Carbohydrate Research 345 (2010) 1901–1908

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Thermal decomposition of β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-hexopyranoses under neutral conditions

Kazuhiro Chiku, Mamoru Nishimoto, Motomitsu Kitaoka *

National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

ARTICLE INFO

Article history:
Received 16 March 2010
Received in revised form 3 June 2010
Accepted 9 June 2010
Available online 15 June 2010

Keywords:
β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-hexopyranose
2-Acetamido-2,3-dideoxy-hex-2-enofuranose
2-Acetamido-3,6-anhydro-2-deoxy-hexofuranose, bicyclic
Peeling reaction
Decomposition under neutral conditions
Reducing aldose, 3-O-linked

ABSTRACT

β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucose (LNB) and β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose (GNB) decompose rapidly upon heating into D-galactose and mono-dehydrated derivatives of the corresponding 2-acetamido-2-deoxy-D-hexoses, including 2-acetamido-2,3-dideoxy-hex-2-enofuranoses and bicyclic 2-acetamido-3,6-anhydro-2-deoxy-hexofuranoses. The decomposition is conducted under neutral conditions where glycosyl linkages are generally believed to be stable. The half-lives of LNB and GNB were 8.1 min and 20 min, respectively, at 90 °C and pH 7.5. The pH dependency of decomposition rates suggests that the instabilities are an extension of the conditions for the peeling reaction, often observed with glycans of O-linked glycoproteins under alkaline conditions. Such decomposition under the neutral conditions is commonly observed with 3-O-linked reducing aldoses.

1. Introduction

β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-hexoses [Galβ(1→3)HexNAc] are common building units of biologically functional sugar chains. β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucose [lacto-*N*-biose I (LNB)] is often found in sugar lipids, blood-type antigens, and human milk oligosaccharides.[1–5] The core 1 structure of O-linked glycans of glycoproteins contains α-linked β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-galactose [galacto-*N*-biose (GNB)] to serine/threonine (Ser/Thr).[6–9]

We reported one-pot enzymatic process to produce LNB[10] and GNB[11] from sucrose and the corresponding 2-acetamido-2-deoxy-hexoses [HexNAc; 2-acetamido-2-deoxy-D-glucose (GlcNAc) and 2-acetamido-2-deoxy-D-galactose (GalNAc), respectively]. During the development of this method, we observed a strange phenomenon wherein LNB and GNB completely decomposed when the solution was heated at neutral pH for a short time, despite the general belief that glycosyl linkages are stable under neutral conditions.

It is known that β-elimination under alkaline conditions to liberate glycans from Ser/Thr often causes the removal of GalNAc from glycan by another β-elimination.[12–15] The reductive alkaline cleavage reaction to form oligosaccharide alditols is required to liberate the glycans without decomposition. In addition, some oligosaccharides containing 3-O-linked HexNAc as the reducing end show further decomposition, and unsaturated sugars are formed.[16–19] This undesirable reaction is sometimes called 'peeling', and it proceeds until the alkali-stable glycosidic linkage appears at the reducing end. The products of the peeling reaction of HexNAc can undergo a color change with the addition of *p*-dimethylaminobenzaldehyde in strong hydrochloric acid (Ehrlich reagent); the coloring reaction is known as the Morgan–Elson reaction.[20] The reaction has been used as a method of detecting 3-O-linked HexNAc residues in carbohydrates and in their decomposition products. Based on these methods, Kuhn et al.[21,22] demonstrated that LNB is easily degraded under alkaline conditions. In addition, some 3-methylated derivatives of GlcNAc have also shown facile decomposition under alkaline conditions.[23,24] Therefore, it is commonly thought that 3-O-linked HexNAc possesses alkaline instability.

In this study we found that the thermal instabilities of LNB and GNB under neutral conditions are an extension of their alkaline instabilities, and that the phenomena commonly occur with free 3-O-linked reducing aldoses. Herein, we show a kinetic analysis of the decomposition of LNB and GNB by heat treatment.

2. Results

2.1. Isolation of decomposition products of LNB and GNB

LNB and GNB mostly decomposed within 1 h at 90 °C under neutral conditions when reacted with 100 mM sodium phosphate

* Corresponding author. Tel.: +81 29 838 8071; fax: +81 29 838 7321.
E-mail address: mkitaoka@affrc.go.jp (M. Kitaoka).

0008-6215/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2010.06.003

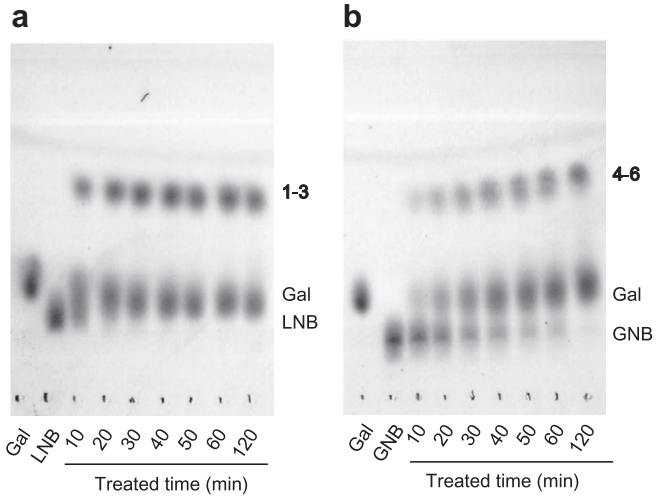
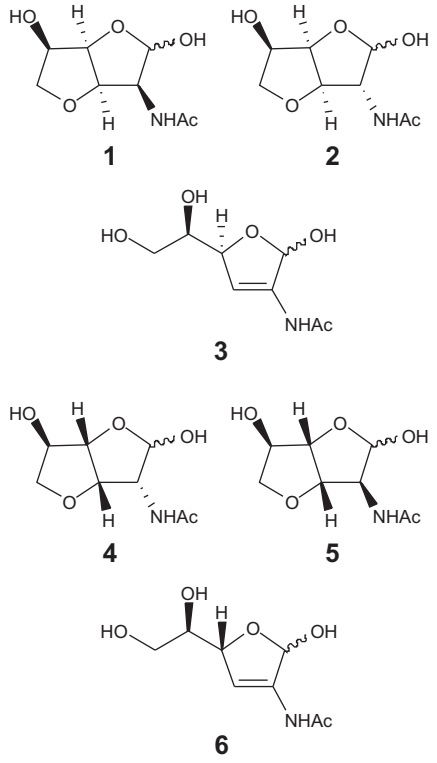


Figure 1. Heat treatment of (a) LNB and (b) GNB with sodium phosphate buffer (pH 7.5) at 90 °C for 0–120 min.

buffer (pH 7.5). In both cases, two spots were detected on thin-layer chromatography (TLC) (Fig. 1). One spot showed the same R_f value as D-galactose (Gal), while the other product showed a higher R_f value than the corresponding HexNAc in each case. The reaction products showing the higher R_f values were separated by two-column chromatography using a Shodex Asahipak ODP-50 and NH2P50-4E column to give three LNB-decomposition products **1**–**3**, and three GNB-decomposition products **4**–**6**. Mass spectrometry (MS) showed that all products **1**–**6** could be represented by the molecular formula $C_8H_{13}NO_5$ that corresponds to mono-dehydrated HexNAc. Nuclear magnetic resonance (NMR) spectra suggested that the products are equilibrium mixtures of α and β anomers.



