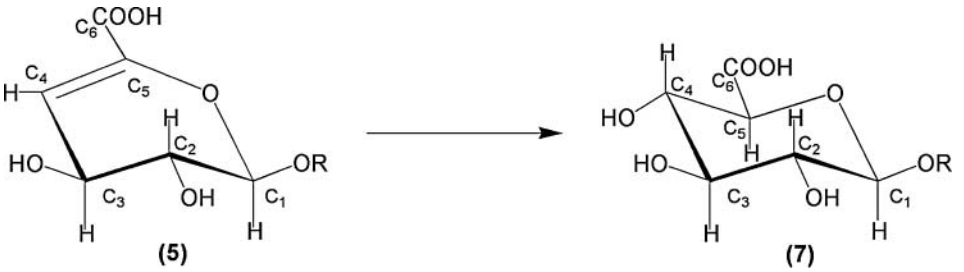
Third analysis

In the ^1H NMR spectrum of the synthesized regenerated disaccharide, the C-3 and C-4 protons of GlcA measured at δ 3.63 (dd, $J_{2,3} = 8.4$, $J_{3,4} = 7.2$ Hz, C-3) and 3.88 (dd, $J_{3,4} = 7.2$, $J_{4,5} = 10.8$ Hz, C-4) indicated that the 4,5-unsaturated HexUA was converted to GlcA (Table 2).[16]

Final analysis

The synthesized disaccharide was digested with β-glucuronidase, and then the resulting products were analyzed by ESI-MS spectrometry in the negative ion mode (Fig. 3). The expected significant ions measured at m/z 193.1 $[M\text{-}H]^-$ and 300.1 $[M\text{-}H]^-$ were derived from GlcA and sulfated *N*-acetyl galactosamine, respectively. These analyses indicated that the 4,5-unsaturated

Table 2: Comparison of the selected ^1H NMR data for H-3 and H-4 of the 4,5-unsaturated hexuronic acid and glucuronic acid



Compound No. (Conformation)	**5** d (multiplicity, *J* (Hz))	**7** d (multiplicity, *J* (Hz))
H-3	3.95 (dd, $J_{2,3} = 1.0$, $J_{3,4} = 4.5$)	3.63 (dd, $J_{2,3} = 8.4$, $J_{3,4} = 7.2$)
H-4	5.97 (d, $J_{3,4} = 4.5$)	3.88 (dd, $J_{3,4} = 7.2$, $J_{4,5} = 10.8$)

Measured at 400 MHz in D_2O.

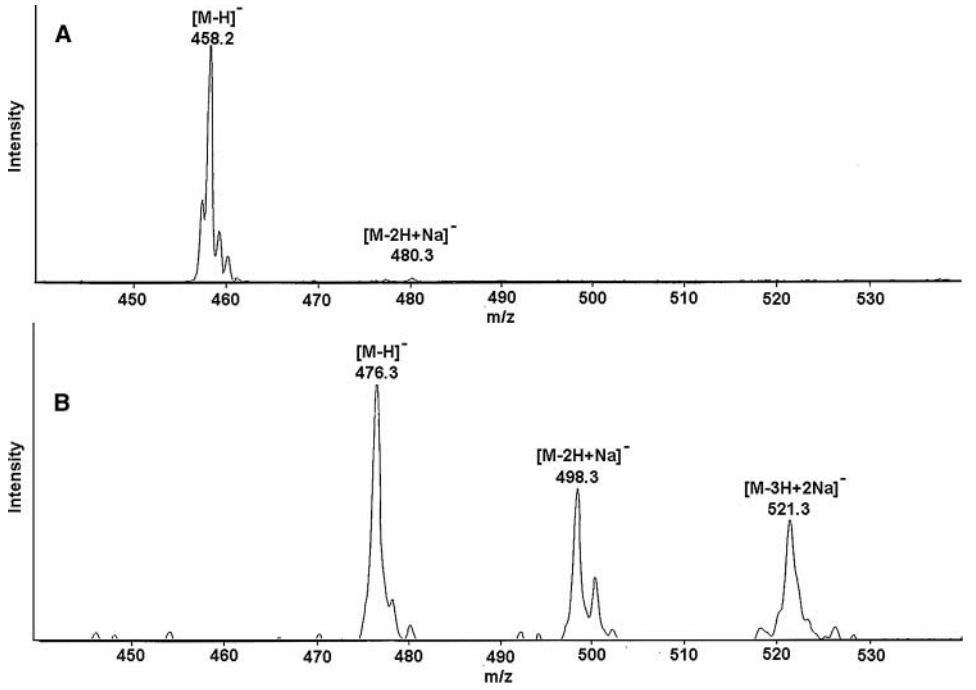


Figure 2: ESI-MS spectra of **5** (chart A) and **7** (chart B) in the negative-ion mode.

