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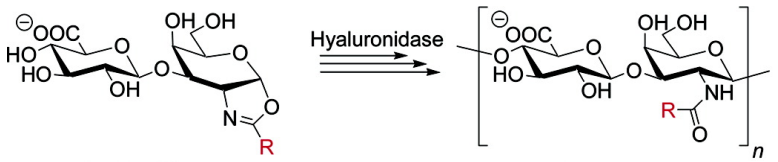
J. Am. Chem. Soc., **2003**, 125 (47), 14357-14369• DOI: 10.1021/ja036584x • Publication Date (Web): 04 November 2003



1a; R=-CH_3
1b; R=-CH_2CH_3
1c; R=-$(CH_2)_2CH_3$
1d; R=-$CH(CH_3)_2$
1e; R=-Ph
1f; R=-$CH{=}CH_2$

2a; R=-CH_3 (Synthetic Chondroitin)
2b; R=-CH_2CH_3
2c; R=-$(CH_2)_2CH_3$
2d; R=-$CH(CH_3)_2$
2e; R=-Ph
2f; R=-$CH{=}CH_2$

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Enzymatic Synthesis of Chondroitin and Its Derivatives Catalyzed by Hyaluronidase

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Abstract: The enzymatic polymerization to provide synthetic chondroitin and its derivatives is reported here, the first example of such in vitro synthesis to date. *N*-Acetylchondrosine (GlcAβ(1→3)GalNAc) oxazoline (**1a**) and its derivatives (**1b**−**1f**) were designed and synthesized as novel transition state analogue substrate monomers for catalysis by hyaluronidase. Hyaluronidase is a hydrolysis enzyme of chondroitin that also catalyzes the formation of repeated glycosidic bonds in in vitro synthesis, rather than in the catabolic direction. Monomers of 2-methyl (**1a**), 2-ethyl (**1b**), and 2-vinyl (**1f**) oxazoline derivatives were polymerized using this enzyme, via ring-opening polyaddition with total control of regioselectivity and stereochemistry. These reactions provided the corresponding synthetic chondroitin (natural type; *N*-acetyl, **2a**) and the derivatives (unnatural type) with *N*-propionyl (**2b**) and *N*-acryloyl (**2f**) functional groups at the C2 position of all the galactosamine units, in good yields. Monomers of 2-*n*-propyl (**1c**) and 2-isopropyl (**1d**) oxazoline derivatives were polymerized to produce **2c** and **2d** in low yield. The 2-phenyl oxazoline derivative (**1e**) did not afford any enzyme-catalyzed products. M_n values of **2a** and **2b** reached 4800 and 4000, respectively. The M_n value of **2a** corresponds to that of the naturally occurring chondroitin. Thus, hyaluronidase catalysis allows the in vitro production of not only natural type but also the formation of unnatural type chondroitins.

Introduction

Chondroitin (Ch) and chondroitin sulfate (ChS) are naturally occurring heteropolysaccharides belonging to the family of glycosaminoglycans (GAGs), which also includes hyaluronan (HA), heparin/heparan sulfate, and dermatan sulfate (DS). Ch is a nonsulfated derivative of ChS, consisting of a β-D-glucuronyl-(1→3)-*N*-acetyl-D-galactosamine (*N*-acetylchondrosine, GlcAβ(1→3)GalNAc) disaccharide repeating unit connected through β(1→4) glycosidic linkages. ChS exists predominantly as polysaccharide side chains in proteoglycans (PGs) in extracellular matrixes (ECMs), where it plays an important role in the bioactivities of living systems,[1] such as controlling morphogenesis by regulation of topology and function.[2] Ch is widely used as a therapeutic material for the prevention or alleviation of symptoms of diseases, such as rheumatoid arthritis[3] and in food supplements.[4]

Biosynthesis of Ch is catalyzed by glycosyl transferases with UDP-GlcA and UDP-GlcNAc as glycosyl donors in the Golgi apparatus.[5] Recently, human cDNA encoding a single polypeptide, chondroitin synthase, with the potential to synthesize Ch has been cloned. This possesses the two glycosyl transferase activities required for chain polymerization.[6] Some microbial Ch synthases have been identified[7] and have been found to produce Ch as capsular polysaccharides surrounding microbes, which contain no sulfate groups or core proteins. In mammalian cells, Ch is regioselectively sulfated at C4, C6, and/or C2′ of the *N*-acetylchondrosine repeating unit, by the action of specific sulfotransferases[8] during chain elongation of Ch backbone. This process results in the structural diversity of ChS.[9]

A number of reports have been published which describe the functions of ChS at a molecular level.[10] It has been found to play a critical role in the brain matrix, promoting neurite cell growth[11] and neural cell migration.[12] Nematodes are animals

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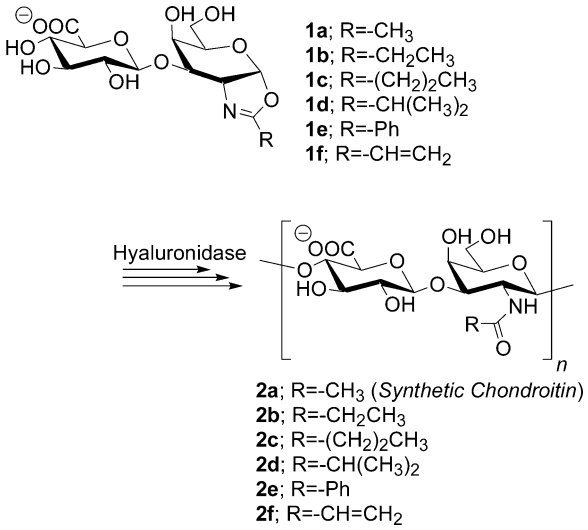
which produce nonsulfated Ch;[13] however, their function has not yet been identified.

Structurally defined polysaccharide samples are essential for the investigation of the function of Ch and ChS at a molecular level. Such an approach has been achieved over the last two decades using synthetic chemistry.[14] The synthesis of ChS has proved challenging, with hexasaccharides containing a 4- or 6-*O*-sulfate group on GalNAc units being the longest derivatives synthesized to date.[15] Therefore, a facile and efficient method for the synthesis of Ch and ChS is urgently required.

Enzymatic polymerization catalyzed by a glycosyl hydrolase has been demonstrated as an effective nonbiosynthetic approach for constructing structurally well-defined oligo- and polysaccharides.[16] This methodology has the advantage that the polymeric products are generated by a single-step polymerization reaction of designed activated substrate monomers in a regio- and stereoselective manner. For example, not only natural homopolysaccharides of cellulose,[17] xylan,[18] and chitin,[19] but also unnatural heteropolysaccharides of alternatingly 6-*O*-methyl-cellulose[20] and a cellulose−xylan hybrid polysaccharide[21] have been synthesized by this method. A mutant hydrolase was also effective for synthesis of β(1→4)-oligo- and polysaccharides.[22] Furthermore, we have recently achieved enzymatic polymerization to form synthetic HA which has a number average molecular weight of (M_n) $\sim 2 \times 10^4$ catalyzed by hyaluronidases (HAases; endo-β-*N*-acetylhexosaminidases[23]). In this case, *N*-acetylhyalobiuronate (GlcAβ(1→3)GlcNAc) disaccharide oxazoline derivative, an activated GlcNAc form of the repeating unit in HA, was used as a transition state analogue substrate monomer.[24,25] In these reactions, the hydrolase enzyme which catalyzes the glycosidic bond cleavage of the polysaccharides by hydrolysis in living systems, catalyzed repeated glycosidic bond formation by transglycosylation of the monomer

Scheme 1. Enzymatic Polymerization to Synthetic Ch and Its Derivatives



in vitro. Two of the above enzymatic polymerizations utilize hydrolysis enzymes as catalysts, Chitinase (family 18) for chitin synthesis and HAases (family 56) for HA synthesis. Both reactions involve the glycosidic bond cleavage of the (1→4)-β-*N*-acetyl-D-glucosaminide linkage.[19,24,26]

Ch, ChS, and DS are known to be hydrolyzed at their (1→4)-β-*N*-acetyl-D-galactosaminide linkage with HAase catalysis.[23] Here, we report a facile and efficient synthesis of synthetic Ch (natural type) by enzymatic ring-opening polyaddition. The reaction is catalyzed by the HAases with an *N*-acetylchondrosine oxazoline derivative, a repeating disaccharide form of Ch with an activated GalNAc, which has the potential to serve as a transition state analogue substrate monomer for the enzyme. The polymerization proceeded in a perfect regio-selective and stereo-controlled fashion. This reaction has been extended to the synthesis of Ch derivatives (unnatural type) with *N*-propionyl, *N*-butyryl, *N*-isobutyryl, *N*-benzoyl, and *N*-acryloyl groups in place of the *N*-acetyl group in natural Ch, by using the corresponding 2-ethyl, 2-*n*-propyl, 2-isopropyl, 2-phenyl, and 2-vinyl oxazoline derivatives as novel monomers for the enzyme (Scheme 1). The present reactions provide the first successful synthesis of natural type Ch and its unnatural type derivatives by nonbiosynthetic pathways. These polymeric products may serve as a novel class of substrates for use in various scientific fields such as medicinal chemistry, organic chemistry, biochemistry, enzymology, and polymer chemistry.

Results and Discussion

Monomer Design. It is generally accepted that an enzyme-catalyzed reaction proceeds at an accelerated rate relative to that of the uncatalyzed reaction by the reduction of the activation energy. This is achieved by stabilization of the reaction transition state, which occurs on formation of the enzyme−substrate complex.[27] Furthermore, all enzymatic reactions are reversible, including complex formation.[28] These observations imply that the formation of a desired enzyme−substrate complex in a

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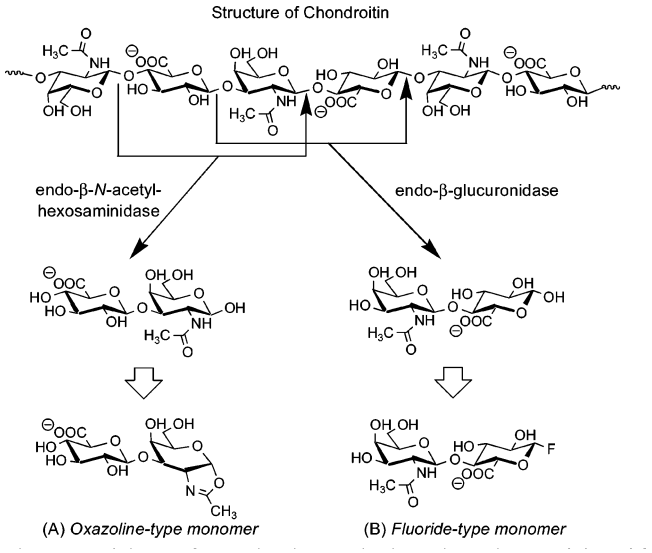
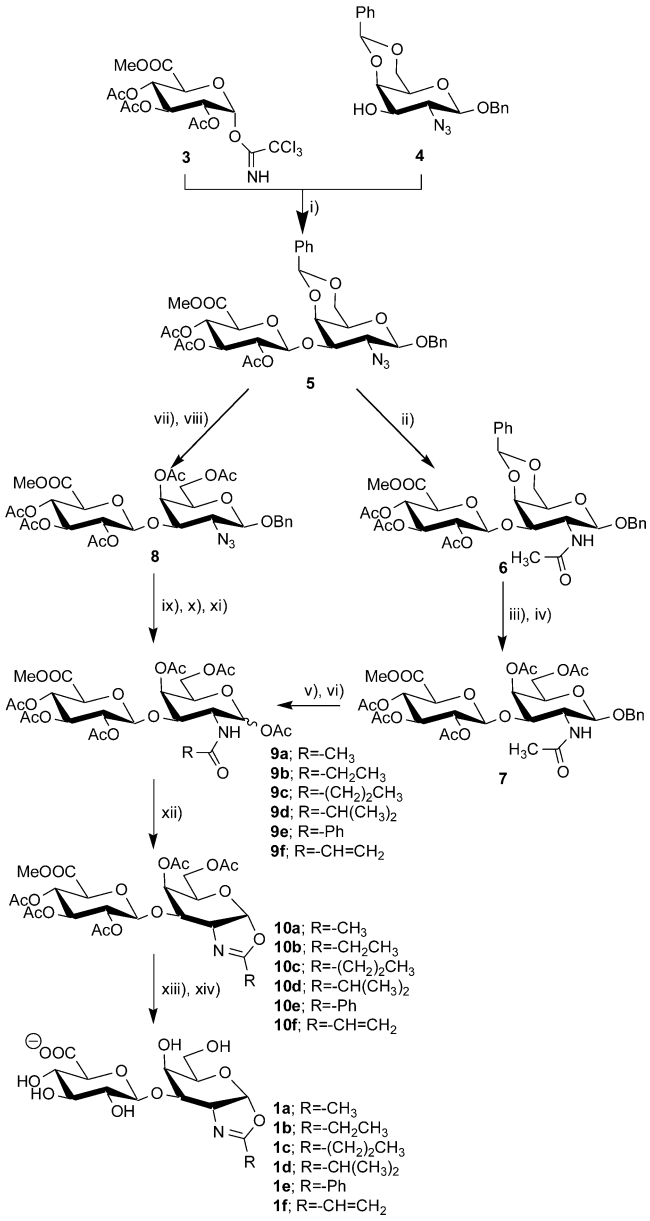