The compounds **1** and **2** were identified to be C-2 epimers of bicyclic 2-acetamido-3,6-anhydrohexofuranoses with the D-manno and D-gluco configurations, respectively. The compound **3** was 2-acetamido-2,3-dideoxy-D-*erythro*-2-enofuranoses. These compounds were previously reported by Ogata et al.[25] and Beau et al.[26]

Bicyclic 3,6-anhydrohexofuranoses were suggested for the structures of **4** and **5** based on the long-range C–H coupling correlations of C-3–H-6, C-4–H-1, and C-6–H-3 that appeared on HMBC spectra like **1** and **2**. The relative stereoconfiguration of the compounds was deduced by determining cross peaks of the protons at C-1–C-3 using nuclear Overhauser effect spectroscopy (NOESY) (Table 1). Finally **4** and **5** were identified to be C-2 epimers of 2-acetamido-3,6-anhydrohexofuranoses with the D-gulo and D-ido configurations, respectively. The inversion of the conformation at C-3 from GalNAc should be noted.

The anomeric proton (H-1) of **6** with a 2-enol structure gave a doublet of doublets (dd, H-1α, $J_{1,3}$ 1.0 Hz, $J_{1,4}$ 4.0 Hz) and an apparent triplet (H-1β, $J_{1,3}$ 1.0 Hz, $J_{1,4}$ 1.0 Hz, α:β = 1:0.6). In addition, the heteronuclear multiple-bond correlation (HMBC) spectrum exhibited a long-range C–H coupling correlation of C-4–H-1, corresponding to the adapted 2-acetamido-2,3-dideoxy-D-*threo*-2-enofuranose.

High performance liquid chromatography (HPLC) analysis showed that the decomposition of LNB/GNB proceeds concomitantly with the release of an equimolar amount of Gal and mono-dehydrated derivatives of the corresponding HexNAc. The time-course of the LNB decomposition (Fig. 2a) showed that **1**–**3** were generated in yields of about 18%, 32%, and 46%, respectively, after heat treatment for 60 min. In contrast, GNB decomposition (Fig. 2b) gave two major bicyclic 3,6-anhydrohexofuranoses, **4** (37%) and **5** (47%), after heat treatment for 100 min. In these reactions, levels of the hex-2-enofuranosides **3** or **6** increased at an early stage (reaching about 48% or 33%, respectively), and then remained steady or decreased gradually. These results suggest that the bicyclic 3,6-anhydrohexofuranoses **1**–**2** and **4**–**5** could also be generated from the hex-2-enofuranoses **3** and **6**.

2.2. Temperature- and pH-dependence on decomposition of LNB and GNB

We investigated the stabilities of LNB and GNB under several pH and temperature conditions by determining the decomposition rate using the Gal 1-P assay.[27] The rate constant (k) of LNB decomposition was 0.085 ± 0.002 min^{-1} with 100 mM sodium phosphate buffer (pH 7.5) at 90 °C, which is about 2.5 times higher than the rate constant of GNB decomposition (k = 0.034 ± 0.001 min^{-1}). The half-lives of LNB and GNB under the above-mentioned conditions were 8.1 min and 20 min, respectively. LNB and GNB decompositions were undetectable at temperatures below 55 °C and 65 °C, respectively, in 100 mM sodium phosphate buffer (pH 7.5) for 1 h. The activation energy (E_a) values calculated for the decomposition of LNB and GNB were 125 ± 5 kJ mol^{-1} and 132 ± 2 kJ mol^{-1}, respectively (Fig. 3). The concentration of phosphate buffer between 20 mM and 250 mM did not significantly affect the reaction rate of LNB decomposition.

The pH dependence of LNB decomposition was studied by treating LNB at 90 °C for 0–60 min with the various above-mentioned buffers at pH values in the range 4.0–10.5. LNB and GNB were mostly stable at pH conditions below 5.5 at 90 °C for 1 h (remaining amount >95%). The rate constants of the decomposition of LNB and GNB increased exponentially with pH. Linear relationships between $\log_{10} k$ and pH observed for both LNB and GNB (Fig. 4) indicated that the decomposition was a function of the hydroxyl ion concentration. The reaction was not significantly affected by the variation in buffer, and depended on the pH value (concentration of hydroxide ions) and temperature. The identical compounds were detected under various conditions on HPLC analysis at the early stage of the reaction. Simple acid hydrolysis was not observed under the conditions examined.

Table 1
NOESY spectral data of 3,6-anhydro-HexNAc furanoses

	Cross peak on NOESY spectrum[a]		Identification of 3,6-anhydro-HexNAc furanose
	H-1–H-2	H-2–H-3	
Compound **1**			
1a	–	+	*N*-Acetyl-α-D-mannosamine form
1b	+	+	*N*-Acetyl-β-D-mannosamine form
Compound **2**			
2a	+	–	*N*-Acetyl-α-D-glucosamine form
2b	–	–	*N*-Acetyl-β-D-glucosamine form
Compound **4**			
4a	+	+	*N*-Acetyl-α-D-gulosamine form
4b	–	+	*N*-Acetyl-β-D-gulosamine form
Compound **5**			
5a	–	–	*N*-Acetyl-α-D-idosamine form
5b	+	–	*N*-Acetyl-β-D-idosamine form

[a] Cross peaks of NOESY spectrum were clearly (+) or undetectable (–).

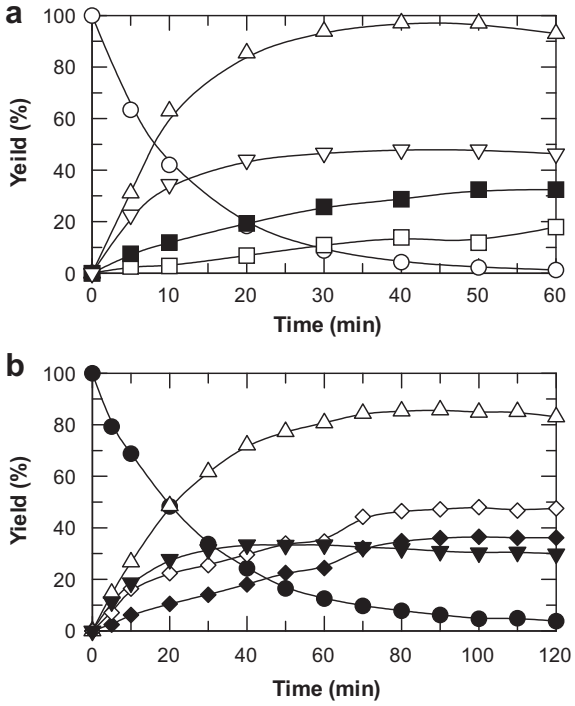


Figure 2. Time course of the decomposition reaction of (a) LNB and (b) GNB in sodium phosphate buffer (pH 7.5). The symbols shown are as follows: ○, LNB; ●, GNB; △, Gal; □, **1**; ■, **2**; ▽, **3**; ◇, **4**; ♦, **5**; ▼, **6**. GlcNAc and GalNAc were not detected.