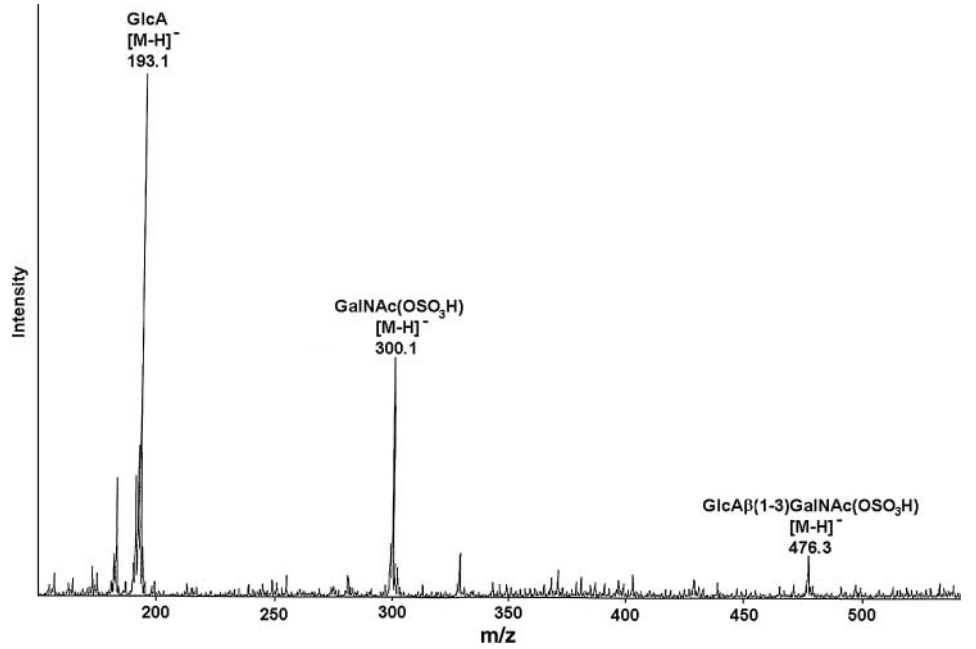


Figure 3: ESI-MS spectrum of β-glucuronidase digestion products in the negative-ion mode.

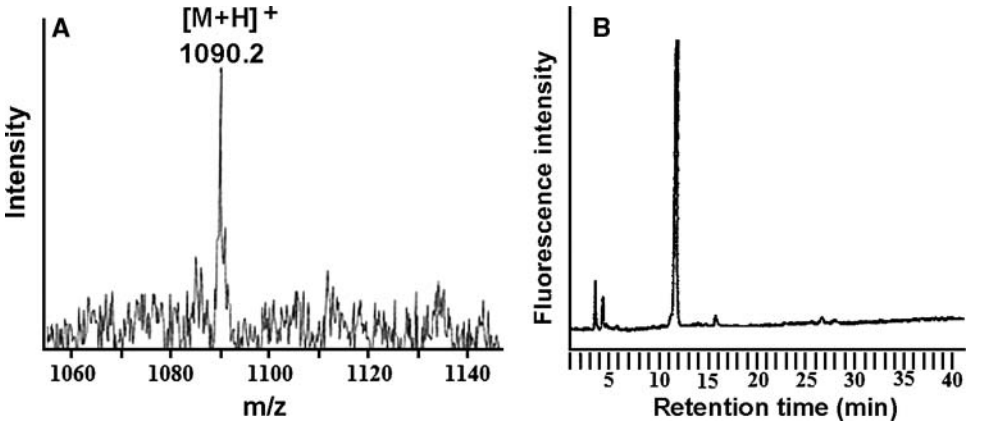


Figure 4: ESI-MS spectra (A) and HPLC chromatograms (B) of compound **6**. The conditions for HPLC and mass spectrometry are described in the Experimental section.