Figure 1. Two possibilities of the monomer design for chondroitin synthesis.

reaction pathway can be controlled whether proceeding in the forward or reverse direction. Therefore, an appropriate combination of monomer and enzyme is crucial for controlling the pathway, so that the enzyme−monomer complex may form, leading irreversibly to the desired product. Hydrolase enzymes have been used as catalysts for polymerization in the synthesis of various polysaccharides, where glycosidic bond formation is repeatedly induced by the hydrolysis enzyme.[16−21,24] In these reactions, the enzyme that inherently catalyzes the glycosidic bond cleavage (the forward direction), also catalyzes in vitro bond formation (the reverse direction). These results strongly suggest that the enzyme and designed substrate monomer formed the complex expected, which is considered very close in structure for both hydrolysis and polymerization, and thus was able to produce the target polysaccharide. To achieve such a complex, the monomer substrate must be recognized by the enzyme. Therefore, a monomer was designed with a structure thought to be similar to that involved in the transition state of the enzyme catalyzed hydrolysis. We named the monomers designed as "transition state analogue substrate monomers".[16−21,24]

We have designed a monomer for Ch synthesis, based on the concept of enzymatic polymerization with hydrolase catalysis.There are two possibilities for monomer design (Figure 1); an oxazoline-type disaccharide monomer (A) designed for HAase (endo-*β*-*N*-acetylhexosaminidase; EC3.2.1.35) catalysis[23,24] cleaving the (1→4)-*β*-*N*-acetyl-D-galactosaminide linkage, and a fluoride-type disaccharide monomer (B) designed for endo-*β*-glucuronidase catalysis cleaving the (1→3)-*β*-D-glucuronide linkage.[29] The oxazoline structure of monomer (A) is analogous to that used in the syntheses of chitin[19] and hyaluronan.[24] These natural polysaccharides contain the *N*-acetyl group at the C2 position of the D-glucosamine and D-galactosamine units, respectively, and their hydrolysis by Chitinase and hyaluronidase is considered to involve an oxazolinium transition state. Combination of the former monomer (A) and enzyme is feasible since HAases are commercially available;

Scheme 2. Syntheses of Substrate Monomers **1a**−**1f**



(i) TMSOTf, MS4A/CH_2Cl_2, 91%, (ii) AcSH, 86%, (iii) 80% aqAcOH, Δ, (iv) Ac_2O/pyridine, 81% (2 steps), (v) $Pd(OH)_2$−C, H_2/MeOH, (vi) Ac_2O/pyridine, quant. (2 steps), (vii) 80% aqAcOH, Δ, (viii) Ac_2O/pyridine, 87% (2 steps), (ix) $Pd(OH)_2$−C, H_2/MeOH, (x) $(R^1CO)_2O$ (**b**, **d**) or R^1COCl (**c**, **e**, **f**), Et_3N/MeOH, (xi) Ac_2O/pyridine, (**b**) 38%, (**c**) 57%, (**d**) 41%, (**e**) 58%, (**f**) 38% (3 steps), (xii) TMSOTf/CH_2Cl_2, (**a**) 89%, (**b**) 82%, (**c**) 64%, (**d**) 76%, (**e**) 64%, (**f**) 77%, (xiii) MeONa/MeOH, (xiv) carbonate buffer (50 mM, pH 10.6), (**a**) 82%, (**b**) 93%, (**c**) 86%, (**d**) 83%, (**e**) 87%, (**f**) 86% (2 steps).

however, the latter (B) is not possible due to the difficulty in obtaining a supply of the enzyme. The specific endo-*β*-glucuronidase for Ch degradation has been identified in the rat liver only[29] and is not commercially available. Therefore, in the present study, we selected the combination of the A monomer and the enzyme for the syntheses of natural type Ch and also unnatural type Chs.

Synthesis of Oxazoline Monomers. All substrate monomers (**1a**−**1e**) were successfully synthesized according to the reactions outlined in Scheme 2. The key intermediate (**5**) was readily prepared through a glycosylation reaction of 2-azidogalactoside derivative (**4**) with trichloroacetimidate of peracetylated methyl

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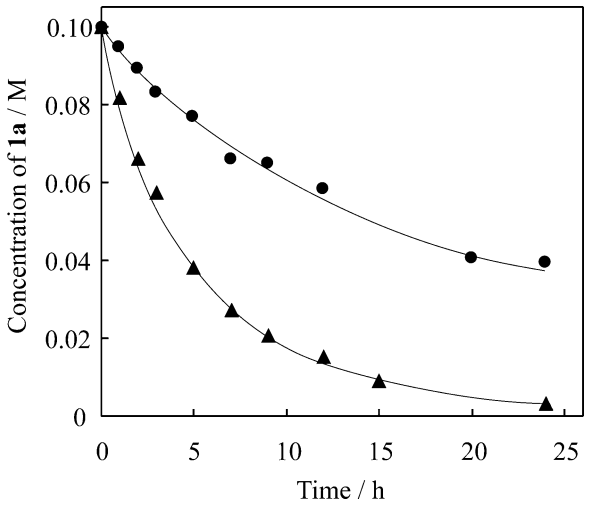

Figure 2. Reaction-time courses of **1a** with H−OTH (▲) and without the enzyme (●).

glucuronate (**3**). For the synthesis of monomer **1a** for natural type Ch, the 2-azido group of **5** was converted to acetamido group by treatment with thioacetic acid. The 4,6-*O*-benzylidene group of **6** was removed by acetolysis followed by acetylation with acetic anhydride in pyridine to give compound **7**. The 1-*O*-benzyl group was removed by hydrogenation followed by acetylation to afford **9a**.

For the syntheses of monomers **1b-1e**, for unnatural Chs, compound **5** was also treated with aqueous acetic acid to remove the 4,6-*O*-benzylidene group, followed by acetylation with acetic anhydride in pyridine to provide **8**. Both the 2-azido and 1-*O*-benzyl groups of **8** were hydrogenated to amino and hydroxyl groups, respectively. Compounds **9b**−**9f** were prepared by modification of the amino group to various amido groups through the reaction with the corresponding acid anhydrides (**9b**, **9d**) or acid chlorides (**9c**, **9e**, **9f**) in methanol prior to acetylation of the anomeric hydroxyl group.

Formation of the oxazoline ring in **9a**−**9f** was performed using trimethylsilyl triflate. All *O*-acetyl groups of **10a**−**10f** were then removed by sodium methoxide in methanol followed by hydrolysis of the methyl ester in carbonate buffer (pH 10.6) which afforded the substrate monomers (**1a**−**1f**).

Synthesis of Ch (natural type) via Enzymatic Polymerization of 1a. Substrate monomer **1a** designed for natural type Ch was polymerized with the ovine testicular HAase (H−OTH: hydrolysis activity, 2160 units/mg) in phosphate buffer (pH 7.5). Figure 2 illustrates the reaction-time courses of **1a** with H−OTH (▲) and without the enzyme (●). Without the enzyme, **1a** was gradually consumed due to nonenzymatic hydrolysis to produce a disaccharide, GlcAβ(1→3)GalNAc.[30] On addition of H−OTH, **1a** was consumed more rapidly due to the enzyme-catalyzed reaction as well as the nonenzymatic hydrolysis. On reaction completion, the enzyme was thermally inactivated and the resulting mixture purified by Sephadex G-10 size exclusion chromatography (SEC) to afford the polymeric product.

Figure 3 shows the ^{1}H (A) and ^{13}C (B) NMR spectra of the product. An overlapping broad signal around δ 4.33 in A (corresponding to two anomeric protons of GlcA and GalNAc),

(30) Structure was identified by HPLC and MALDI-TOF/MS measurements; *m*/*z* 396.96 [M−H]$^-$.

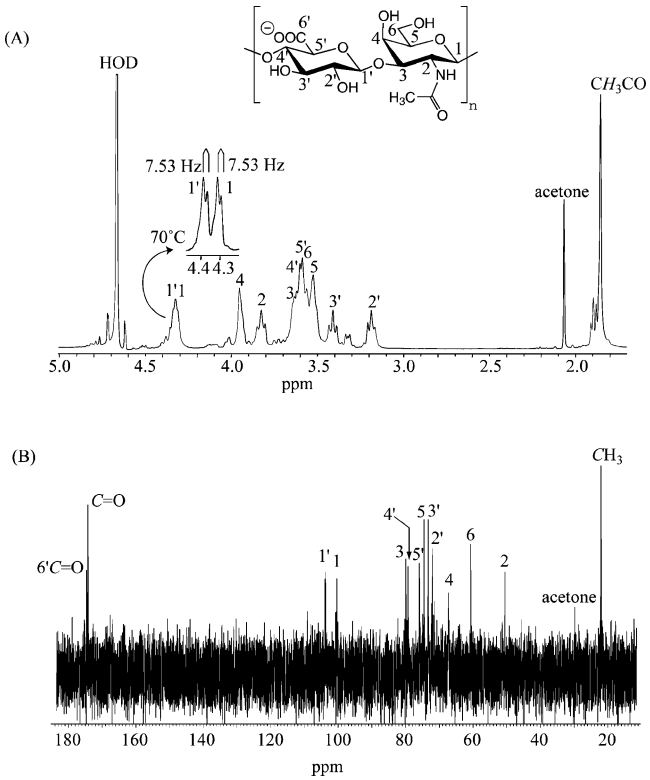

Figure 3. (A) ^{1}H and (B) ^{13}C NMR spectra of synthetic Ch.

Table 1. ^{13}C NMR Chemical Shift Values[a] of Synthetic Ch and Natural Ch

GalNAc

	C1	C2	C3	C4	C5	C6	C=O	CH_3
synthetic Ch[b]	100.38	50.55	79.91	67.23	74.52	60.61	174.17	22.04
natural Ch[c]	100.36	50.50	79.82	67.24	74.49	60.61	174.14	22.02

GlcA

	C1	C2	C3	C4	C5	C6
synthetic Ch	103.87	72.01	73.24	79.23	75.90	174.54
natural Ch	103.83	71.97	73.19	79.17	75.88	174.51

[a] All chemical shift values are indicated by ppm from the methyl carbon of acetone as an internal standard (at 29.8 ppm in D_2O). [b] Obtained in this study. [c] Purchased from Seikagaku Co.

measured at 23 °C, became two characteristic doublet signals at δ 4.37 and 4.30 with the same coupling constant of 7.53 Hz, on measurement at 70 °C. The former signal was assigned to the anomeric proton derived from GlcA, and the latter from GalNAc, showing the existence of glycosidic linkages with the β-orientation only. In B, the specific signals derived from the anomeric carbon atoms of GlcA and GalNAc were observed at δ 103.87 and 100.38, respectively. In addition, the signals at δ 79.91 and 79.23 were assigned to C3 of GalNAc and C4 of GlcA by C−H correlation spectroscopy. These results indicate the formation of a GlcAβ(1→3)GalNAcβ(1→4) repeating structure, natural type (synthetic) Ch **2a**. All of the chemical shift values were closely similar to those of the natural Ch sample (Table 1). The yield and molecular weight of synthetic Ch were 50% and M_n 2100, respectively (M_w 2500; mainly decasaccharide) as determined by SEC measurements.