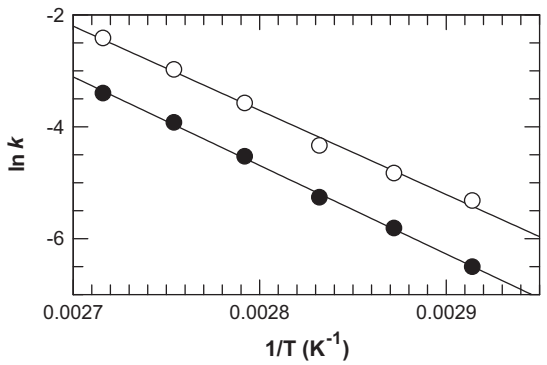


Figure 3. The Arrhenius plot of the decompositions of LNB and GNB. ○, LNB; ●, GNB.

2.3. Heat treatment of various LNB- and GNB-related sugars

The heat stabilities of 17 sugars were examined under neutral conditions. Only the free 3-O-linked hexoses showed decomposition. The rate constants and the half-lives are summarized in Table 2. All compounds whose structures can be described as 3-O-linked reducing hexoses were decomposed to remove the reducing hexoses under these conditions. No decomposition of lacto-*N*-tetraose was detected under these conditions, suggesting that the instability required the reducing end of LNB to be free. *N*-Acetyllactosamine, lactose, and cellobiose were also stable under these conditions, suggesting that the 4-O-linked reducing hexoses were stable. The rate constant of β-D-galactopyranosyl-(1→3)-2-deoxy-D-glucose[†] [Galβ(1→3)2dGlc] was comparable to that of LNB, whereas β-D-galactopyranosyl-(1→3)-D-glucose [Galβ(1→3)Glc] and laminaribiose decomposed much more slowly. Lewis[a] trisaccharide[28] decomposed with the release of Gal slightly faster than LNB, indicating that the additional 4-O-linkage did not stabilize LNB. Nigerose decomposed at a similar rate to laminaribiose, indicating that cleavage was not affected whether the linkage was α or β. It should be noted that laminaribiose and nigerose produced an equimolar amount of glucose (not double molar), probably from their non-reducing ends. *N*-Acetylmuramic acid was found to decompose, indicating that the decomposition did not require a glycosyl linkage.

3. Discussion

GNB and LNB are often found in mammalian biologically functional carbohydrates. We report herein that the free forms of GNB and LNB are actually easily decomposed by heating under neutral conditions, where glycosyl linkages are generally considered to be stable. The pH dependencies of the decomposition rate clearly indicate that the instabilities are an extension of the conditions for the peeling reaction, which is often observed under alkaline conditions. We also demonstrated that instability under neutral conditions commonly occurs with 3-O-linked reducing hexoses.

The products of the peeling reaction have been predicted but not determined earlier. This is probably due to two reasons; the low availability of LNB and GNB, and the fact that the alkaline conditions of the peeling reaction cause further decomposition of the products. The large-scale production of the compounds and the

[†] IUPAC name is β-D-galactopyranosyl-(1→3)-2-deoxy-D-*arabino*-hexose.

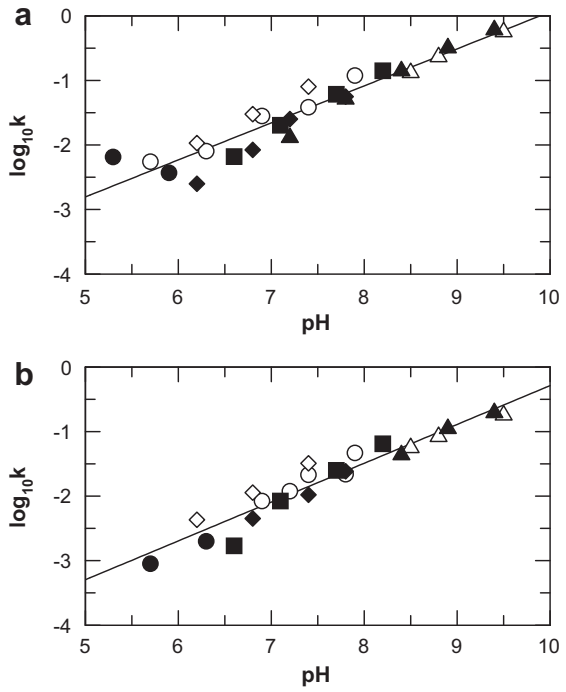


Figure 4. Relationship between rate constant (k) and pH on LNB and GNB decompositions. The following relationships on (a) LNB and (b) GNB decomposition were derived from linear plot with a correlation coefficient greater than 0.91 and 0.92, respectively: (a) $y = 0.57x - 5.7$ (x = [pH value]; $y = \log_{10}[k]$); (b) $y = 0.60x - 6.3$ (x = [pH value]; $y = \log_{10}[k]$). Buffers used are as follows: ○, 100 mM sodium phosphate buffer (pH 5.7, 6.3, 6.9, 7.4, and 7.9); ●, 100 mM citrate–NaOH buffer (pH 5.3, 5.7, and 6.3); ◇, 100 mM TAPS–NaOH buffer (pH 6.2, 6.8, and 7.4); ♦, 100 mM HEPES–NaOH buffer (pH 6.8, 7.4, and 7.8); △, 100 mM CHES–NaOH buffer (pH 8.5, 8.8, and 9.5); ▲, 100 mM glycine–NaOH buffer (pH 7.2, 7.8, 8.4, 8.9, and 9.4); and ■, 100 mM tricine–NaOH buffer (pH 6.6, 7.1, 7.7, and 8.2).

much milder conditions for the degradation used in this study made detailed analyses of the reaction easier.

The HexNAc parts of LNB and GNB are transferred to the furanoses of 3-deoxy-2,3-unsaturated compounds (**3** and **6**) and bicyclic 3,6-anhydro compounds (**1**–**2** and **4**–**5**), losing the configuration at the C-2 position of GlcNAc and GalNAc, respectively.