HexUA at the nonreducing terminal had changed into GlcA, and not into galacturonic acid or other moieties.

HPLC and Mass Analysis of Synthesized PA-Hexasaccharide: GlcAβ(1→3)GalNAcβ(1→4)GlcAβ(1→3)Galβ(1→3) Galβ(1→4)Xyl1-PA 8

The prepared PA-hexsasaccharide **6**, bearing 4,5-unsaturated HexUA at the nonreducing terminal, was subjected to the oxymercuration-demercuration reaction. The resulting product was then subjected to HPLC (Polyamine II column) and analyzed by ESI-MS spectrometry in the positive ion mode. The molecular weight of compound **6** was shown at m/z 1090.2 $[M+H]^+$ (Fig. 4) and the reactant hexasaccharide at m/z 1108.4 $[M+H]^+$ (Fig. 5), which coincided with the saturated hexasaccharide **8**. Judging from these results, the reaction was suggested to be broadly applicable for unsaturated sugars located at the nonreducing terminal.

Enzymatic Synthesis of Glycosaminoglycan Chains 9

Hyaluronic acid as a donor and the PA-hexasaccharide GlcAβ(1→3) GalNAcβ(1→4)GlcAβ(1→3)Galβ(1→3)Galβ(1→4)Xyl1-PA **8** as an acceptor were incubated with BTH. The transglycosylation reaction underwent the optimized condition, and the reaction products were examined by tracing the fluorescence of the PA-hexasaccharide acceptor by HPLC (Amide 80 column).[6]

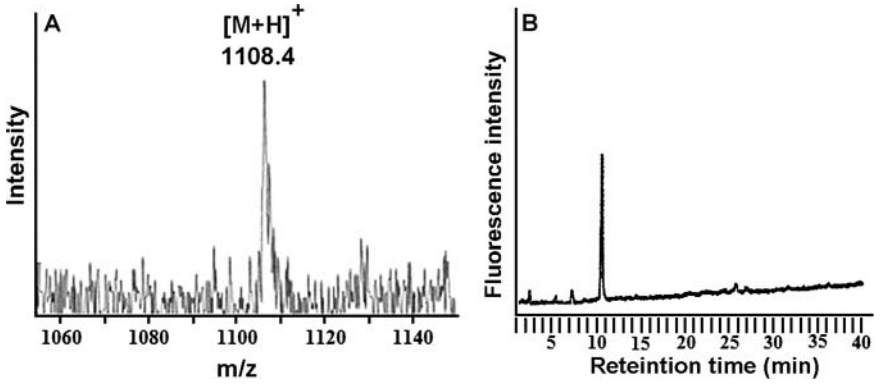


Figure 5: ESI-MS spectra (A) and HPLC chromatograms (B) of compound **8**. The conditions for HPLC and mass spectrometry are described in the Experimental section.

Seven peaks of newly synthesized products were observed, which were identified as octa-, deca-, dodeca-, tetradeca-, hexadeca-, octadeca-, and icosasaccharide by HPLC (Fig. 6). In the case of using chondroitin sulfate as a donor, octa-, deca-, and dodecasaccharide were identified (data not shown).

DISCUSSION

O-Linked sugar chains united with specific serine or threonine residues of peptides or proteins are known to have specific biological functions.[1] To construct native-type glycopeptides and proteins artificially, it is necessary to develop technology that can introduce these saccharide chains to specific amino acid residues.

To achieve these purposes in glycosaminoglycans, we have established several original techniques for glycosaminoglycan glycotechnology. [7,17–22] The first method utilized the transglycosylation activity of endo-β-xylosidase. This enzyme hydrolyzed the xylosyl serine linkage between a peptide and GAG chain, and at the same time introduced the released GAG chain into the purpose acceptor in accordance with its transglycosylation activity. Utilizing this method, we have succeeded in synthesizing GAG-containing peptides and proteins. [20–22] However, we have not yet achieved selective introduction of a GAG chain into the specific serine residue of an acceptor peptide containing a number of serine residues.