Enzymatic Polymerization of 1a under Various Reaction Conditions. Polymerization of **1a** was performed under varying pH conditions, in phosphate buffer, for different reaction times using H−OTH as the catalyst (entries 1−7, Table 2). The

Table 2. Enzymatic Polymerization of Monomer **1a** under Various Reaction Conditions

	polymerization[a]						polymer (**2a**)		
entry	enzyme[b]	pH	monomer concentration/M	enzyme amount/ wt % for **1a**	temp /°C	time /h	yield[c] / %	M_n[d]	M_w[d]
1	H−OTH	6.0	0.10	10	30	1	28	1600	1700
2	H−OTH	7.0	0.10	10	30	9	47	1900	2200
3	H−OTH	7.5	0.10	10	30	23	50	2100	2500
4	H−OTH	8.0	0.10	10	30	6	15	4600	6700
5	H−OTH	8.0	0.10	10	30	33	49	2200	2700
6	H−OTH	8.5	0.10	10	30	6	9	4600	6500
7	H−OTH	9.0	0.10	10	30	72	0		
8	H−OTH	7.5	0.05	10	30	3	19	4100	6000
9	H−OTH	7.5	0.10	10	30	2	19	4600	6800
10	H−OTH	7.5	0.20	10	30	3	16	4800	7100
11	H−OTH	7.5	0.10	5	30	2	6	4800	7000
12	H−OTH	7.5	0.10	20	30	1	16	4400	6600
13	H−OTH	7.5	0.10	10	20	3	30	4300	6400
14	H−OTH	7.5	0.10	10	40	1	10	4300	6300
15	OTH	7.5	0.10	10	30	6	10	4300	6600
16	OTH	7.5	0.10	10	30	23	35	2500	3200
17	H−BTH	7.5	0.10	10	30	40	29	2600	3400
18	BTH	7.5	0.10	10	30	40	10	2800	3600
19	bee venom	7.5	0.10	10	30	40	~1		
20	H−OTH	7.5[e]	0.10	10	30	19	12	1700	1800
21	H−OTH	7.5[f]	0.10	10	30	22	22	1800	2100

[a] In a phosphate buffer: 50 mM. [b] OTH: ovine testicular hyaluronidase (560 units/mg), BTH: bovine testicular hyaluronidase (330 units/mg), H−OTH: ovine testicular hyaluronidase (2160 units/mg from ICN Biochemicals, Inc.), H−BTH: bovine testicular hyaluronidase (1010 units/mg from SIGMA). [c] Determined by HPLC containing products with molecular weight higher than tetrasaccharides. [d] Determined by SEC calibrated with hyaluronan standards. [e] In a phosphate buffer: 500 mM. [f] In a phosphate buffer (50 mM) containing sodium chloride (500 mM).

monomer consumption became slower when increasing the pH from 6.0 to 9.0. In these reactions, monomer consumption was complete within the respective reaction times except for entries 4 and 6. These observations are in agreement with the reported phenomena that the optimal pH range for hydrolysis of natural Ch is between 4 and 6;[23] the hydrolysis activity of the enzyme is notably higher in the regions with relatively low pH. Recent reports have investigated the hydrolysis activities of hyaluronidase enzymes in detail, in terms of pH dependency versus enzyme preparation and their structure.[31]

The optimum polymer yield was obtained at pH 7.5, indicating that at this pH the polymerization proceeded effectively prior to the formation of the hydrolysis product. The M_n of synthetic Ch (**2a**) reached 4600 (22−24 saccharides) (the M_n value of which is similar to that of naturally occurring Ch)[32] for shorter reaction times of 6 h at pH 8.0 and 8.5 (entries 4 and 6). At pH 6.0, the monomer was consumed completely via enzymatic polymerization as well as nonenzymatic hydrolysis within 1 h, giving rise to a 28% yield of synthetic Ch. This indicates that the polymerization and hydrolysis of **1a** was fast, as was the hydrolysis of the product Ch. At pH 9.0 synthetic Ch was not obtained, which was most likely to be due to a complete suppression of the glycosylation reaction leading to synthetic Ch.

As H−OTH catalysis was optimal at pH 7.5, polymerization reactions for the investigation of other reaction parameters were performed at this pH. Monomer concentration did not significantly affect the yield and molecular weight of polymer **2a** with relatively short reaction times of 2 or 3 h (entries 8−10). Varying the amount of H−OTH enzyme (entries 11, 9, and 12) did not confer a large effect, with reaction times of 2 h. Reaction temperature was also investigated for the same enzyme at 20 °C for 3 h, 30 °C for 2 h, and 40 °C for 1 h (entries 13, 9, and 14, respectively). It was found that temperature did not greatly effect the yield and molecular weight for reaction times less than 3 h. However, it is notable that synthetic Ch **2a** with a higher molecular weight was obtained with shorter reaction times in the pH range of 7.5 to 8.5 (entries 4, 6, 8−14).

Catalytic activity was examined for other enzymes, OTH (hydrolysis activity, 560 units/mg), bovine testicular HAase (BTH: 330 units/mg, H−BTH: 1010 units/mg), and bee venom containing HAase, at pH 7.5. OTH and BTH had a hydrolysis activity lower than H−OTH or H−BTH, and although polymerization proceeded, synthetic Ch was produced in lower yields (entries 3 vs 16 and 17 vs 18, respectively). Shorter reaction times conferred a yield reduction of **2a** but a higher molecular weight was observed (entry 15). Bee venom showed a reduced catalytic activity for the polymerization reaction (entry 19).

Increasing the buffer concentration or the addition of NaCl to the solution resulted in a decrease in both the yield and molecular weight (entries 20 and 21) The effect of organic solvents such as methanol, acetonitrile, acetone, and tetrahydrofuran were investigated. Monomer **1a** was polymerized in phosphate buffer (pH 7.0)/methanol (2:1 v/v) using OTH at 30 °C and afforded the tetra- and hexasaccharides in low yield, as determined by MALDI-TOF/MS. Similar results were obtained with the three other organic solvents, suggesting that the use of an organic cosolvent drastically reduces the catalytic activity.

Polymerization Mechanism of 1a. Figure 4 illustrates the postulated reaction mechanism catalyzed by a hyaluronidase of family 56. Hydrolysis of Ch occurs by the following steps as shown in A and B.[24,25] Protonation of the oxygen atom in the $\beta(1\rightarrow4)$ glycosidic linkage occurs after the recognition of the substrate Ch by the enzyme. Subsequently, the carbonyl oxygen atom of GalNAc at the donor site attacks its own anomeric carbon atom from the α-side to assist in cleavage of the

(31) (a) Oettl, M, Hoechstetter, J.; Asen, I.; Bernhardt, G.; Buschauer, A. *Eur. J. Pharm. Sci.* **2003**, *18*, 267−277. (b) Frost, G. I.; Stern, R. *Anal. Biochem.* **1997**, *251*, 263−269.
(32) Poole, A. R. *Biochem. J.* **1986**, *236*, 1−14.

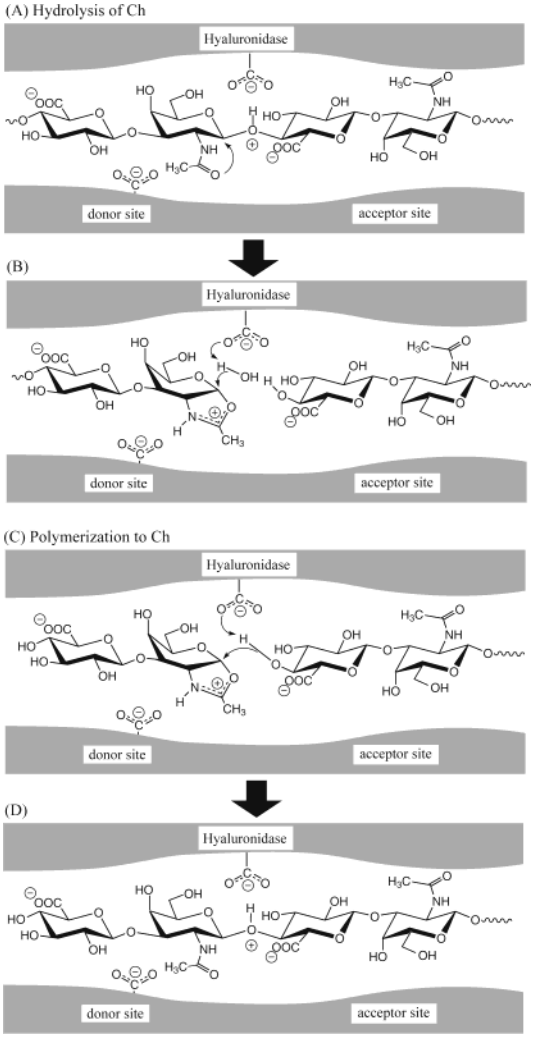

Figure 4. Postulated reaction mechanisms of HAase catalysis for the hydrolysis of Ch (A and B) and for the polymerization of monomer **1a** (C and D).

glycosidic bond, which results in a high-energy oxazolinium ion species in B. Water nucleophilically attacks the oxazolinium anomeric carbon to open the oxazolinium ring resulting in the formation of the hydrolysis products of natural Ch, GlcAβ-(1→3)GalNAc and/or Ch chains with GlcA and GalNAc end structures.

In the polymerization, monomer **1a** is readily recognized by the enzyme and is activated by protonation at the donor site in C, because the protonated monomer structure in C is identical to that of the oxazolinium transition state in B. Therefore, **1a** can be regarded as a transition state analogue substrate monomer in an activated form. The structure of **1a** facilitates recognition and further activation by the enzyme, with lowering the activation energy for the subsequent reactions. The 4-hydroxyl group of GlcA in another molecule of **1a**, or in the nonreducing end of the growing chain placed in the acceptor site, regioselectively adds to the anomeric carbon of the oxazolinium ion of **1a** from the β-side with ring-opening to form a β(1→4) glycosidic linkage between GalNAc and GlcA, as shown in D. Repetition of this regio- and stereoselective glycosylation, ring-opening polyaddition reaction of **1a** is catalyzed by the enzyme, giving rise to synthetic Ch; thus, the monomer formula of **1a** is the same as that of the product Ch.[19,24]

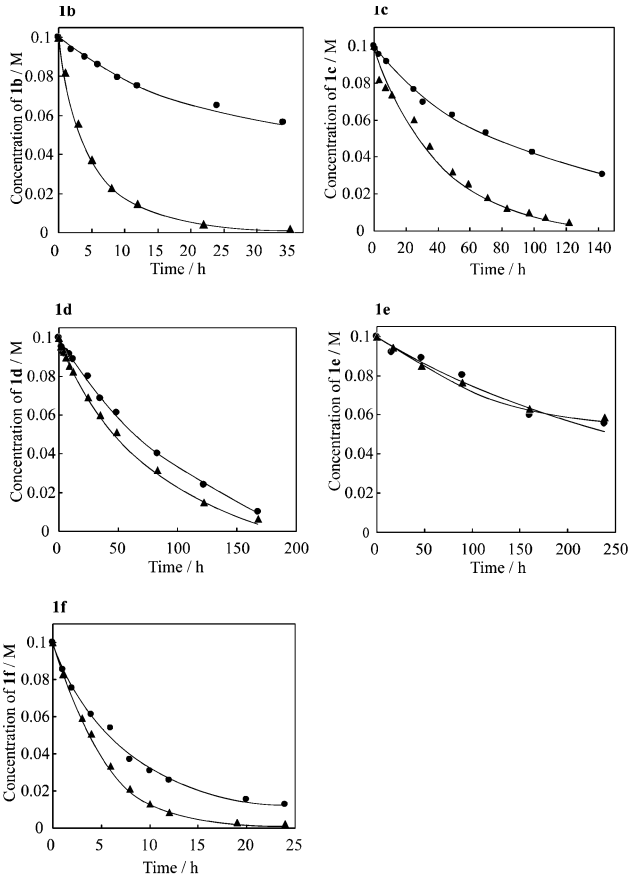

Figure 5. Reaction-time courses of monomers **1b**-**1f** with H−OTH (▲) and without the enzyme (●).

Enzymatic Polymerization to Ch Derivatives (unnatural type) with Various Amido Groups. Newly designed monomers **1b**−**1f** for the synthesis of Ch derivatives were subjected to enzymatic polymerization. The reaction with H−OTH was performed under the previously identified optimal conditions of pH 7.5 and 30 °C. Figure 5 shows the reaction-time courses for five substrate monomers. In all reactions performed in the absence of the enzyme (control), the disaccharide was produced via hydrolysis from the corresponding monomers **1b**−**1f**. Interestingly, monomers of **1b**, **1c**, **1d**, and **1f** for unnatural type Ch were significantly catalyzed by the enzyme, as transition state analogue substrates, resulting in ring-opening polyaddition of the oxazoline monomers. Notably, the 2-ethyl oxazoline monomer (**1b**) was consumed at an almost identical rate as the 2-methyl derivative (**1a**). However, no notable difference was observed for compound **1e** in monomer consumption in the presence or absence of the enzyme. The catalysis of these oxazoline derivatives at the donor site of the HAase is strongly dependent on the nature of the 2-substituent in the oxazoline-ring.[33]

Table 3 illustrates the results of the polymerization reactions of substrates **1b**−**1f**. Unnatural *N*-propionyl (**2b**) and *N*-acryloyl (**2f**) derivatives of Ch were obtained from **1b** and **1f** in a 46% yield with M_n of 2700 (mainly 12−14 saccharides) and in a

(33) From the time-conversion courses of these monomers in Figures 2 and 5, reaction rate constants for the control experiments (without enzyme) could be evaluated. Rate constants of the pseudo-first order (k_1 at 30 °C in a phosphate buffer at pH 7.5) were as follows; 1.26×10^{-5} s^{-1} (**1a**), 4.98×10^{-6} s^{-1} (**1b**), 2.41×10^{-6} s^{-1} (**1c**), 3.26×10^{-6} s^{-1} (**1d**), 6.59×10^{-7} s^{-1} (**1e**), and 2.86×10^{-5} s^{-1} (**1f**).