Judging from the compounds formed, the reaction is initiated from the linear form of HexNAc. Scheme 1 illustrates the cleavage of LNB. Initially, the enol form of the aldehyde is generated as the intermediate, and the C–O linkage at position 3 is cleaved to form the 2-deoxy-2,3-unsaturated sugar (the linear form of compound **3**) with the release of galactose through β-elimination. The hydroxy group at the C-6 position attacks the double bond at the C-3 position to form a furan ring of the enol compound. The stereochemical configuration at the C-2 position is lost through the formation of an aldehyde.[29,30] It should be noted that the reverse reaction, the formation of the bicyclic compound, is identical to the peeling reaction where compounds **1** and **2** are intramolecular 3-O-substituted 2-acetamido-2-deoxy-hexoses.

This mechanism explains the difference in the rate of decomposition of LNB, Galβ(1→3)2dGlc, and Galβ(1→3)Glc. Only Galβ(1→3)Glc can quench the enol intermediate by the formation of a carbonyl group at the C-2 position (ketose). Kainuma et al.[31] reported that glucose was transformed into fructose in 200 mM sodium phosphate buffer (pH 6.5–7.5) at 97 °C. We confirmed that the treatment of 50 mM glucose in 100 mM sodium phosphate buffer (pH 7.5) at 90 °C for 1 h caused transformation into fructose at a yield of 11%. This is also evidence for the formation of the enol structure under neutral conditions.

N-Acetylmuramic acid decomposed much slower than LNB did. This can be explained by the reaction mechanism in which the C–O linkage is stabilized from cleavage by the acidity of the lactic acid. It should be noted that *N*-acetylmuramic acid 6-phosphate hydrolase (or etherase), an enzyme that hydrolyzes *N*-acetylmuramic acid 6-phosphate into GlcNAc 6-phosphate and lactic acid, is considered to catalyze the reaction via a similar mechanism in which the elimination of lactate gives a 2,3-unsaturated aldehyde and is followed by the addition of water to give the saturated product.[32]

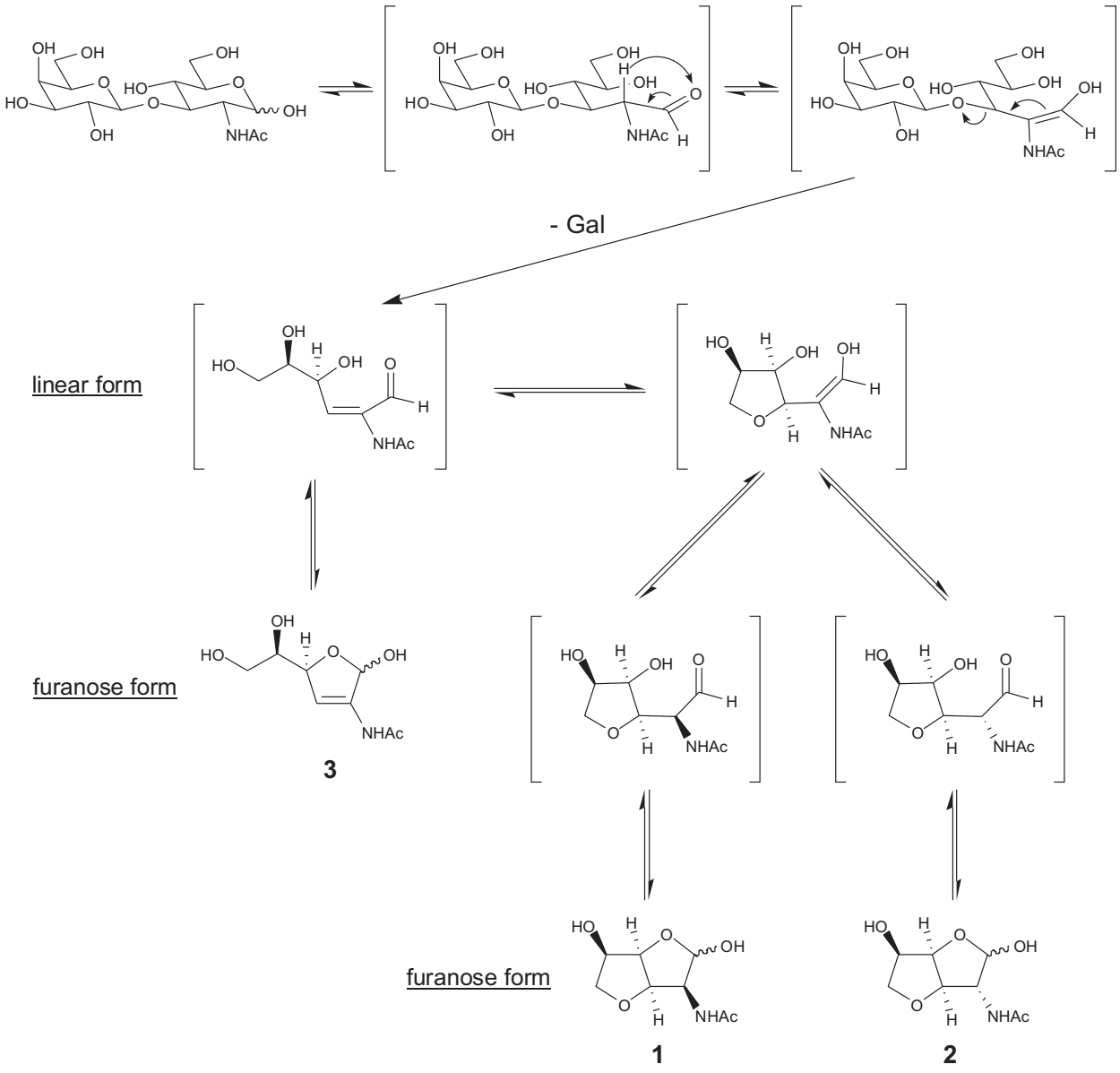
The reactions which transformed LNB and GNB into **1**–**3** and **4**–**6** in total yields of approximately 100% in the reaction mixture are economical and simple methods to give libraries of 2,3-unsaturated furanose and bicyclic furanoses. These compounds have effectively not been isolated from the peeling reaction under alkaline conditions because the intermediates are easily decomposed under the severe conditions.