The second method we employed utilized the transglycosylation reaction activity of BTH. We have been investigating the use of BTH with the aim of performing enzymatic degradation and synthesis of GAGs. [6,23–25] This approach makes it possible to synthesize GAG chains that are bound to specific serine residues. Thus, a GAG chain united with a specific serine residue in a peptide or protein can be exhaustively digested by BTH except for its linkage

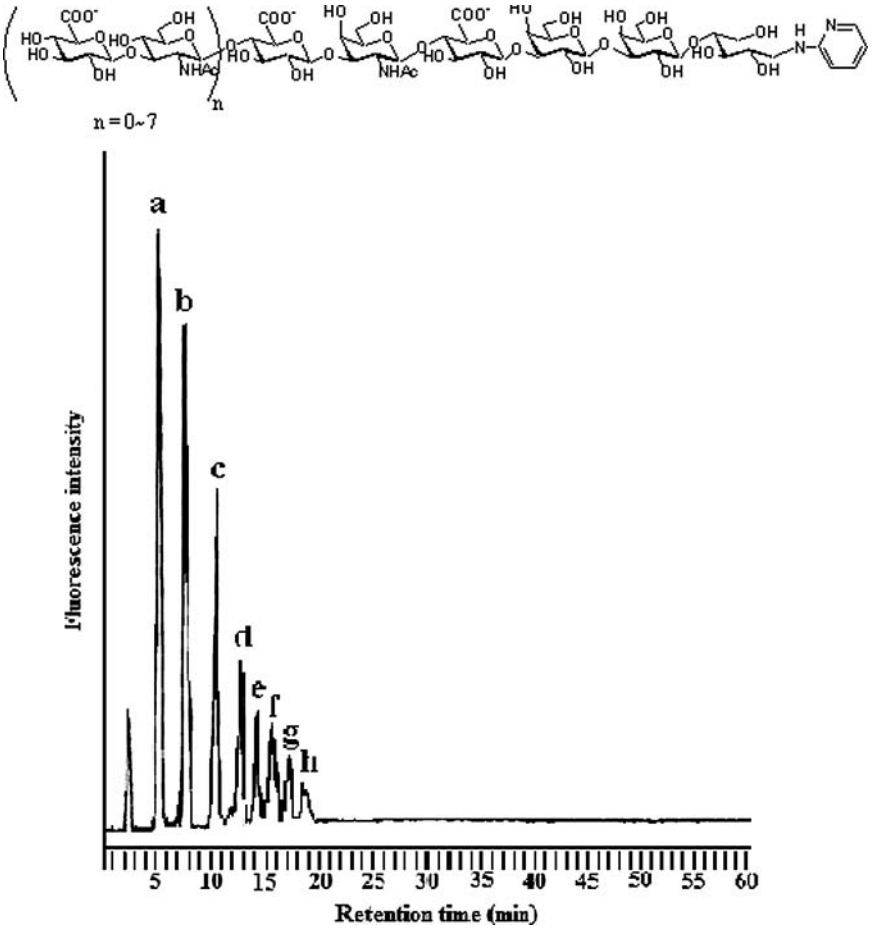


Figure 6: Structure and HPLC chromatogram (Amido 80 column) of the synthesized glycosaminoglycan chains **9**. a, PA-hexasaccharide acceptor **8**; b, octasaccharide; c, decasaccharide; d, dodecasaccharide; e, tetradecasaccharide; f, hexadecasaccharide; g, octadecasaccharide; h, icosasaccharide.

region tetrasaccharide [i.e., GlcAβ(1→3)Galβ(1→3)Galβ(1→4)Xyl], and the remaining saccharide utilized as an acceptor for the transglycosylation reaction of BTH. The introduction of a new oligosaccharide to an acceptor allows the reconstruction of peptides or proteins containing GAG chains. With this method, however, it is difficult to control the length of the acceptor GAG chain, because the hyaluronidase lacks structural specificity for GAG digestion.

Therefore, we have come to advocate the third method and utilized it in the present study. If it were possible to prepare acceptor carbohydrate chains using the chondroitinase family, long GAG chains could be attached to proteins because of their structural specificity for GAG digestion.[26–29] In addition, there is a possibility that some bioactive clusters might be retained in the resulting

GAG chains. However, the GAG chains produced by digestion with these enzymes cannot use acceptors for the transglycosylation reaction of BTH because these lyases produce disaccharide, which contains 4,5-unsaturated HexUA at its non-reducing terminal.