Table 3. Enzymatic Polymerization of Monomers **1b**−**1f**

	polymerization[a]			polymer			
entry	monomer	enzyme	time /h	structure	yield[d] / %	M_n[e]	M_w[e]
1	**1b**	H−OTH[b]	35	**2b**	46	2700	3600
2	**1c**	H−OTH[b]	122	**2c**	∼1		
3	**1d**	H−OTH[b]	168	**2d**	∼1		
4	**1e**	H−OTH[c]	239	-	-		
5	**1f**	H−OTH[c]	24	**2f**	19	3400	4600

[a] In a phosphate buffer at pH 7.5: 50 mM, monomer concentration: 0.1 M, amount of enzyme:10 wt % for monomer, reaction temperature: 30 °C. [b] H−OTH (2502 unit/mg) from ICN Biochemicals, Inc. [c] H−OTH (1870 unit/mg) from SIGMA. [d] Determined by HPLC containing products with molecular weight higher than tetrasaccharides. [e] Determined by SEC calibrated with hyaluronan standards.

19% yield with M_n 3400 (mainly 16−18 saccharides) respectively (entries 1 and 5 in Table 3). Ring-opening polyaddition of **1b** and **1f** proceeded in a regio- and stereoselective fashion to produce polymers **2b** and **2f**. Their structures were confirmed by ^{1}H and ^{13}C NMR as having a *β*-D-glucuronyl-(1→3)-*β*-*N*-propionyl-D-galactosaminyl-(1→4) repeating unit and a *β*-D-glucuronyl-(1→3)-*β*-*N*-acryloyl-D-galactosaminyl-(1→4) repeating unit for **2b** and **2f**, respectively. Monomers **1c** and **1d** provided oligomers of the *N*-butyryl (**2c**) and *N*-isobutyryl (**2d**) derivatives of Ch up to decasaccharide and octasaccharide respectively, as determined by MALDI-TOF/MS (entries 2 and 3). No polymeric or oligomeric products were formed through the reaction of **1e** with the enzyme (entry 4).

It should be emphasized that catalysis by the natural enzyme allowed production of not only the natural type Ch but also unnatural type Ch such as the *N*-propionyl (**2b**) and *N*-acryloyl (**2f**) derivatives. The substrate oxazoline structure is important for monomer design; 2-substituents in the oxazoline-ring can be varied in the approximate order 2-methyl > 2-ethyl > 2-vinyl ≫ 2-*n*-propyl > 2-isopropyl. 2-Phenyl oxazoline monomer **1e** was not recognized by the enzyme.

Polymerization Behaviors of Monomers 1a and 1b. Correlation among consumption of monomers, yields of polymeric products and M_n was examined in more detail for **1a** and **1b** (Figure 6, A and B). The yield of product **2a** of natural type Ch increased gradually with monomer consumption and then reached a plateau at around 50% after about 10 h, as shown in A. It is notable that the molecular weight value (M_n) of **2a** at 1 h was 4700, larger than the value following reaction completion. It had already been observed in Table 2 that shorter reaction times produced **2a** with higher M_n values. The product Ch is an intact substrate for the catalyst HAase, therefore the M_n value of **2a** was gradually reduced to 2100 due to enzymatic hydrolysis. In B, the yield of the product **2b** gradually increased with a decrease in monomer concentration and the M_n of **2b** after 1 h was 4000. Polymer **2b** with a *N*-propionyl group in the galactosamine unit is an unnatural substrate for the enzyme HAase, however, the value of M_n gradually decreased to 2700. The rate of decrease of M_n for **2a**, was less than that of **2b,** indicating that **2b** was hydrolyzed more slowly by the enzyme. The natural enzyme HAase served as a catalyst for both polymerization of monomer **1b** and hydrolysis of the unnatural substrate **2b**. These observations suggest that the M_n value can be controlled to some extent by selecting reaction conditions.

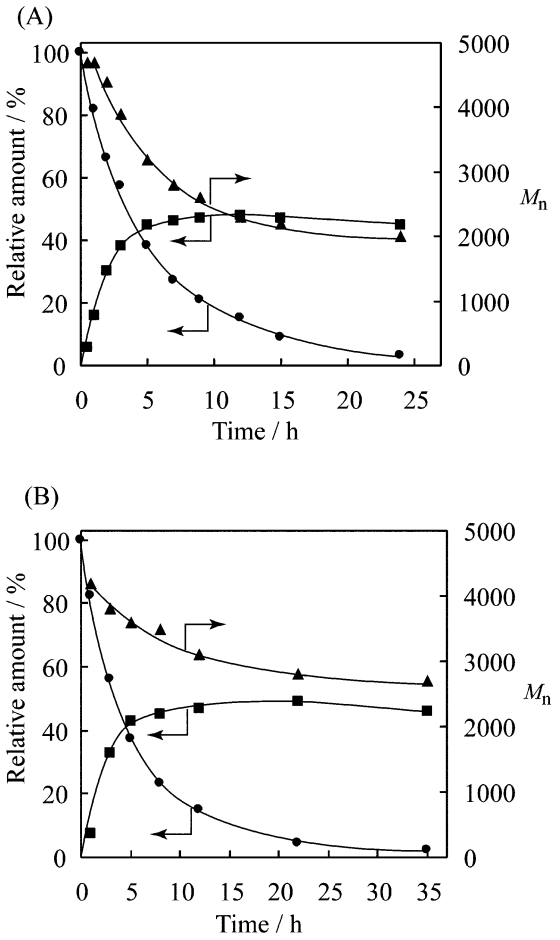

Figure 6. Polymerization behavior of **1a** (A) and **1b** (B); monomer consumption (●), polymer yields (■) and molecular weight (M_n) (▲).

Conclusion

Synthetic Ch **2a** (natural type) was successfully prepared for the first time via enzymatic ring-opening polyaddition of *N*-acetylchondrosine oxazoline derivative **1a**, a novel transition state analogue substrate monomer, using HAase as catalyst. The polymerization reactions catalyzed by both BTH and OTH proceeded smoothly at 30 °C in the pH range of 6.0 to 8.5 with total control of regioselectivity and stereochemistry. M_n values of **2a** ranged from 1600 (octasaccharide) to 4800 (24 saccharide) (M_w, 1700−7100). The higher M_n value of **2a** corresponds to that of naturally occurring Ch. Thus, synthetic Ch **2a** provides a natural type Ch sample with a well-defined structure for use in the present study. Unnatural type Chs with *N*-propionyl (**2b**) and *N*-acryloyl (**2f**) groups were also prepared by utilizing natural HAase as a catalyst for polymerization of the novel substrate monomers of 2-ethyl (**1b**) and 2-vinyl (**1f**) oxazoline derivatives, respectively. These unnatural Chs cannot be produced through biosynthetic pathways and are therefore expected to be potential materials for use in various scientific fields, such as polymeric drugs, biomaterials, tissue engineering, and glycotechnology. Furthermore, the fact that the vinyl oxazoline monomer **1f** is a good substrate for the HAase implies possible applications for the preparation of functionalized Ch utilizing the reactive vinyl group. This would permit syntheses of structurally defined new macromonomers, telechelics and gels, which have natural or unnatural Ch chains. Fundamental work

toward these applications, including the preparation of sulfated derivatives of ChS and DS is now in progress in our laboratory.

Experimental Section

Measurements. NMR spectra were recorded with a Bruker DPX-400 spectrometer. FABMS spectra were obtained on a JEOL XPS spectrometer using 2,4-dinitrobenzyl alcohol or dithiothreitol/thioglycerol (1/1, v/v) as matrix. Optical rotations were measured with a JASCO P-1010 polarimeter. Melting points were determined with a YAMATO MP-21. Concentrations of oxazoline monomers in the reaction mixtures were calculated by HPLC measurements (LC8020 system; TOSOH) with Shodex Asahipak NH2P-50 4E column (4.6 × 250 mm) eluting with phosphate buffer (10 mM, pH 7.0)-acetonitrile mixed solution (30:70 (v/v), flow rate; 0.5 mL/min, 30 °C). 3 μL of the reaction mixture was sampled, then injected to HPLC. Yields and molecular weight values of the products given after the reactions were determined by SEC measurements (GPC-8020 system; TOSOH) with Shodex Ohpak SB-803HQ column (8.0 × 300 mm) eluting with 0.1 M aqueous sodium nitrate (flow rate; 0.5 mL/min, 40 °C) calibrated by hyaluronan standards (M_n 800, 2000, 4000) and chondroitin sodium salt (M_n 4000, Seikagaku Co.). MALDI-TOF/MS analysis of the product was performed with a JEOL JMS-ELITE spectrometer by using 2,5-dihydroxybenzoic acid as a matrix on Nafion-coated plate[34] under negative ion mode.

Materials. Hyaluronidases from ovine testes were purchased from ICN Biochemicals Inc. (Lot No. 9303B, OTH, 560 units/mg; 6830B, H−OTH, 2160 units/mg). Hyaluronidases from bovine testes were purchased from SIGMA (Lot No. 30K7049, BTH, 330 units/mg; 38H7026, H−BTH, 1010 units/mg). Bee venom was purchased from SIGMA (Lot No. 79H1017). All enzymes were used without further purification.

Benzyl 2-azido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-*β*-D-galactopyranoside (5). A mixture of benzyl 2-azido-4,6-*O*-benzylidene-2-deoxy-*β*-D-galactopyranoside (**4**) (334 mg, 0.871 mmol) and methyl (2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate)uronate (**3**) (500 mg, 1.05 mmol) in anhydrous dichloromethane (9.0 mL) was stirred at −20 °C under argon atmosphere in the presence of molecular sieves 4A (MS4A; 1.80 g). After 30 min, trimethylsilyl trifluoromethanesulfonate (TMSOTf; 190 μL, 1.05 mmol) in anhydrous dichloromethane (1.0 mL) was added dropwise. After 1 h, the reaction mixture was quenched with triethylamine (0.1 mL), filtered through diatomaceous earth (Celite), diluted with chloroform, washed with saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, then filtered through diatomaceous earth, and concentrated. The residue was purified by silica gel chromatography (toluene-ethyl acetate 4:1−2:1) followed by crystallization from diethyl ether to give pure **5** (555 mg, 0.793 mmol, 91%) as a white crystal. $[\alpha]_D$ −10° (*c* 1.0, $CHCl_3$); mp 232 °C (decomposition); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.54−7.52 (2H, m, aromatic), 7.38−7.31 (8H, m, aromatic), 5.56 (1H, s, PhC*H*), 5.26−5.22 (2H, m, H-3′, H-4′), 5.06 (1H, dd, H-2′, $J_{1',2'}$ = 7.52 Hz, $J_{2',3'}$ = 8.52 Hz), 4.99 (1H, d, PhCH_2, J = 11.60 Hz), 4.92 (1H, d, H-1′, $J_{1',2'}$ = 7.52 Hz), 4.69 (1H, d, PhCH_2, J = 11.60 Hz), 4.37−4.29 (3H, m, H-6a, H-1, H-4), 4.09−4.02 (2H, m, H-6b, H-5′), 3.88 (1H, dd, H-2, $J_{1,2}$ = 8.04 Hz, $J_{2,3}$ = 10.5 Hz), 3.72 (3H, s, COOCH_3), 3.49 (1H, dd, H-3, $J_{2,3}$ = 10.50 Hz, $J_{3,4}$ = 3.52 Hz), 3.37 (1H, s, H-5), 2.06−2.01 (9H, m, Ac); HRMS (FAB) calcd. for $C_{33}H_{38}N_3O_{14}$ $[M+H]^+$ 700.2354, found 700.2357.

Benzyl 2-acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-*β*-D-galactopyranoside (6). Compound **5** (250 mg, 0.358 mmol) in thioacetic acid (2.5 mL) was stirred at room temperature under dry atmosphere for 24 h. The reaction mixture was then evaporated, and the residue was purified by silica gel chromatography (toluene-ethyl acetate 1:0−1:3) followed by crystallization from ethanol to give pure **6** (220 mg, 0.307 mmol, 86%) as a white crystal. $[\alpha]_D$ −9.0° (*c* 1.0, $CHCl_3$); mp 230 °C (decomposition); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.57−7.52 (2H, m, aromatic), 7.37−7.30 (8H, m, aromatic), 5.75 (1H, d, N*H*, $J_{NH,2}$ = 7.03 Hz), 5.57 (1H, s, PhC*H*), 5.23−5.19 (3H, m, H-4′, H-3′, H-1), 5.01 (1H, t, H-2′, $J_{1',2'} = J_{2',3'}$ = 8.29 Hz), 4.96−4.91 (2H, m, PhCH_2, H-1′), 4.79 (1H, dd, H-3, $J_{2,3}$ = 11.5 Hz, $J_{3,4}$ = 3.53 Hz), 4.56 (1H, d, PhCH_2, J = 12.10 Hz), 4.38−4.35 (2H, m, H-6a, H-4), 4.10 (1H, dd, H-6b, $J_{5,6b}$ = 1.51 Hz, $J_{6a,6b}$ = 11.80 Hz), 4.01 (1H, d, H-5′, $J_{4',5'}$ = 9.54 Hz), 3.69 (3H, s, COOCH_3), 3.52−3.46 (2H, m, H-2, H-5), 2.02−2.01 (9H, m, CH_3CO), 1.92 (3H, s, CH_3CONH); HRMS (FAB) calcd. for $C_{35}H_{42}NO_{15}$ $[M+H]^+$ 716.2554, found 716.2554.