The bicyclic furanoses **1** and **2** have been reported as intermediates in the synthesis of the furanodictines A and B, which show a neuronal differentiation activity in rat PC12 cells.[33] Ogata et al.[25] reported the transformation of GlcNAc into **1**–**3** by treating

Table 2
Thermal decomposition of various sugars under neutral conditions

Substrate	Structure	Rate constant (k) (min^{-1})	Half-life ($t_{1/2}$) (min)
Lacto-*N*-biose I (LNB)	Galβ(1→3)GlcNAc	0.085	8.1
Lacto-*N*-tetraose (LNT)	Galβ(1→3)GlcNAcβ(1→3)Galβ(1→4)Glc	N.D.[a]	–
Lewis[a] trisaccharide	Galβ(1→3)[Fucα(1→4)]GlcNAc	0.13	5.3
Galacto-*N*-biose (GNB)	Galβ(1→3)GalNAc	0.034	20
β-D-Galactopyranosyl-(1→3)-D-glucose	Galβ(1→3)Glc	0.0056	124
β-D-Galactopyranosyl-(1→3)-2-deoxy-D-glucose	Galβ(1→3)2dGlc	0.069[b]	10
Laminaribiose	Glcβ(1→3)Glc	0.0059	117
Laminaritriose	Glcβ(1→3)Glcβ(1→3)Glc	0.0081	86
β-Methyl laminaribioside	Glcβ(1→3)Glcβ-*O*-Me	N.D.[a]	–
Nigerose	Glcα(1→3)Glc	0.0052	133
Cellobiose	Glcβ(1→4)Glc	N.D.[a]	–
β-D-Glucopyranosyl-(1→4)-2-deoxy-D-glucose	Glcβ(1→4)2dGlc	N.D.[a,b]	–
Lactose	Galβ(1→4)Glc	N.D.[a]	–
N-Acetyllactosamine	Galβ(1→4)GlcNAc	N.D.[a]	–
N-Acetylmuramic acid	GlcNAc 3-*O*-lactyl ether	0.010[c]	72
N-Acetylglucosamine (GlcNAc)	GlcNAc	N.D.[a]	–
N-Acetylgalactosamine (GalNAc)	GalNAc	N.D.[a]	–

[a] N.D., not detected under the conditions.
[b] The rate constant calculated by quantifying Gal or Glc produced.
[c] The rate constant calculated by quantifying the total yield of compounds **1**–**3**.



Scheme 1. Proposed mechanism of thermal decomposition of LNB into compounds **1**–**3**.

100 mM GlcNAc in 400 mM borate buffer (pH 7.0) at 100 °C for 2 h at an approximately 80% conversion yield. The thermal decomposition of LNB presented here may be another practical method of synthesizing **1**–**3** by improving the procedures for the isolation of each compound, since it gives a high conversion yield and does not require excess amounts of ionic compounds. It should also be noted that LNB can be easily prepared in a one-pot enzymatic system from sucrose and GlcNAc.[11]

We conclude that 3-O-substituted reducing aldoses are generally decomposed under neutral conditions by heating.

4. Experimental

4.1. Sugars used

LNB and GNB were synthesized from sucrose using GNB/LNB phosphorylase as described previously.[11,12] Laminaribiose, laminaritriose, and methyl β-laminaribioside were synthesized using a laminaribiose phosphorylase.[34,35] β-D-Galactopyranosyl-(1→3)-D-glucose [Galβ-(1→3)-Glc] and β-D-galactopyranosyl-(1→3)-2-deoxy-D-glucose [Galβ-(1→3)-2dGlc] were prepared by the method given by Nakajima et al.[36] β-D-Glucopyranosyl-(1→4)-2-deoxy-D-glucose [Glcβ-(1→4)-2dGlc] was prepared as described previously.[37] Lacto-*N*-tetraose and *N*-acetylmuramic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Nigerose and cellobiose were purchased from Wako Pure Chemicals (Osaka, Japan). *N*-Acetyllactosamine was purchased from Seikagaku Kogyo (Tokyo, Japan). Lewis[a] trisaccharide {Galβ-(1→3)[Fucα-(1→4)]GlcNAc} was purchased from Dextra Laboratories (Reading, UK).

4.2. Decomposition of LNB and GNB

LNB or GNB was dissolved to approximately 50 mM with the following buffers: 100 mM citrate–NaOH buffer (pH 4.0, 4.5, 5.0, 5.5, and 6.0), 100 mM sodium phosphate buffer (pH 6.0, 6.5, 7.0, 7.5,

8.0, and 8.5), 100 mM HEPES–NaOH buffer (pH 7.0, 7.5, 8.0, and 8.5), 100 mM Tricine–NaOH buffer (pH 7.5, 8.0, 8.5, and 9.0), 100 mM TAPS–NaOH buffer (pH 8.0, 8.5, and 9.0), 100 mM glycine–NaOH buffer (pH 8.5, 9.0, 9.5, 10.0, and 10.5), and 100 mM CHES–NaOH buffer (pH 9.7, 10.0, and 10.5). The solutions (20 μL) were placed into a 200-μL thermal tube and subjected to heat treatment at 90 °C for 0–60 min using a GeneAmp® PCRSystem 9700 (Applied Biosystems, CA, USA). The products were periodically analyzed by TLC, HPLC, and colorimetric quantification employing carbohydrate enzymes (each of the measurement methods are described below).

4.3. Thin-layer chromatography (TLC)

TLC was performed on Silica Gel-60 F_{254} (E. Merck) using 80% MeCN in distilled water as a solvent. The products were visualized by heating the plate after dipping it in 20% H_2SO_4 in MeOH.

4.4. HPLC analysis

Both the substrate and the products of heat treatment were analyzed by HPLC systems (Shimadzu, Kyoto, Japan) with a Corona Charged Aerosol Detector (ESA Inc., Chelmsford, MA) using a Shodex Asahipak NH2P50-4E column (4.6 mm i.d. × 250 mm) with 75% MeCN, 90% MeCN (for separation of 3,6-anhydro compounds), and 70% MeCN (for LNT and Lewis^a trisaccharide) in distilled water as the solvent at a flow rate of 1.0 mL/min.

4.5. Spectrum analysis

Electrospray-ionization mass spectrometry (ESIMS) spectra of the purified decomposition products were recorded in the positive-ion mode on a Bruker APEX II 70e Fourier-transform ion-cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA, USA). One-dimensional (^{1}H and ^{13}C) and two-dimensional [double-quantum-filtered correlation spectroscopy (DQF-COSY), NOESY, heteronuclear single quantum coherence (HSQC), and HMBC] NMR spectra of the purified products were recorded with a Bruker Avance 800 or Avance 500 spectrometer (Bruker Biospin, Rheinstetten, Germany) in D_2O with *t*-BuOH as the internal standard.