Our present approach solved this problem by transforming the produced 4,5-unsaturated HexUA into GlcA utilizing the optimized oxymercuration-demercuration reaction. The procedure was applicable to disaccharide and oligosaccharide containing 4,5-unsaturated HexUA, and no structural degradation occurred during the reaction process. The resulting oligosaccharide can be used as an acceptor for the transglycosylation reaction of BTH. Thus, our proteoglycan glycotechnology technique (second method) is applicable for the preparation of bioactive glycosaminoglycan chains.

In conclusion, we have succeeded in synthesizing glycosaminoglycan chains using the chemically converted oligosaccharide as an acceptor. Our present hybrid synthetic method using bacterial lyase, bovine testicular hyaluronidase, and the oxymercuration-demercuration reaction makes it possible to create further diverse GAG oligosaccharides and expands both the application of glycoengineering and the study of proteoglycans.

EXPERIMENTAL SECTION

General Methods

Endo-β-xylosidase was isolated from the *Patinopecten* midgut gland using the procedure described in our previous report.[7] Actinase E was purchased from Kaken Pharmaceutical Co. (Tokyo, Japan). Chondroitinase ABC (from *Proteus vulgaris*) and β-glucuronidase (from bovine liver) were purchased from Seikagaku (Tokyo, Japan). We confirmed that the β-glucuronidase could act on disaccharide *N*-acetyl chondroisne.[30] Bovine testicular hyaluronidase (type 1-S) was purchased from Sigma-Aldrich (St. Louis, MO). Hyaluronic acid (Mr = 80,000) was purchased from KIBUN Food Chemifa. (Tokyo, Japan). Sephadex G-25 was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). All other chemicals were obtained from commercial sources.

HPLC: A high-performance liquid chromatography (Hitachi L-6200, Hitachi, Japan) connected to a fluorescence detector (Hitachi F-1050) or a UV detector (Hitachi L-4200) was used. Analysis of PA-GAG **4** was carried out with a size exclusion chromatography Shodex OH pack SB-803 HQ (300 $\times$ 8.0 mm, Showa Denko., Kawasaki, Japan), which was eluted with 0.2 M NaCl at a flow rate of 1 mL/min and column temperature of 40°C. The eluates were monitored at excitation and emission wavelengths of 330 and 400 nm, respectively.

Analysis of disaccharides **5** and **7** and PA-hexasaccharides **6** and **8** was carried out with a Polyamine II column (4.6 $\times$ 250 mm; YMC, Tokyo. Japan). The

eluates were monitored by UV absorbance at 215 nm and 232 nm (disaccharide) or fluorescence excitation and emission wavelengths of 320 and 400 nm, respectively (PA-hexasaccharide). The elution conditions were as follows: eluent A: 10 mM NaH_2PO_4; eluent B: 1 M NaH_2PO_4. The column was equilibrated with eluent A, and the ratio of eluent B to eluent A was increased linearly to 40% over a 40-min period at a flow rate of 1.0 mL/min at 40°C.

Analysis of transglycosylated glycosaminoglycan chains **9** was carried out with a TSKgel Amide 80 column (4.6 × 250 mm; TOSOH, Tokyo, Japan). The eluates were monitored at fluorescence excitation and emission wavelengths of 320 and 400 nm, respectively. The elution conditions were as follows: eluent A: containing 3% acetic acid, adjusted to pH 7.3 with triethlyamine and acetonitrile at a ratio of 20:80; eluent B: containing the same reagents at a ratio of 50:50. The column was equilibrated with eluent A, and the ratio of eluent B to eluent A was increased linearly to 100% over a 60-min period at a flow rate of 1.0 mL/min at 30°C.

Mass spectra were obtained on an API-III triple-quadrupole mass spectrometer (Sciex, Thornhill, Ontario, Canada). The samples were dissolved in 0.1% formic acid containing acetonitrile (50:50) and injected at 3 μL/min with a micro-HPLC syringe pump. The ^{1}H NMR spectrum was recorded at 300 K with a Bruker AV500 spectrometer. The values (ppm) are given relative to HOD as the internal standard.