Benzyl 2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-*β*-D-galactopyranoside (7). Compound **6** (960 mg, 1.34 mmol) in 80% aqueous acetic acid (15.0 mL) was stirred at 80 °C for 1 h to remove benzylidene acetal. The reaction mixture was then evaporated to dryness, and the residue was subjected to silica gel chromatography (chloroform-methanol 10:1) to give benzyl 2-acetamido-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-*β*-D-galactopyranoside (760 mg, 1.21 mmol, 90%). Acetic anhydride (470 μL, 4.84 mmol) was then added dropwise to the solution of this compound (760 mg, 1.21 mmol) in pyridine (10.0 mL) at 0 °C. The mixture was stirred for 3 h at room temperature under dry atmosphere, and then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate, and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, filtered through diatomaceous earth, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:2−1:4) to afford pure **7** (700 mg, 0.984 mmol, 81%) as a white crystal (from ether). $[\alpha]_D$ −20° (*c* 1.0, $CHCl_3$); mp 173−174 °C;^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.37−7.29 (5H, m, aromatic), 5.64 (1H, d, N*H*, $J_{NH,2}$ = 7.03 Hz), 5.39 (1H, d, H-4, $J_{3,4}$ = 3.01 Hz), 5.22−5.14 (2H, m, H-4′, H-3′), 5.02 (1H, d, H-1, $J_{1,2}$ = 8.03 Hz), 4.97 (1H, t, H-2′, $J_{1',2'} = J_{2',3'}$ = 8.53 Hz), 4.89 (1H, d, PhCH_2, J = 11.80 Hz), 4.70−4.65 (2H, m, H-1′, H-3), 4.58 (1H, d, PhCH_2, J = 11.80 Hz), 4.17 (1H, dd, H-6a, $J_{5,6a}$ = 6.28 Hz, $J_{6a,6b}$ = 11.29 Hz), 4.08 (1H, dd, H-6a, $J_{5,6a}$ = 6.28 Hz, $J_{6a,6b}$ = 11.29 Hz), 3.98 (1H, d, H-5′, $J_{4',5'}$ = 9.54 Hz), 3.87 (1H, t, H-5, $J_{5,6a} = J_{5,6b}$ = 6.28 Hz), 3.75 (3H, s, COOCH_3), 3.46 (1H, ddd, H-2, $J_{1,2}$ = 8.03 Hz, $J_{NH,2}$ = 7.03 Hz, $J_{2,3}$ = 9.28 Hz), 2.09−2.00 (15H, m, CH_3CO), 1.89 (3H, s, CH_3CONH); HRMS (FAB) calcd. for $C_{32}H_{42}NO_{17}$ 712.2453 $[M+H]^+$, found 712.2453.

2-Acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-*β*-D-galactopyranosyl acetate (9a). A mixture of **7** (700 mg, 0.984 mmol) and 20% palladium hydroxide on activated carbon (200 mg) in methanol (20.0 mL) was stirred at room temperature under hydrogen atmosphere for 3 h. The reaction mixture was then filtered through diatomaceous earth and evaporated. The residue was dried by vacuum pump for overnight. Then, to a solution of this compound in pyridine (10.0 mL) was added acetic anhydride (320 μL, 3.39 mmol) at 0 °C. The reaction mixture was stirred at room temperature under dry atmosphere for 3 h, and then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate. The solution was then filtered through diatomaceous earth, and evaporated. The residue was purified by silica gel chromatography (ethyl acetate) to give **9a** (680 mg, 1.03 mmol, quant., α / β = 1/1) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 6.34−6.32 (2H, m, H-1α, N*H*α), 5.89 (1H, d, H-1β, $J_{1,2}$ = 8.53 Hz), 5.78 (1H, d, N*H*β, $J_{2,NH}$ = 8.03 Hz), 5.49 (1H, d, H-4β, $J_{3,4}$ = 3.01 Hz), 5.29−5.13 (7H, m, H-4α, H-3′αβ, H-4′αβ, H-2′α), 5.02 (1H, dd, H-2′β, $J_{1',2'}$ = 8.03 Hz, $J_{2',3'}$ = 8.53 Hz), 4.86 (1H, d, H-1′α, $J_{1',2'}$ = 8.03 Hz), 4.77 (1H, d, H-1′β, $J_{1',2'}$ = 8.03 Hz), 4.55 (1H, ddd, H-2α, $J_{1,2}$ = 3.52 Hz, $J_{2,NH}$ = 7.03 Hz, $J_{2,3}$ =

(34) Jacobs, A.; Dahlman, O. *Anal. Chem.* **2001**, *73*, 405−410.

8.53 Hz), 4.38 (1H, dd, H-3β, $J_{2,3}$ = 10.79 Hz, $J_{3,4}$ = 3.01 Hz), 4.24−3.97 (11H, m, H-3α, H-6αβ, H-5′ αβ, H-2β, H-5αβ), 3.76 (6H, s, COOCH_3), 2.19−2.02 (36H, m, CH_3CO), 1.93−1.90 (6H, d, CH_3-CONH); HRMS (FAB) calcd. for $C_{27}H_{38}NO_{18}$ $[M+H]^+$ 664.2089, found 664.2092.

2-Methyl-[4,6-di-*O*-acetyl-1,2-di-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (10a). To a solution of **9a** (200 mg, 0.301 mmol) in anhydrous dichloromethane (10.0 mL) was added dropwise TMSOTf (110 μL, 0.603 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature for 12 h, quenched with triethylamine (200 μL) at 0 °C, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:2−2:5, containing 0.5% (v/v) triethylamine) to give pure **10a** (161 mg, 0.268 mmol, 89%) as white amorphous. $[\alpha]_D$ +34° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.93 (1H, d, H-1, $J_{1,2}$ = 6.53 Hz), 5.40 (1H, d, H-4, $J_{3,4}$ = 3.51 Hz), 5.30−5.20 (2H, m, H-3′, H-4′), 5.07 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 4.99 (1H, t, H-2′, $J_{1',2'} = J_{2',3'}$ = 8.03 Hz), 4.18−4.12 (3H, m, H-6, H-5), 4.08 (1H, d, H-5′, $J_{4',5'}$ = 9.53 Hz), 3.93 (1H, dd, H-3, $J_{2,3}$ = 6.53 Hz, $J_{3,4}$ = 3.51 Hz), 3.83 (1H, t, H-2, $J_{1,2} = J_{2,3}$ = 6.53 Hz), 3.76 (3H, s, COOCH_3), 2.08−2.02 (18H,, m, CH_3CO, CH_3C of oxazoline); HRMS (FAB) calcd. for $C_{25}H_{34}NO_{16}$ $[M+H]^+$ 604.1877, found 604.1879.

2-Methyl-[1,2-di-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (1a). To a solution of **10a** (240 mg, 0.398 mmol) in methanol (1.0 mL) was added about 28% (w/v) sodium methoxide in methanol (420 mg) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature under argon atmosphere for 30 min, and evaporated. The residue was dissolved with carbonate buffer (50 mM, pH 10.6, 4.0 mL) and stirred at room temperature for 1 h. The reaction mixture was then lyophilized to give **1a** (160 mg, purity 82%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 5.92 (1H, d, H-1, $J_{1,2}$ = 7.03 Hz), 4.47 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 4.00 (1H, d, H-4, $J_{3,4}$ = 3.52 Hz), 3.80−3.73 (2H, m, H-2, H-5), 3.64−3.52 (4H, m, H-3, H-6, H-5′), 3.34−3.32 (2H, m, H-4′, H-3′), 3.21 (1H, dd, H-2′, $J_{1',2'}$ = 8.03 Hz, $J_{2',3'}$ = 9.03 Hz), 1.84 (3H, s, CH_3C of oxazoline); HRMS (FAB) calcd. for $C_{14}H_{20}NNaO_{11}$ $[M+H]^+$ 402.1012, found 402.1018.

Benzyl 4,6-di-*O*-acetyl-2-azido-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-β-D-galactopyranoside (8). A solution of **5** (555 mg, 0.793 mmol) in 80% aqueous acetic acid (15.0 mL) was stirred at 80 °C for 1 h. The reaction mixture was then evaporated, and the residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:2−1:4) to give benzyl 2-azido-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-β-D-galactopyranoside (453 mg, 0.741 mmol, 93%). Then, to a solution of this compound (453 mg, 0.741 mmol) in dry pyridine (10.0 mL) was added dropwise acetic anhydride (430 μL, 4.46 mmol) at 0 °C. The reaction mixture was stirred at room temperature under dry atmosphere for 4 h followed by evaporation. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, filtered through diatomaceous earth, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:1) followed by crystallization from diethyl ether to give pure **8** (478 mg, 0.687 mmol, 93%) as a white crystal. $[\alpha]_D$ +0.12° (*c* 1.0, $CHCl_3$); mp 144−145 °C;^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.39−7.31 (5H, m, aromatic), 5.33 (1H, d, H-4, $J_{3,4}$ = 3.01 Hz), 5.23−5.21 (2H, m, H-4′, H-3′), 4.99−4.93 (2H, m, H-2′, PhCH_2), 4.80 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 4.70 (1H, d, PhCH_2, *J* = 12.05 Hz), 4.29 (1H, d, H-1, $J_{1,2}$ = 8.03 Hz), 4.17 (1H, dd, H-6a, $J_{5,6a}$ = 5.52 Hz, $J_{6a,6b}$ = 11.54 Hz), 4.08 (1H, dd, H-6a, $J_{5,6a}$ = 7.02 Hz, $J_{6a,6b}$ = 11.54 Hz), 3.98 (1H, d, H-5′, $J_{4',5'}$ = 10.04 Hz), 3.75 (3H, s, COOCH_3), 3.73−3.64 (2H, m, H-5, H-2), 3.53 (1H, dd, H-3, $J_{2,3}$ = 10.54 Hz, $J_{3,4}$ = 3.01 Hz), 2.11−2.01 (15H, m, CH_3CO); HRMS (FAB) calcd. for $C_{30}H_{38}N_3O_{16}$ $[M+H]^+$ 696.2252, found 696.2249.

4,6-Di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-2-propanamide-β-D-galactopyranosyl acetate (9b). A mixture of **8** (100 mg, 0.144 mmol) and 20% palladium hydroxide on activated carbon (50.0 mg) in methanol (10.0 mL) was stirred at room temperature under hydrogen atmosphere for 4 h. The reaction mixture was filtered through diatomaceous earth and evaporated. To a solution of the residue in methanol (10.0 mL) was added triethylamine (1.0 mL) and propionic anhydride (180 μL, 1.44 mmol) at 0 °C under dry atmosphere. The reaction mixture was stirred at room temperature for 2 h, quenched with pyridine (1.00 mL), and evaporated. The residue was concentrated by vacuum pump for overnight. To a solution of the residue in pyridine (10.0 mL) was added acetic anhydride (84 μL, 0.863 mmol) at 0 °C. The reaction mixture was stirred at room temperature under dry atmosphere for 3 h, and then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, filtered through diatomaceous earth, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:4) to give **9b** (37.0 mg, 0.0546 mmol, 38%, α / β = 5/1) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 6.34 (1H, d, H-1α, $J_{1,2}$ = 3.51 Hz), 6.16 (1H, d, NHα, $J_{1,2}$ = 8.03 Hz), 5.90 (1H, d, H-1β, $J_{1,2}$ = 8.52 Hz), 5.62 (1H, d, NHβ, $J_{2,NH}$ = 8.52 Hz), 5.27 (1H, s, H-4α), 5.25−5.13 (3H, m, H-3′α, H-4′α, H-2′α), 4.85 (1H, d, H-1′α, $J_{1',2'}$ = 8.04 Hz), 4.57 (1H, ddd, H-2α, $J_{1,2}$ = 3.51 Hz, $J_{2,NH}$ = 8.03 Hz, $J_{2,3}$ = 8.53 Hz), 4.23−4.19 (2H, m, H-3α, H-6aα), 4.11−4.07 (2H, m, H-5′α, H-6bα), 4.00 (1H, dd, H-5α, $J_{5,6a}$ = 6.53 Hz, $J_{5,6b}$ = 11.54 Hz), 3.76 (3H, s, COOCH_3), 2.19−2.01 (20H, m, CH_3CO, CH$_3$CH_2CONH), 1.09 (3H, t, CH_3CH$_2$CONH, *J* = 8.03 Hz); HRMS (FAB) calcd. for $C_{28}H_{40}NO_{18}$ $[M+H]^+$ 678.2245, found 678.2248.