4.6. Enzymatic quantification of LNB, GNB, and Gal

The amounts of LNB, GNB, and Gal were monitored by quantifying the produced Gal 1-P[27] from Gal by galactokinase (GalK) and from LNB and GNB by GNB/LNB phosphorylase.[38] Working reagent A for the Gal 1-P assay was prepared from the following mixture: 0.5 IU/mL of UDP-glucose hexose-1-phosphate uridylyltransferase, 2.5 IU/mL of phosphoglucomutase, 2.0 IU/mL of glucose 6-phosphate dehydrogenase, 20 μM α-D-glucose 1,6-bisphosphate, 2.0 mM thio-NAD^+, and 2.0 mM UDP-D-glucose in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The determination of LNB and GNB was performed by the preparation of a 100-μL sample in 96-well microtiter plate. The solution was added to 50 μL of reagent A and reagent B containing 2 IU/mL of GNB/LNB phosphorylase in 100 mM sodium phosphate buffer (pH 7.5) and incubated at 37 °C for 1 h. It is important that the reaction solution containing reagent A and B be prepared daily because magnesium phosphate precipitates upon storage. The concentration of Gal 1-P produced was quantified by measuring absorbance at 400 nm derived from thio-NADH (ε_{398} = 11,700 cm^{-1} M^{-1}). The determination of Gal was performed via the same operation by replacing the reagent B with reagent C containing 2 IU/mL mutarotase, 4 IU/mL GalK, and 2 mM ATP in 50 mM Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$. The LNB/GNB or Gal calibration curve was linear in the concentration range from 0 to 0.25 mM.

4.7. Determination of kinetic parameters

Rate constant (k) of the decomposition was calculated by regressing data with Eq. 1 or 2 using Grafit version 4 (Erithacus Software, Middlesex, United Kingdom):

$$[\text{Substrate}]_t = [\text{Substrate}]_0 \cdot \exp(-k \cdot t) \quad (1)$$

$$[\text{Product}]_t = [\text{Substrate}]_0 \cdot [1 - \exp(-k \cdot t)] \quad (2)$$

The apparent energy of activation (E_a) was obtained by regressing data with the Arrhenius Eq. 3 using Grafit version 4:

$$\ln k = \ln A - E_a/RT \ (A: \text{ frequency factor}) \quad (3)$$

4.8. Influence of temperature, pH, and treatment times on decomposition of LNB and GNB

LNB and GNB were treated under various conditions of pH (the previously mentioned buffers at pH 4.0–10.5 were used), at temperatures of 25–95 °C and the treatment time was between 0 and 60 min, and the products were detected by TLC. In addition, the concentration of the LNB or GNB reduced with the heat treatment was sequentially measured using Gal 1-P assay.[27]

4.9. Heat treatment of various sugars

The sugars were dissolved to approximately 50 mM with 100 mM sodium phosphate buffer (pH 7.5). The pH value in the solution of MurNAc was readjusted to 7.5 with NaOH solution. The mixture was incubated at 90 °C for 0–180 min, and the concentrations of the sugars and the products were sequentially analyzed by HPLC.

4.10. Isolation of LNB decomposition products (1–3)

LNB (768 mg, 2.00 mmol) was dissolved in 40 mL of 100 mM sodium phosphate buffer (pH 7.5) and incubated at 90 °C for 2 h. The product was desalted with Amberlite MB-3 (Organo) and concentrated. The solution was purified using a Shodex Asahipak ODP-50 column (10 mm i.d. × 250 mm, distilled water), and the products were lyophilized. A significant amount of a new compound that was not detected in the reaction mixture and which showed a lower R_f value than **1**–**3** on TLC with exact mass corresponding to [2HexNAc−4H_2O] {ESIMS: *m/z* 393.12 $[M+Na]^+$ (calcd for $C_{16}H_{22}N_2NaO_8$, 393.13)} was formed during this stage causing the drastic decreases in the isolation yields of **1**–**3**. The residue was dissolved in acetone–water, and the solution was further purified by Shodex Asahipak NH2P50-4E column chromatography (4.6 mm i.d.× 250 mm; 37:3 acetone–water) to give the LNB decomposition products **1** (9.6 mg) and **2** (13 mg), and product **3** (24 mg).

4.11. Isolation of GNB decomposition products (4–6)

GNB (768 mg, 2.00 mmol) was dissolved in 40 mL of 100 mM sodium phosphate buffer (pH 7.5) and incubated at 90 °C for 3 h. The purification of the decomposition products was performed using the same operation as for the purification of the LNB decomposition products, to give the GNB decomposition products **4** (26 mg) and **5** (37 mg), and product **6** (11 mg). A significant amount of a new compound showing lower R_f value than **4**–**6** on TLC formed during the first stage of the purification, causing the drastic decreases in the isolation yields of **4**–**6**.

4.12. 2-Acetamido-3,6-anhydro-2-deoxy-D-mannofuranose (1)

Compound **1** was obtained in a yield of 2.4% as a colorless powder, and included two anomers (α:β = 1:1.5) in the mixture; ^{1}H

(D_2O, 500 MHz) α anomer: δ 5.51 (d, 1H, $J_{1,2}$ 5.5 Hz, H-1), 4.68 (t, 1H, $J_{3,4}$ 4.8 Hz, $J_{4,5}$ 4.8 Hz, H-4), 4.63 (dd, 1H, $J_{2,3}$ 5.5 Hz, H-3), 4.37–4.40 (m, H-5), 4.34 (t, 1H, H-2), 3.93 (dd, 1H, $J_{5,6}$ 6.8 Hz, $J_{6,6'}$ 8.4 Hz, H-6), 3.89 (t, 1H, $J_{5,6'}$ 8.4 Hz, H-6′), 2.06 (s, 3H, CH_3CONH–); β anomer: δ 5.28 (d, 1H, $J_{1,2}$ 6.3 Hz, H-1), 4.79 (H-4), 4.61 (dd, 1H, $J_{2,3}$ 5.6 Hz, $J_{3,4}$ 4.8 Hz, H-3), 4.37–4.40 (m, H-5), 4.23 (dd, 1H, H-2), 4.01 (dd, 1H, $J_{5,6}$ 6.5 Hz, $J_{6,6'}$ 8.9 Hz, H-6), 3.53 (dd, 1H, $J_{5,6'}$ 8.3 Hz, H-6′), 2.04 (s, 3H, CH_3CONH–); ^{13}C NMR (D_2O, 125 MHz) α anomer: δ 175.9 (CH_3CONH–), 97.2 (C-1), 83.6 (C-4), 81.9 (C-3), 72.9 (C-5), 72.4 (C-6), 56.4 (C-2), 23.3 (CH_3CONH–); β anomer: δ 176.2 (CH_3CONH–), 102.5 (C-1), 82.4 (C-4), 81.6 (C-3), 73.2 (C-5), 72.8 (C-6), 60.7 (C-2), 23.4 (CH_3CONH–); ESIMS: *m/z* 226.07 $[M+Na]^+$ (calcd for $C_8H_{13}NNaO_5$, 226.07).