Preparation of pyridylaminated glycosaminoglycan (PA-GAG) 4

PA-GAG **4**, containing the linkage region tetrasaccharide GlcAβ(1→3)Galβ(1→3)Galβ(1→4)Xyl, was prepared from proteoglycan **1** (0.5 g) derived from salmon nose cartilage. The procedure was as follows: the proteoglycan **1** was digested with actinase E (50 mg) in 100 mM Tris-HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ at 40°C in a total volume of 10 mL. The reaction mixture was incubated for 48 h at 40°C, and the reaction was terminated by immersion in a boiling water bath for 3 min. After centrifugation, the resulting supernatants were passed through a C18-Sep-Pack cartridge column to remove soluble hydrophobic components. The resulting eluate was cooled to 0°C, and 4 volumes of NaCl-saturated ethanol (40 mL) were added. The resulting precipitate, peptide-chondroitin sulfate **2** (380 mg), was digested with endo-β-xylosidase in 100 mM sodium acetate buffer (pH 4.0) at 37°C for 24 h in a total volume of 10 mL. The reaction was terminated by immersion in a boiling water bath for 3 min. The precipitate, chondroitin sulfate **3** bearing the linkage region tetrasaccharide (367 mg), was obtained by addition of 4 volumes of NaCl-saturated ethanol (40 mL). Compound **3** (100 mg) was labeled by PA at the reducing terminal in accordance with our previous report, based on the method of Hase et al., [31,32] to afford PA-GAG **4** (78 mg). This is the first report preparing a large quantity of PA-GAG from salmon nose cartilage.

SEC-HPLC: t_R = 7.38 min. The average molecular weight of **4** was estimated to be approximately 9,000 using the calibration curve derived from chondroitin sulfate chain standards of known molecular weight.

Simultaneous preparation of compounds 5 and 6

Degradation of PA-GAG **4** with chondroitinase ABC was performed as follows: the incubation mixture contained **4** (51 mg), 21 μL of 400 mM Tris-HCl buffer (pH 8.0), 25 μmol of 400 mM sodium acetate buffer (pH 5.0), 42 μg of BSA, and 0.3 U of chondroitinase ABC in a total volume of 420 μL. The mixture was incubated for 48 h at 37°C. The reaction was terminated by immersion in a boiling water bath for 3 min and then filtered. The filtrate was applied to a Sephadex G-25 column (4 × 60 cm) with an eluent of H_2O:ethanol = 9:1, and this afforded a mixture of compounds **5** and **6**. The mixture was then purified by HPLC (Polyamine II column). Fractions corresponding to **5** and **6** were then desalted by Sephadex G-25 column chromatography (4 × 120 cm) with an eluent of H_2O:ethanol = 9:1 to give **5** (4-*O*-sulfate, 6-*O*-sulfate) and **6**, respectively. Compound **5**: HPLC (Polyamine II column): t_R = 20.80 min, 6-*O*-sulfate; t_R = 22.86 min, 4-*O*-sulfate. ESI-MS calcd. for $C_{14}H_{21}NO_{14}S$ $[M\text{-}H]^-$ 458.07, found 458.2 (Figs. 1A and 2A). Compound **6**: HPLC (Polyamine II column): t_R = 10.62 min. ESI-MS calcd. for $C_{42}H_{63}N_3O_{30}$ $[M+H]^+$ 1090.3, found 1090.2 (Fig. 4).

Preparation of Compound 7 Using the Oxymercuration-Demercuration Reaction

To a solution of compound **5** (290 mM) in H_2O:THF = 2:1 was added $(CH_3COO)_2Hg$ (522 mM) at 0°C. The reaction mixture was stirred for 10 min at 0°C, and then $NaBH_4$ (435 mM) was added at 0°C in a total volume of 138 μL. The reaction mixture was stirred for 1 h at 0°C. The mixture was then filtered, and the filtrate was passed through a cation-exchange column (AG 50W × 8, H^+ form, bed volume 1 mL), followed by elution with H_2O (1.5 mL) to remove the Hg^{2+} ions. The resulting eluate was subjected to Sephadex G-25 column chromatography to give compound **7**.