2-Ethyl-[4,6-di-*O*-acetyl-1,2-di-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (10b). To a solution of **9b** (100 mg, 0.148 mmol) in anhydrous dichloromethane (5.0 mL) was added TMSOTf (40 μL, 0.220 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature for 18 h, quenched with triethylamine (200 μL) at 0 °C, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:2−2:5, containing 0.5% (v/v) triethylamine) to give pure **10b** (75.0 mg, 0.121 mmol, 82%) as white amorphous. $[\alpha]_D$ +20° (*c* 0.75, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.93 (1H, d, H-1, $J_{1,2}$ = 7.02 Hz), 5.38 (1H, d, H-4, $J_{3,4}$ = 3.51 Hz, $J_{4,5}$ = 2.00 Hz), 5.31−5.20 (2H, m, H-3′, H-4′), 5.09 (1H, d, H-1′, $J_{1',2'}$ = 7.52 Hz), 5.00 (1H, dd, H-2′, $J_{1',2'}$ = 7.52 Hz, $J_{2',3'}$ = 8.53 Hz), 4.16−4.11 (3H, m, H-6, H-5), 4.05 (1H, d, H-5′, $J_{4',5'}$ = 9.53 Hz), 3.94 (1H, dd, H-3, $J_{2,3}$ = 7.02 Hz, $J_{3,4}$ = 3.51 Hz), 3.83 (1H, t, H-2, $J_{1,2} = J_{2,3}$ = 7.02 Hz), 3.76 (3H, s, COOCH_3), 2.32 (2H, q, CH$_3$CH_2 of oxazoline, *J* = 7.53 Hz), 2.08−2.02 (15H, m, CH_3CO), 1.18 (3H, t, CH_3CH$_2$ of oxazoline, *J* = 7.53 Hz); HRMS (FAB) calcd. for $C_{26}H_{36}NO_{16}$ $[M+H]^+$ 618.2034, found 618.2035.

2-Ethyl-[1,2-di-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (1b). To a solution of **10b** (75.0 mg, 0.121 mmol) in methanol (1.21 mL) added about 28% (w/v) sodium methoxide in methanol (2.3 mg) at 0 °C under argon atmosphere. The reaction mixture was stirred at 0 °C for 30 min then at room temperature for 30 min followed by evaporation. The residue was dissolved with carbonate buffer (50 mM, pH 10.6, 1.2 mL) and stirred for 1 h at room temperature. The mixture was freeze-dried to give **1b** (56.0 mg, purity 93%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 5.91 (1H, d, H-1, $J_{1,2}$ = 7.53 Hz), 4.47 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 4.00 (1H, s, H-4), 3.78−3.73 (2H, m, H-2, H-5), 3.63−3.52 (4H, m, H-3, H-6, H-5′), 3.34−3.32 (2H, m, H-4′, H-3′), 3.22 (1H, t, H-2′, $J_{1',2'} = J_{2',3'}$ = 8.03 Hz), 2.16 (2H, q, CH$_3$CH_2 of oxazoline, *J* = 7.53

Hz), 0.96 (3H, t, CH_3CH$_2$ of oxazoline, J = 7.53 Hz); HRMS (FAB) calcd. for $C_{15}H_{23}NO_{11}Na$ $[M+H]^+$ 416.1169, found 416.1161.

4,6-Di-*O*-acetyl-2-butanamide-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-β-D-galactopyranosyl acetate (9c). A mixture of **8** (200 mg, 0.288 mmol) and 20% palladium hydroxide on activated carbon (100 mg) in methanol (20.0 mL) was stirred at room temperature under hydrogen atmosphere for 3.5 h. The reaction mixture was filtered through diatomaceous earth and evaporated. To a solution of the residue in methanol (20.0 mL) was added triethylamine (1.0 mL) and butyryl chloride (60 μL, 0.575 mmol) at 0 °C under dry atmosphere. The reaction mixture was stirred at 0 °C for 2 h, quenched with pyridine (1.0 mL), and evaporated. The residue was dried by vacuum pump for overnight. Then, to a solution of this residue in pyridine (20.0 mL) was added acetic anhydride (168 μL, 1.73 mmol) at 0 °C. The mixture was stirred at room temperature under dry atmosphere for 12 h, and then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, then filtered through diatomaceous earth, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:1−1:3) to give pure **9c** (113 mg, 0.163 mmol, 57%, α / β = 50/9) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 6.34 (1H, d, H-1α, $J_{1,2}$ = 3.52 Hz), 6.23 (1H, d, N*H*α, $J_{1,2}$ = 7.53 Hz), 5.89 (1H, d, H-1β, $J_{1,2}$ = 8.53 Hz), 5.27−5.13 (4H, m, H-4α, H-3′α, H-4′α, H-2′α), 4.85 (1H, d, H-1′α, $J_{1',2'}$ = 8.53 Hz), 4.56 (1H, dt, H-2α, $J_{1,2}$ = 3.52 Hz, $J_{2,NH}$ = $J_{2,3}$ = 7.53 Hz), 4.25−4.11 (2H, m, H-3α, H-6aα), 4.09−4.06 (2H, m, H-5′α, H-6bα), 4.00 (1H, dd, H-5a, $J_{5,6a}$ = 6.53 Hz, $J_{5,6b}$ = 11.04 Hz), 3.76 (3H, s, COOCH_3), 2.19−2.01 (20H, m, CH_3CO, CH$_3$CH$_2$CH_2CONH), 1.62−1.59 (2H, m, CH$_3$CH_2CH$_2$CONH), 0.883 (3H, t, CH_3CH$_2$CH$_2$CONH, J = 7.03 Hz); HRMS (FAB) calcd for $C_{29}H_{42}NO_{18}$ $[M+H]^+$ 692.2402, found 692.2402.

2-Propyl-[4,6-di-*O*-acetyl-1,2-di-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (10c). To a solution of **9c** (113 mg, 0.163 mmol) in anhydrous dichloromethane (5.0 mL) was added TMSOTf (45 μL, 0.245 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature for 6 h, then quenched with triethylamine (0.5 mL) at 0 °C, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:1, containing 0.5% (v/v) triethylamine) to give pure **10c** (66.0 mg, 0.105 mmol, 64%) as white amorphous. $[\alpha]_D$ +25° (*c* 0.66, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.93 (1H, d, H-1, $J_{1,2}$ = 7.03 Hz), 5.38 (1H, t, H-4, $J_{3,4}$ = $J_{4,5}$ = 3.51 Hz), 5.29 (1H, t, H-3′, $J_{2',3'}$ = $J_{3',4'}$ = 9.03 Hz), 5.22 (1H, m, H-4′, $J_{3',4'}$ = $J_{4',5'}$ = 9.03 Hz), 5.10 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 5.00 (1H, dd, H-2′, $J_{1',2'}$ = 8.03 Hz, $J_{2',3'}$ = 9.03 Hz), 4.13 (3H, m, H-6, H-5), 4.05 (1H, d, H-5′, $J_{4',5'}$ = 9.54 Hz), 3.93 (1H, dd, H-3, $J_{2,3}$ = 7.03 Hz, $J_{3,4}$ = 3.51 Hz), 3.82 (1H, t, H-2, $J_{1,2}$ = $J_{2,3}$ = 7.03 Hz), 3.75 (3H, s, COOCH_3), 2.28 (2H, t, CH$_3$CH$_2$CH_2CONH, J = 7.03 Hz), 2.08−2.02 (15H, m, CH_3CO), 1.70−1.62 (2H, m, CH$_3$CH_2CH$_2$CONH), 0.973 (3H, t, CH_3CH$_2$CH$_2$CONH, J = 7.53 Hz); HRMS (FAB) calcd for $C_{27}H_{38}NO_{16}$ $[M+H]^+$ 632.2190, found 632.2191

2-Propyl-[1,2-di-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (1c). To a solution of **10c** (66.0 mg, 0.105 mmol) in methanol (1.1 mL) was added about 28% (w/v) sodium methoxide in methanol (2.0 mg) at 0 °C under argon atmosphere. The reaction mixture was stirred at 0 °C for 20 min then at room temperature for 10 min followed by concentration in vacuo. The residue was dissolved with carbonate buffer (50 mM, pH 10.6, 1.1 mL) and stirred at room temperature for 1 h, then freeze-dried to give **1c** (46.0 mg, purity 86%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 5.98 (1H, d, H-1, $J_{1,2}$ = 7.03 Hz), 4.52 (1H, d, H-1′, $J_{1',2'}$ = 7.53 Hz), 4.06 (1H, d, H-4, $J_{3,4}$ = 2.51 Hz), 3.84−3.79 (2H, m, H-2, H-5), 3.71−3.57 (4H, m, H-3, H-6, H-5′), 3.40−3.38 (2H, m, H-4′, H-3′), 3.28 (1H, t, H-2′, $J_{1',2'}$ = $J_{2',3'}$ = 7.53 Hz), 2.21 (2H, t, CH$_3$CH$_2$CH_2CONH, J = 7.53 Hz), 1.56−1.47 (2H, m, CH$_3$CH_2CH$_2$CONH), 0.813 (3H, t, CH_3CH$_2$CH$_2$CONH, J = 7.53 Hz); HRMS (FAB) calcd for $C_{16}H_{24}NNa_2O_{11}$ $[M+Na]^+$ 452.1145, found 452.1141.

4,6-Di-*O*-acetyl-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-2-(2-methylpropanamido)-β-D-galactopyranosyl acetate (9d). A mixture of **8** (315 mg, 0.453 mmol) and 20% palladium hydroxide on activated carbon (150 mg) in methanol (30.0 mL) was stirred at room temperature under hydrogen atmosphere for 3.5 h. The reaction mixture was filtered through diatomaceous earth and evaporated. To a solution of the residue in methanol (30.0 mL) was added triethylamine (1.0 mL) and isobutyric anhydride (240 μL, 1.44 mmol) at 0 °C under dry atmosphere. The mixture was stirred at room temperature for 2 h, then quenched with pyridine (3.0 mL), and evaporated. The residue was dried by vacuum pump for overnight. Then, to a solution of the residue in pyridine (30.0 mL) was added acetic anhydride (265 μL, 2.72 mmol) at 0 °C. The mixture was stirred at room temperature under dry atmosphere for 6 h, then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, then filtered through diatomaceous earth and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:2−1:3) to give pure **9d** (129 mg, 0.187 mmol, 41%, α / β = 20/3) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 6.35 (1H, d, H-1α, $J_{1,2}$ = 3.51 Hz), 6.10 (1H, d, N*H*α, $J_{1,2}$ = 7.53 Hz), 5.90 (1H, d, H-1β, $J_{1,2}$ = 8.54 Hz), 5.27 (1H, d, H-4α, $J_{3,4}$ = 2.00 Hz), 5.26−5.13 (3H, m, H-3′α, H-4′α, H-2′α), 4.84 (1H, d, H-1′α, $J_{1',2'}$ = 8.53 Hz), 4.55 (1H, ddd, H-2α, $J_{1,2}$ = 3.51 Hz, $J_{2,NH}$ = 7.53 Hz, $J_{2,3}$ = 8.53 Hz), 4.25−4.20 (2H, m, H-3α, H-6aα), 4.11−4.07 (2H, m, H-5′α, H-6bα), 4.00 (1H, dd, H-5a, $J_{5,6a}$ = 6.52 Hz, $J_{5,6b}$ = 11.04 Hz), 3.76 (3H, s, COOCH_3), 2.27 (1H, m, (CH$_3$)$_2$C*H*CONH), 2.19−1.95 (18H, m, CH_3CO), 1.10−1.04 (6H, m, (CH_3)$_2$CHCONH); HRMS (FAB) calcd. for $C_{29}H_{42}NO_{18}$ $[M+H]^+$ 692.2402, found 692.2402.

2-Isopropyl-[4,6-di-*O*-acetyl-1,2-di-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (10d). To a solution of **9d** (118 mg, 0.171 mmol) in anhydrous dichloromethane (6.0 mL) was added TMSOTf (47 μL, 0.256 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature for 15 h, then quenched with triethylamine (100 μL) at 0 °C followed by concentration in vacuo. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:1, containing 0.5% (v/v) triethylamine) to give pure **10d** (82.0 mg, 0.130 mmol, 76%) as white amorphous. $[\alpha]_D$ +17° (*c* 0.82, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.91 (1H, d, H-1, $J_{1,2}$ = 7.03 Hz), 5.36 (1H, t, H-4, $J_{3,4}$ = $J_{4,5}$ = 2.51 Hz), 5.31−5.19 (2H, m, H-3′, H-4′), 5.09 (1H, d, H-1′, $J_{1',2'}$ = 8.53 Hz), 5.00 (1H, t, H-2′, $J_{1',2'}$ = $J_{2',3'}$ = 8.53 Hz), 4.15−4.09 (3H, m, H-6, H-5), 4.03 (1H, d, H-5′, $J_{4',5'}$ = 9.54 Hz), 3.94 (1H, dd, H-3, $J_{2,3}$ = 7.03 Hz, $J_{3,4}$ = 2.51 Hz), 3.83 (1H, t, H-2, $J_{1,2}$ = $J_{2,3}$ = 7.03 Hz), 3.75 (3H, s, COOCH_3), 2.58 (1H, m, (CH$_3$)$_2$C*H*CONH), 2.08−2.02 (15H, m, CH_3CO), 1.20−1.18 (6H, m, (CH_3)$_2$CHCONH). HRMS (FAB) calcd. for $C_{27}H_{38}NO_{16}$ $[M+H]^+$ 632.2190, found 632.2186.