4.13. 2-Acetamido-3,6-anhydro-2-deoxy-D-glucofuranose (2)

Compound **2** was obtained in a yield of 3.2% as a colorless powder, and included two anomers (α:β = 1:0.4) in the mixture; ^{1}H (D_2O, 800 MHz) α anomer: δ 5.59 (d, 1H, $J_{1,2}$ 4.4 Hz, H-1), 4.73 (t, 1H, $J_{4,5}$ 5.4 Hz, H-4), 4.63 (dd, 1H, $J_{2,3}$ 4.4 Hz, $J_{3,4}$ 5.7 Hz, H-3), 4.28 (ddd, 1H, $J_{5,6}$ 5.9 Hz, $J_{5,6'}$ 7.5 Hz, H-5), 4.24 (t, 1H, H-2), 3.97 (dd, 1H, $J_{6,6'}$ 9.3 Hz, H-6), 3.63 (dd, 1H, H-6′), 2.03 (s, 3H, CH_3CONH–); β anomer: δ 5.41 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.76 (H-4), 4.49 (dd, 1H, $J_{2,3}$ 1.3 Hz, $J_{3,4}$ 4.7 Hz, H-3), 4.33–4.39 (m, H-5), 4.15 (br s, 1H, H-2), 3.93 (dd, 1H, $J_{5,6}$ 6.5 Hz, $J_{6,6'}$ 7.5 Hz, H-6), 3.86 (t, 1H, $J_{5,6'}$ 7.5 Hz, H-6′), 1.99 (s, 3H, CH_3CONH–); ^{13}C NMR (D_2O, 200 MHz) α anomer: δ 176.0 (CH_3CONH–), 99.3 (C-1), 87.4 (C-3), 80.6 (C-4), 71.9 (C-5, C-6), 60.4 (C-2), 23.4 (CH_3CONH–); β anomer: δ 175.7 (CH_3CONH–), 104.0 (C-1), 87.4 (C-3), 84.5 (C-4), 72.7 (C-6), 72.4 (C-5), 63.5 (C-2), 23.4 (CH_3CONH–); ESIMS: *m/z* 226.07 $[M+Na]^+$ (calcd for $C_8H_{13}NNaO_5$, 226.07).

4.14. 2-Acetamido-2,3-dideoxy-D-*erythro*-hex-2-enofuranose (3)

Compound **3**, called Chromogen I, was obtained in a yield of 6.0% as a colorless oil, and included two anomers (α:β = 1:0.6) in the mixture; ^{1}H NMR (D_2O, 500 MHz) α anomer: δ 6.14 (br s, 1H, H-3), 6.01 (dd, 1H, $J_{1,3}$ 0.9 Hz, $J_{1,4}$ 4.0 Hz, H-1), 5.03 (dt, 1H, $J_{3,4}$ 1.6 Hz, $J_{4,5}$ 4.0 Hz, H-4), 3.80 (dt, 1H, $J_{5,6}$ 4.0 Hz, H-5), 3.69 (dd, 1H, $J_{6,6'}$ 11.9 Hz, H-6), 3.55 (dd, 1H, $J_{5,6'}$ 7.2 Hz, H-6′), 2.10 (s, 3H, CH_3CONH–); β anomer: δ 6.20 (dd, 1H, $J_{3,4}$ = 1.6 Hz, H-3), 5.97 (d, 1H, $J_{1,3}$ = 1.0 Hz, H-1β), 4.81 (H-4), 3.75–3.71 (m, 2H, H-5 and H-6), 3.59–3.64 (m, 1H, H-6′), 2.10 (s, 3H CH_3CONH–); ^{13}C NMR (D_2O, 125 MHz): α anomer: δ 176.5 (CH_3CONH–), 137.3 (C-2), 112.2 (C-3), 102.4 (C-1), 87.9 (C-4), 76.4 (C-5), 65.4 (C-6), 25.6 (CH_3CONH–); β anomer: δ 176.5 (CH_3CONH–), 136.8 (C-2), 113.1 (C-3), 102.3 (C-1), 87.5 (C-4), 76.8 (C-5), 65.5 (C-6), 25.6 (CH_3CONH–); ESIMS: *m/z* 226.07 $[M+Na]^+$ (calcd for $C_8H_{13}NNaO_5$, 226.07).

4.15. 2-Acetamido-3,6-anhydro-2-deoxy-D-gulofuranose (4)

Compound **4** was obtained in a yield of 6.3% as a colorless powder, and included two anomers (α:β = 1:0.9) in the mixture; ^{1}H (D_2O, 500 MHz) α anomer: δ 5.22 (d, 1H, $J_{1,2}$ 5.6 Hz, H-1), 4.73–4.76 (H-3, H-4), 4.37–4.40 (m, 1H, H-5), 4.23 (t, 1H, $J_{2,3}$ 5.6 Hz, H-2), 3.90–3.94 (m, 1H, $J_{5,6}$ 6.8 Hz, H-6), 3.89 (dd, 1H, $J_{5,6'}$ 3.0 Hz, $J_{6,6'}$ 10.5 Hz, H-6′), 2.06 (s, 3H, CH_3CONH–); β anomer: δ 5.43 (d, 1H, $J_{1,2}$ 5.6 Hz, H-1), 4.73–4.76 (H-3), 4.59 (d, 1H, $J_{3,4}$ 4.0 Hz), 4.40 (d, 1H, $J_{5,6}$ 3.6 Hz, H-5), 4.34 (dd, 1H, $J_{6,6'}$ 10.1 Hz, H-6), 4.31 (t, 1H, $J_{2,3}$ 5.6 Hz, H-2), 3.83 (d, 1H, H-6′), 2.04 (s, 3H, CH_3CONH–); ^{13}C NMR (D_2O, 125 MHz) α anomer: δ 176.1 (CH_3CONH–), 102.2 (C-1), 87.5 (C-4), 81.7 (C-3), 76.7 (C-5), 76.1 (C-6), 60.7 (C-2), 23.4 (CH_3CONH–); β anomer: δ 175.9 (CH_3CONH–), 97.2 (C-1), 89.0 (C-4), 81.6 (C-3), 77.6 (C-5), 75.6 (C-6), 56.3 (C-2), 23.3 (CH_3CONH–); ESIMS: *m/z* 226.07 $[M+Na]^+$ (calcd for $C_8H_{13}NNaO_5$, 226.07).

4.16. 2-Acetamido-3,6-anhydro-2-deoxy-D-idofuranose (5)

Compound **5** was obtained in a yield of 9.1% as a colorless oil and included two anomers (α:β = 1:2) in the mixture; ^{1}H (D_2O, 800 MHz) α anomer: δ 5.31 (d, 1H, $J_{1,2}$ 1.8 Hz, H-1), 4.64–4.66 (m, H-4), 4.62 (dd, 1H, $J_{2,3}$ 1.0 Hz, $J_{3,4}$ 4.6 Hz, H-3), 4.42 (m, 1H, H-5), 4.27 (m, 1H, $J_{5,6}$ 3.6 Hz, $J_{6,6'}$ 10.2 Hz, H-6), 4.13 (br s, 1H, H-2), 3.83–3.84 (m, 1H, H-6′), 2.00 (s, 3H, CH_3CONH–); β anomer: δ 5.50 (d, 1H, $J_{1,2}$ 4.4 Hz, H-1), 4.75–4.76 (H-3), 4.64–4.66 (m, H-4), 4.32 (dd, 1H, *J* 1.5 Hz, 0.7 Hz, H-5), 4.17 (t, 1H, $J_{2,3}$ 4.4 Hz, H-2), 3.9 (m, 2H, H-6 and H-6′), 2.03 (s, 3H, CH_3CONH–); ^{13}C NMR (D_2O, 200 MHz) α anomer: δ 175.7 (CH_3CONH–), 103.8 (C-1), 89.8 (C-4), 87.0 (C-3), 76.8 (C-5), 75.5 (C-6), 63.1 (C-2), 23.5 (CH_3CONH–); β anomer: δ 176.1 (CH_3CONH–), 98.3 (C-1), 87.1 (C-3), 86.6 (C-4), 76.0 (C-5), 74.1 (C-6), 59.6 (C-2), 23.4 (CH_3CONH–); ESIMS: *m/z* 226.07 $[M+Na]^+$ (calcd for $C_8H_{13}NNaO_5$, 226.07).