HPLC (Polyamine II column): t_R = 12.03 min, 6-*O*-sulfate; t_R = 12.84 min, 4-*O*-sulfate (Fig. 1C). H^1 NMR: 400 MHz (D_2O) of 4-*O*-sulfate of α-isomer: δ = 5.17 (d, 1 H, J = 2.8 Hz), 4.59 (br-d, 1 H), 4.56 (d, 1 H, J = 3.2 Hz), 4.41 (m, 1 H), 4.05 (m, 2 H), 3.97 (dd 1 H, J = 8.8, 3.2 Hz), 3.89 (dd, 1 H, J = 7.2, 10.0 Hz), 3.78 (dd, 1 H, J = 6.4, 8.4 Hz), 3.75 (dd, 1 H), 3.67 (dd, 1 H), 3.63 (dd, 1 H, J = 8.4, 7.2 Hz), 1.82 (s, 3 H). ESI-MS calcd. for $C_{14}H_{23}NO_{15}S$ $[M\text{-}H]^-$ 476.0, found 476.3 (Fig. 2B).

Conditions for β-Glucuronidase Digestion of Compound 7

Before the present experiment, it was confirmed that β-glucuronidase could act on disaccharide *N*-acetyl chondrosine. The GlcAβ(1→3)GalNAc 4-*O*-sulfate 7 (70 mM) was incubated with 0.5 U of β-glucuronidase in 10 mM sodium acetate buffer (pH 5.0) at 37°C in a total volume of 45 μL. The reaction mixture was incubated for 24 h, and the reaction was then terminated by immersion in a boiling water bath for 3 min. The resulting solution was passed through a C18-Sep-Pac cartridge column and eluted with H_2O (1 mL) to remove the hydrophobic components resulting from the enzyme reaction. The resulting eluate was subsequently subjected to PD-10 column chromatography to afford the enzyme digestion products. These products were analyzed by ESI-MS spectrometry, and the results are shown in Figure 3.

Preparation of Compound 8 Using the Oxymercuration-Demercuration Reaction

To a solution of compound **6** (32.7 mM) in H_2O:THF = 2:1 was added $(CH_3COO)_2Hg$ (65.3 mM) at 0°C. The reaction mixture was stirred for 10 min, and then $NaBH_4$ (49.0 mM) was added at 0°C in a total volume of 48 μL. The reaction mixture was stirred for 1 h at 0°C and then filtered. The filtrate was passed through a cation-exchange column (AG 50W × 8, H^+ form, bed volume 1 mL) and eluted with H_2O (1.5 mL) to remove the Hg^{2+} ions, followed by lyophilization. The residue was again dissolved in 20 μL of deionized water, and the solution was subjected to HPLC (Polyamine II column). Then, the fraction corresponding to **8** was desalted by Sephadex G-25 column chromatography (4 × 120 cm) with an eluent of H_2O:ethanol = 9:1 to give compound **8**.

HPLC (Polyamine II column): t_R = 9.96 min. ESI-MS calcd. for $C_{42}H_{65}N_3O_{31}$ $[M+H]^+$ 1107.3, found 1108.4 (Fig. 5).

Conditions for the Transglycosylation Reaction Catalyzed by Testicular Hyaluronidase

Hyaluronic acid (5 μg) as a source of oligosaccharide donors, PA-hexasaccharide GlcAβ(1→3)GalNAcβ(1→4)GlcAβ(1→3)Galβ(1→3)Galβ(1→4)Xyl1-PA **8** (2 μM) as an acceptor, and 1.0 NFU of BTH dissolved in 50 μL of 150 mM Tris-HCl buffer (pH 7.0) were incubated at 37°C for 1 h. The reaction was terminated by immersion in a boiling water bath for 3 min. HPLC (Amido 80 column) elution times were t_R = 5.22 min, PA-hexasaccharide (acceptor); 7.45 min, octasaccharide; 10.27 min, decasaccharide; 12.92 min, dodecasaccharide; 14.91 min, tetradecasaccharide; 16.07 min, hexadecasaccharide; 17.72 min, octadecasaccharide; and 19.22 min, icosasaccharide (Fig. 6).

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ABBREVIATIONS

BSA, bovine serum albumin; BTH, bovine testicular hyaluronidase; $(CH_3COO)_2Hg$, mercury (II) acetate; ESI-MS, electrospray ionization mass spectrometry; GAG, glycosaminoglycan; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcA, glucuronic acid; HexUA, hexuronic acid; THF, tetrahydrofuran; HPLC, high-performance liquid chromatography; PA, 2-aminopyridine; SEC, size exclusion chromatography; Xyl, xylose.

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