2-Isopropyl-[1,2-di-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (1d). To a solution of **10d** (82.0 mg, 0.130 mmol) in methanol (1.3 mL) added about 28% (w/v) sodium methoxide in methanol (2.5 mg) at 0 °C under argon atmosphere. The reaction mixture was stirred at 0 °C for 1 h then at room temperature for 1 h. To the mixture was added about 28% (w/v) sodium methoxide in methanol (2.5 mg) and stirred for 1 h followed by concentration in vacuo. The residue was dissolved with carbonate buffer (50 mM, pH 10.6, 1.3 mL) at room temperature and stirred for 1 h. The reaction mixture was lyophilized to give **1d** (65.0 mg, purity 83%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 5.91 (1H, d, H-1, $J_{1,2}$ = 7.03 Hz), 4.46 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 4.01 (1H, d, H-4, $J_{3,4}$ = 2.01 Hz), 3.77−3.73 (2H, m, H-2, H-5), 3.65−3.52 (4H, m, H-3, H-6, H-5′), 3.36−3.30 (2H, m, H-4′, H-3′), 3.23 (1H, t, H-2′, $J_{1',2'}$ = $J_{2',3'}$ =

8.03 Hz), 2.45 (1H, m, $(CH_3)_2CHCONH$), 1.01−0.97 (6H, m, $(CH_3)_2$-CHCONH); HRMS (FAB) calcd. for $C_{16}H_{25}NO_{11}Na$ $[M+H]^+$ 430.1325, found 430.1323.

4,6-Di-*O*-acetyl-2-benzamido-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-D-galactopyranosyl acetate (9e). A mixture of **8** (300 mg, 0.431 mmol) and 20% palladium hydroxide on activated carbon (150 mg) in methanol (30.0 mL) was stirred at room temperature under hydrogen atmosphere for 3.5 h. The reaction mixture was then filtered through diatomaceous earth and evaporated. To a solution of the residue in methanol (30.0 mL) was added triethylamine (1.0 mL) and benzoyl chloride (100 μL, 0.863 mmol) at 0 °C under dry atmosphere. The mixture was stirred at 0 °C for 1 h, quenched with pyridine (3.0 mL), and evaporated. The residue was dried by vacuum pump for overnight. Then, to a solution of the residue in pyridine (30.0 mL) was added acetic anhydride (252 μL, 2.59 mmol) at 0 °C. The reaction mixture was kept stirring at room temperature under dry atmosphere for 3 h, then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, then filtered through diatomaceous earth, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:1−1:2) to give pure **9e** (180 mg, 0.248 mmol, 58%, $\alpha / \beta = 5/2$) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.72−7.37 (5H, m, aromatic), 6.55 (1H, d, N*H*α, $J_{1,2} = 7.53$ Hz), 6.44 (1H, d, H-1α, $J_{1,2} = 3.01$ Hz), 6.16 (1H, d, H-1*β*, $J_{1,2} = 9.04$ Hz), 5.37 (1H, d, H-4α, $J_{3,4} = 2.00$ Hz), 5.23−5.10 (3H, m, H-3′α, H-4′α, H-2′α), 4.87 (1H, d, H-1′α, $J_{1',2'} = 8.03$ Hz), 4.83 (1H, ddd, H-2α, $J_{1,2} = 3.01$ Hz, $J_{2,NH} = 7.53$ Hz, $J_{2,3} = 8.54$ Hz), 4.37 (1H, dd, H-3α, $J_{2,3} = 8.54$ Hz, $J_{3,4} = 2.00$ Hz), 4.28 (1H, dd, H-6aα, $J_{5,6a} = 5.52$ Hz, $J_{6a,6b} = 11.04$ Hz), 4.14 (1H, dd,, H-6bα, $J_{5,6b} = 7.03$ Hz, $J_{6a,6b} = 11.04$ Hz), 4.06−4.00 (2H, dd, H-5′α, H-5α), 3.62 (3H, s, COOC*H*$_3$), 2.20−1.91 (18H, m, C*H*$_3$CO); HRMS (FAB) calcd. for $C_{32}H_{40}NO_{18}$ $[M+H]^+$ 726.2245, found 726.2248.

2-Phenyl-[4,6-di-*O*-acetyl-1,2-di-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (10e). To a solution of **9e** (180 mg, 0.248 mmol) in anhydrous dichloromethane (9.0 mL) was added TMSOTf (68 μL, 0.372 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature for 12 h, then quenched with triethylamine (200 μL) at 0 °C, and evaporated. The residue was purified by silica gel chromatography (toluene-ethyl acetate 3:1−2:1, containing 0.5% (v/v) triethylamine) to give pure **10e** (105 mg, 0.158 mmol, 64%) as white amorphous. $[\alpha]_D$ +30° (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.96−7.43 (5H, m, aromatic), 6.15 (1H, d, H-1, $J_{1,2} = 6.02$ Hz), 5.42 (1H, s, H-4), 5.33 (1H, t, H-3′, $J_{2',3'} = J_{3',4'} = 8.29$ Hz), 5.27−5.19 (2H, m, H-4′, H-1′), 5.04 (1H, t, H-2′, $J_{1',2'} = J_{2',3'} = 8.29$ Hz), 4.17 (3H, m, H-6, H-5), 4.11−4.02 (3H, m, H-5′, H-2, H-3), 3.72 (3H, s, COOC*H*$_3$), 2.10−2.04 (15H, m, C*H*$_3$CO); HRMS (FAB) calcd. for $C_{30}H_{36}NO_{16}$ $[M+H]^+$ 666.2034, found 666.2043.

2-Phenyl-[1,2-di-deoxy-3-*O*-(sodium *β*-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (1e). To a solution of **10e** (105 mg, 0.158 mmol) in methanol (1.6 mL) was added about 28% (w/v) sodium methoxide in methanol (3.0 mg) at 0 °C under argon atmosphere. The reaction mixture was stirred at 0 °C for 0.5 h, then at room temperature for 1 h. To the mixture was added additional sodium methoxide in methanol (3.0 mg) at room temperature, and stirred for 30 min followed by evaporation. The residue was dissolved with carbonate buffer (50 mM, pH 10.6, 1.6 mL) at room temperature, then stirred for 1 h followed by lyophilization to give **1e** (60.0 mg, purity 87%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 7.77−7.34 (5H, m, aromatic), 6.13 (1H, d, H-1, $J_{1,2} = 7.03$ Hz), 4.53 (1H, d, H-1′, $J_{1',2'} = 7.53$ Hz), 4.06 (1H, d, H-4, $J_{3,4} = 2.51$ Hz), 4.00 (1H, dd, H-2, $J_{1,2} = 7.03$ Hz, $J_{2,3} = 7.53$ Hz,), 3.83 (1H, dd, H-5, $J_{5,6a} = 7.53$ Hz, $J_{5,6b} = 11.54$ Hz), 3.74 (1H, dd, H-3, $J_{2,3} = 7.53$, $J_{3,4} = 7.53$ Hz), 3.68−3.52 (3H, m, H-6, H-5′), 3.45−3.32 (2H, m, H-4′, H-3′), 3.27 (1H, t, H-2′, $J_{1',2'} = J_{2',3'} = 7.53$ Hz); HRMS (FAB) calcd. for $C_{19}H_{23}NO_{11}Na$ $[M+H]^+$ 464.1169, found 464.1173.

4,6-Di-*O*-acetyl-2-acrylamido-2-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-*β*-D-galactopyranosyl acetate (9f). A mixture of **8** (502 mg, 0.722 mmol) and 20% palladium hydroxide on activated carbon (250 mg) in methanol (50.0 mL) was stirred at room temperature under hydrogen atmosphere for 3.5 h. The reaction mixture was filtered through diatomaceous earth and evaporated. To a solution of the residue in methanol (50.0 mL) was added triethylamine (1.0 mL) and acryloyl chloride (292 μL, 3.61 mmol) at 0 °C under dry atmosphere. The mixture was kept stirring at 0 °C for 1 h, then added acryloyl chloride (292 μL, 3.61 mmol) additionally. After 1 h, the reaction mixture was quenched with pyridine (2.0 mL) and concentrated in vacuo. The residue was dried under diminished pressure for overnight. Then, to a solution of the residue in pyridine (20.0 mL) was added acetic anhydride (422 μL, 4.33 mmol) at 0 °C. The reaction mixture was stirred at room temperature under dry atmosphere for 4 h, and then evaporated. The residue was diluted with chloroform, washed with 4% (w/v) aqueous potassium hydrogen sulfate, saturated aqueous hydrogen carbonate and saturated aqueous sodium chloride. The organic layer was dried over anhydrous magnesium sulfate, then filtered through diatomaceous earth, and concentrated in vacuo. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 1:1−1:3) to give pure **9f** (183 mg, 0.271 mmol, 38%, $\alpha / \beta = 4/1$) as white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 6.42 (1H, d, N*H*α, $J_{NH,2} = 7.52$ Hz), 6.38 (1H, d, H-1α, $J_{1,2} = 3.52$ Hz), 6.32 (1H, dd, C*H*$_A$H$_B$═CH$_C$, $J_{A,B} = 1.51$ Hz, $J_{A,C} = 17.06$ Hz), 6.07 (1H, dd, CH$_A$H$_B$═C*H*$_C$, $J_{B,C} = 10.04$ Hz, $J_{A,C} = 17.06$ Hz), 5.96 (1H, d, H-1*β*, $J_{1,2} = 9.04$ Hz), 5.61 (1H, dd, CH$_A$*H*$_B$═CH$_C$, $J_{A,B} = 1.51$ Hz, $J_{B,C} = 10.04$ Hz), 5.27−5.14 (4H, m, H-4α, H-3′α, H-4′α, H-2′α), 4.84 (1H, d, H-1′α, $J_{1',2'} = 8.06$ Hz), 4.64 (1H, ddd, H-2α, $J_{1,2} = 3.52$ Hz, $J_{2,NH} = 7.52$ Hz, $J_{2,3} = 7.54$ Hz), 4.28−4.21 (2H, m, H-3α, H-6aα), 4.12−4.08 (2H, m, H-5′α, H-6bα), 4.01 (1H, dd, H-5a, $J_{5,6a} = 6.53$ Hz, $J_{5,6b} = 9.04$ Hz), 3.78 (3H, s, COOC*H*$_3$), 2.18−2.00 (18H, m, C*H*$_3$CO); HRMS (FAB) calcd for $C_{28}H_{38}NO_{18}$ $[M+H]^+$ 676.2089, found 676.2090.

2-Vinyl-[4,6-di-*O*-acetyl-1,2-di-deoxy-3-*O*-(methyl 2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (10f). To a solution of **9f** (100 mg, 0.148 mmol) in anhydrous dichloromethane (5.0 mL) was added TMSOTf (41 μL, 0.222 mmol) at 0 °C under argon atmosphere. The reaction mixture was stirred at room temperature for 3 h, then quenched with triethylamine (0.5 mL) at 0 °C, and evaporated. The residue was purified by silica gel chromatography (*n*-hexane−ethyl acetate 2:1−1:1, triethylamine 0.5%) to give pure **10f** (70.0 mg, 0.114 mmol, 77%) as white amorphous. $[\alpha]_D$ +42° (*c* 0.70, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 6.24 (1H, dd, CH$_A$H$_B$═C*H*$_C$, $J_{B,C} = 10.04$ Hz, $J_{A,C} = 17.57$ Hz), 6.16 (1H, dd, C*H*$_A$H$_B$═CH$_C$, $J_{A,B} = 2.01$ Hz, $J_{A,C} = 17.57$ Hz), 6.01 (1H, d, H-1, $J_{1,2} = 6.52$ Hz), 5.79 (1H, dd, CH$_A$*H*$_B$═CH$_C$, $J_{A,B} = 2.01$ Hz, $J_{B,C} = 10.04$ Hz), 5.41 (1H, s, H-4), 5.29 (1H, t, H-3′, $J_{2',3'} = J_{3',4'} = 9.03$ Hz), 5.22 (1H, t, H-4′, $J_{3',4'} = J_{4',5'} = 9.03$ Hz), 5.09 (1H, d, H-1′, $J_{1',2'} = 7.53$ Hz), 5.00 (1H, dd, H-2′, $J_{1',2'} = 7.53$ Hz, $J_{2',3'} = 9.03$ Hz), 4.15 (3H, m, H-6, H-5), 4.07 (1H, d, H-5′, $J_{4',5'} = 9.03$ Hz), 3.95−3.94 (2H, m, H-3, H-2), 3.76 (3H, s, COOC*H*$_3$), 2.09−2.02 (15H, m, C*H*$_3$-CO); HRMS (FAB) calcd for $C_{26}H_{33}NO_{16}$ $[M+H]^+$ 616.1877, found 616.1893.

2-Vinyl-[1,2-di-deoxy-3-*O*-(sodium *β*-D-glucopyranosyluronate)-α-D-galactopyrano]-[2,1-*d*]-2-oxazoline (1f). To a solution of **10f** (70.0 mg, 0.114 mmol) in methanol (1.2 mL) was added about 28% (w/v) sodium methoxide in methanol (2.2 mg) at 0 °C under argon atmosphere. The reaction mixture was stirred at 0 °C for 0.5 h, then at room temperature for 0.5 h. To the mixture was added again sodium methoxide in methanol (2.2 mg). After 0.5 h, the reaction mixture was concentrated in vacuo. The residue was dissolved with carbonate buffer (50 mM, pH 10.6, 1.2 mL) at room temperature and kept stirring for

1 h followed by lyophilization to afford **1f** (72 mg, purity 86%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 6.14−6.02 (3H, m, CH_AH_B=CH_C, CH_AH$_B$=CH_C, H-1), 5.78 (1H, dd, CH_AH_B=CH_C, $J_{A,B}$ = 1.51 Hz, $J_{B,C}$ = 10.04 Hz), 4.54 (1H, d, H-1′, $J_{1',2'}$ = 7.53 Hz), 4.08 (1H, d, H-4, $J_{3,4}$ = 2.00 Hz), 3.95 (1H, t, H-2, $J_{1,2}$ = 7.03 Hz), 3.84 (1H, dd, H-5, $J_{5,6a}$ = 7.03 Hz, $J_{5,6b}$ = 10.54 Hz), 3.73−3.58 (4H, m, H-3, H-6, H-5′), 3.40−3.38 (2H, m, H-4′, H-3′), 3.28 (1H, t, H-2′, $J_{1',2'}$ = $J_{2',3'}$ = 7.53 Hz); HRMS (FAB) calcd for $C_{15}H_{21}NNaO_{11}$ $[M+H]^+$ 414.1012, found 414.1004.

Enzymatic Polymerization of 1a. A typical polymerization procedure of **1a** is given as follow: **1a** (15.0 mg, 37.5 μmol) in a phosphate buffer (50 mM, pH 7.5, 375 μL) was incubated with H−OTH (1.5 mg) at 30 °C. After 23 h, the mixture was heated at 90 °C for 3 min to inactivate the enzyme and poured into excess amount of tetrahydrofuran. The precipitate was collected by centrifugation. A small part of the precipitate was dissolved in water for SEC measurements (yield 50%, M_n 2100, M_w 2500) and MALDI TOF/MS. The residual precipitate was dissolved in water and subjected to Sephadex G-10 size exclusion chromatography eluting with 0.1 M aqueous sodium chloride to remove hydrolyzed disaccharide fraction, then desalted through Sephadex G-10 column eluting with distilled water. The fractions containing polysaccharides were combined and lyophilized to afford **2a** as a white solid (6.6 mg, 44%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 4.30 (2H, bs, H-1, H-1′), 3.93 (1H, bs, H-4), 3.80 (1H, bt, H-2), 3.61−3.50 (6H, m, H-3, H-4, H-5′, H-6, H-5), 3.38 (1H, bt, H-3′), 3.16 (1H, bt, H-2′), 1.83 (1H, s, CH_3CO). Measurement at 70 °C; 4.37 (1H, d, H-1, $J_{1,2}$ = 7.53 Hz), 4.30 (1H, d, H-1′, $J_{1',2'}$ = 7.53 Hz); ^{13}C NMR (100 MHz, D_2O, acetone) δ 174.54 (C-6′), 174.17 (CH_3*C*O), 103.87 (C-1′), 100.38 (C-1), 79.91 (C-3), 79.23 (C-4′), 75.90 (C-5′), 74.52 (C-5), 73.24 (C-3′), 72.01 (C-2′), 67.23 (C-4), 60.61 (C-6), 50.55 (C-2), 22.04 (*C*H_3CO).

Enzymatic polymerizations of 1b-1f. A solution of **1b** (9.0 mg, 21.6 μmol) in a phosphate buffer (50 mM, pH 7.5, 216 μL) was incubated with H−OTH (0.9 mg) at 30 °C. After 35 h, the resulting suspension was heated at 90 °C for 3 min and poured into excess amount of tetrahydrofuran to inactivate enzyme. The precipitate was collected by centrifugation, dissolved in water, and analyzed by SEC measurement (yield 46%, M_n 2700, M_w 3600) and MALDI-TOF/MS. The reaction mixture was purified by Sephadex G-10 size exclusion chromatography eluting with 0.1 M aqueous sodium chloride, then desalted through Sephadex G-10 column eluting with distilled water to give **2b** as a white solid (3.0 mg, 33%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 4.33 (1H, d, H-1, $J_{1,2}$ = 8.53 Hz), 4.28 (1H, d, H-1′, $J_{1',2'}$ = 8.03 Hz), 3.92 (1H, s, H-4), 3.82 (1H, t, H-2, $J_{1,2}$ = 8.53 Hz), 3.63−3.46 (6H, m, H-3, H-4′, H-5′, H-6, H-5), 3.37 (1H, t, H-3′, $J_{2',3'}$ = 8.03 Hz), 3.16 (1H, t, H-2′, $J_{1',2'}$ = 8.03 Hz), 2.10 (2H, q, CH_3CH_2*C*O, J = 7.53 Hz), 0.91 (3H, t, C$H_3$$CH_2$CO, J = 7.53 Hz). ^{13}C NMR (100 MHz, D_2O, acetone) δ 178.34 (C-6′), 174.15 (CH_3CH_2*C*O), 103.82 (C-1′), 100.21 (C-1), 79.66 (C-3), 78.86 (C-4′), 75.97 (C-5′), 74.49 (C-5), 73.18 (C-3′), 72.06 (C-2′), 67.40 (C-4), 60.65 (C-6), 50.40 (C-2), 28.97 (CH_3*C*H_2CO), 8.76 (*C*$H_3$$CH_2$CO). In the control experiment (without the enzyme), the only product was the disaccharide derived from hydrolysis of **1b**, 2-deoxy-2-propanamido-3-*O*-(sodium β-D-glucopyranosyluronate)-D-galactopyranose: MALDI-TOF/MS; *m*/*z* 410.62 $[M-H]^-$.

A solution of **1c** (5.0 mg, 11.6 μmol) in a phosphate buffer (50 mM, pH 7.5, 116 μL) was incubated with H−OTH (0.5 mg) at 30 °C. After 122 h, the reaction mixture was heated at 90 °C for 3 min and poured into excess amount of tetrahydrofuran to inactivate the enzyme. The resulting precipitate was collected by centrifugation, dissolved in water, and its small part was analyzed by SEC measurement, but no polymeric products were detected. The residual solution was subjected to Sephadex G-10 column chromatography eluting with 0.1 M aqueous sodium chloride, then desalted through Sephadex G-10 column eluting with distilled water. The resulting solution was applied to MALDI-TOF/MS analysis, and the spectrum showed peaks of oligomers of **2c**. MALDI-TOF/MS; *m*/*z* 830.78 $[M-H]^-$ (n = 2, tetrasaccharide), 1238.04 $[M-H]^-$ (n = 3, hexasaccharide), 1645.52 $[M-H]^-$ (n = 4, octasaccharide), 2052.56 $[M-H]^-$ (n = 5, decasaccharide). In the control experiment (without the enzyme), the only product was the disaccharide derived from hydrolysis of **1c**, 2-butanamido-2-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-D-galactopyranose: MALDI-TOF/MS; *m*/*z* 424.21 $[M-H]^-$.

A solution of **1d** (5.0 mg, 11.6 μmol) in a phosphate buffer (50 mM, pH 7.5, 116 μL) was incubated with H−OTH (0.5 mg) at 30 °C. After 168 h, the resulting suspension was heated at 90 °C for 3 min and poured into excess amount of tetrahydrofuran to inactivate the enzyme. The precipitate was collected by centrifugation, dissolved in water, and analyzed by SEC measurement, but no polymeric products were detected by SEC measurement. The reaction mixture was purified through Sephadex G-10 column eluting with 0.1 M aqueous sodium chloride, then desalted through Sephadex G-10 column eluting with distilled water. The resulting solution was applied to MALDI-TOF/MS analysis, and the spectrum showed peaks of oligomers of **2d**. MALDI-TOF/MS; *m*/*z* 830.90 $[M-H]^-$ (n = 2, tetrasaccharide), 1237.17 $[M-H]^-$ (n = 3, hexasaccharide), 1644.54 $[M-H]^-$ (n = 4, octasaccharide). In the control experiment (without the enzyme), the only product was the disaccharide derived from hydrolysis of **1d**, 2-deoxy-2-(2-methylpropanamido)-3-*O*-(sodium β-D-glucopyranosyluronate)-D-galactopyranose: MALDI-TOF/MS; *m*/*z* 424.87 $[M-H]^-$.

A solution of **1e** (5.0 mg, 10.8 μmol) in a phosphate buffer (50 mM, pH 7.5, 108 μL) was incubated with H−OTH (0.5 mg) at 30 °C. After 239 h, the resulting mixture was heated at 90 °C for 3 min and poured into excess amount of tetrahydrofuran to inactivate the enzyme. The precipitate was collected by centrifugation, dissolved in water, and analyzed by SEC measurement and MALDI-TOF/MS, but neither polymeric products nor oligomers were detected. In the control experiment (without the enzyme), the only product was the disaccharide derived from hydrolysis of **1e**, 2-benzamido-2-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-D-galactopyranose: MALDI-TOF/MS; *m*/*z* 458.55 $[M-H]^-$.

A solution of **1f** (25.0 mg, 60.5 μmol), in a phosphate buffer (50 mM, pH 7.5, 605 μL) was incubated with H−OTH (2.5 mg) at 30 °C. After 24 h, the resulting suspension was heated at 90 °C for 3 min and poured into excess amount of tetrahydrofuran to inactivate enzyme. The precipitate was collected by centrifugation, dissolved in water, and analyzed by SEC measurement (yield 19%, M_n 3400, M_w 4600) and MALDI-TOF/MS. The reaction mixture was purified through Sephadex G-10 column eluting with 0.1 M aqueous sodium chloride. Fractions containing the products were collected and subjected to SEC column (Shodex ohpak SB-803HQ, 0.1M sodium nitrate), then desalted through Sephadex G-10 column eluting with distilled water to give **2f** (3.0 mg, 12%). ^{1}H NMR (400 MHz, D_2O, acetone) δ 6.11−5.98 (2H, m, CH_AH_B=CH_C, CH_AH$_B$=CH_C), 5.59 (1H, d, CH_AH_B=CH_C, $J_{B,C}$ = 8.03 Hz), 4.34 (1H, d, H-1, $J_{1,2}$ = 7.03 Hz), 4.25 (1H, d, H-1′, $J_{1',2'}$ = 8.53 Hz), 3.93−3.89 (2H, m, H-4, H-2), 3.64−3.44 (6H, m, H-3, H-4′, H-5′, H-6, H-5), 3.33 (1H, t, H-3′, $J_{2',3'}$ = 9.03 Hz), 3.14 (1H, t, H-2′, $J_{1',2'}$ = 8.53 Hz). ^{13}C NMR (100 MHz, D_2O, acetone) δ 174.25 (C-6′), 168.92 (CH_3CH_2*C*O), 129.78 (CH_2=*C*H), 126.74 (*C*H_2=CH), 103.84 (C-1′), 100.05 (C-1), 80.11 (C-3), 78.62 (C-4′), 75.75 (C-5′), 74.56 (C-5), 73.09 (C-3′), 72.02 (C-2′), 67.30 (C-4), 60.67 (C-6), 50.60 (C-2). In the control experiment (without the enzyme), the only product was the disaccharide derived from hydrolysis of **1f**, 2-acrylamido-2-deoxy-3-*O*-(sodium β-D-glucopyranosyluronate)-D-galactopyranose: MALDI-TOF/MS; *m*/*z* 408.06 $[M-H]^-$.

Enzymatic Polymerization of 1a in an Organic Cosolvent. A typical polymerization procedure of **1a** in methanol as an organic cosolvent is given as follow: **1a** (5.0 mg, 12.5 μmol) in a phosphate buffer (50 mM, pH 7.0, 83 μL) including methanol (42 μL) was

incubated with OTH (0.5 mg) at 30 °C. After 26 h, a small amount of the mixture was subjected to SEC measurement, but no polymeric products were detected. The reaction mixture was then subjected to Dowex 50W−X8 (NH_4^+ form) to remove sodium ion. The resulting solution was applied to MALDI-TOF/MS analysis, and the spectrum showed peaks of oligomers of **2a**. MALDI-TOF/MS; *m*/*z* 775.35 $[M-H]^-$ ($n = 2$, tetrasaccharide), 1154.21 $[M-H]^-$ ($n = 3$, hexasaccharide).

Acknowledgment. We thank Drs. T. Miyoshi and T. Morikawa of Denka Co. (Tokyo, Japan) for supplying hyaluronan samples for calibration standards. This work was partially supported by the Mitsubishi Foundation and by the 21st COE program for a United Approach to New Materials Science in Kyoto University.

JA036584X