4.17. 2-Acetamido-2,3-dideoxy-D-*threo*-hex-2-enofuranose (6)

Compound **6** was obtained in a yield of 2.8% as a colorless powder, and included two anomers (α:β = 1:0.6) in the mixture; ^{1}H NMR (D_2O, 800 MHz): α anomer: δ 6.12 (dd, 1H, $J_{3,4}$ 1.8 Hz, H-3), 5.96 (t, 1H, $J_{1,3}$ 1.0 Hz, $J_{1,4}$ 1.0 Hz, H-1), 4.83 (H-4), 3.68–3.73 (m, 2H, H-5 and H-6), 3.59–3.62 (m, 1H, H-6′), 2.10 (s, 3H CH_3CONH–); β anomer: δ 6.11 (dd, 1H, $J_{3,4}$ 1.6 Hz, H-3), 6.01 (dd, 1H, $J_{1,3}$ 1.0 Hz, $J_{1,4}$ 4.0 Hz, H-1), 5.04 (ddd, 1H, $J_{4,5}$ 3.2 Hz, H-4), 3.71 (ddd, 1H, $J_{5,6}$ 4.3 Hz, H-5), 3.68 (dd, 1H, $J_{6,6'}$ 11.6 Hz, H-6), 3.62 (dd, 1H, $J_{5,6'}$ 7.5 Hz, H-6′), 2.10 (s, 3H, CH_3CONH–); ^{13}C NMR (D_2O, 200 MHz); α anomer: 175.2 (CH_3CONH–), 135.5 (C-2), 111.6 (C-3), 100.9 (C-1), 86.1 (C-4), 75.1 (C-5), 64.3 (C-6), 24.2 (CH_3CONH–); β anomer: 175.1 (CH_3CONH–), 135.5 (C-2), 111.9 (C-3), 101.4 (C-1), 86.4 (C-4), 74.4 (C-5), 65.4 (C-6), 24.2 (CH_3CONH–); ESIMS: *m/z* 226.07 $[M+Na]^+$ (calcd for $C_8H_{13}NNaO_5$, 226.07).

Acknowledgments

We thank the staffs of Instrumental Analysis Center for Food Chemistry of National Food Research Institute for recording NMR and MS spectra. This work was supported in part by a grant from the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan.

References

1. Haeuw-Fievre, S.; Wieruszeski, J. M.; Plancke, Y.; Michalski, J. C.; Montreuil, J.; Strecker, G. *Eur. J. Biochem.* **1993**, *215*, 361–371.
2. Urashima, T.; Saito, T.; Nakamura, T.; Messer, M. *Glycoconjugate J.* **2001**, *18*, 357–371.
3. Kuhn, R.; Gauhe, A.; Baer, H. H. *Chem. Ber.* **1954**, *87*, 289–300.
4. Cote, R. H.; Morgan, W. T. J. *Nature* **1956**, *178*, 1171–1172.
5. Schiffman, G.; Kabat, E. A.; Leskowitz, S. *J. Am. Chem. Soc.* **1962**, *84*, 73–77.
6. Springer, G. F. *Science* **1984**, *224*, 1198–1206.
7. Bhavanandan, V. P.; Zhu, Q.; Yamakami, K.; Dilulio, N. A.; Nair, S.; Capon, C.; Lemoine, J.; Fournet, B. *Glycoconjugate J.* **1998**, *15*, 37–49.
8. Berger, E. G. *Biochim. Biophys. Acta* **1999**, *1455*, 255–268.
9. Hanisch, F. A. *Biol. Chem.* **2001**, *382*, 143–149.
10. Nishimoto, M.; Kitaoka, M. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 2101–2104.
11. Nishimoto, M.; Kitaoka, M. *Carbohydr. Res.* **2009**, *344*, 2573–2576.
12. Jorpes, E. *Biochem. Zool.* **1929**, *204*, 354–360.
13. Anderson, B.; Meyer, K.; Hoffman, P. *Biochim. Biophys. Acta* **1963**, *74*, 309–311.
14. Carlson, D. M. *J. Biol. Chem.* **1968**, *243*, 616–626.
15. Lloyd, K. O.; Kabat, E. A.; Licerio, E. *Biochemistry* **1968**, *7*, 2976–2990.
16. Kabat, E. A.; Bassett, E. W.; Pryzwans, K.; Lloyd, K. O.; Kaplan, M. E.; Layug, E. J. *Biochemistry* **1965**, *4*, 1632–1638.
17. Lloyd, K. O.; Kabat, E. A. *Carbohydr. Res.* **1967**, *4*, 165–177.
18. Lloyd, K. O.; Kabat, E. A. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *61*, 1470–1477.
19. Lloyd, K. O.; Kabat, E. A. *Carbohydr. Res.* **1969**, *9*, 41–48.
20. Morgan, W. T. J.; Elson, L. A. *Biochem. J.* **1934**, *28*, 988–995.

21. Kuhn, R.; Kruger, G. *Chem. Ber.* **1957**, *90*, 264–277.
22. Kuhn, R.; Gauhe, A.; Baer, H. H. *Chem. Ber.* **1956**, *89*, 1027–1033.
23. Aminoff, D.; Morgan, W. T. J.; Watkins, W. M. *Biochem. J.* **1952**, *51*, 379–389.
24. Jeanloz, R. W.; Tremege, M. *Fed. Proc.* **1956**, *15*, 282.
25. Ogata, M.; Hattori, T.; Takeuchi, R.; Usui, T. *Carbohydr. Res.* **2010**, *345*, 230–234.
26. Beau, J. M.; Rollin, P.; Sinaÿ, P. *Carbohydr. Res.* **1977**, *53*, 187–195.
27. Nihira, T.; Nakajima, M.; Inoue, K.; Nishimoto, M.; Kitaoka, M. *Anal. Biochem.* **2007**, *371*, 259–261.
28. Rege, V. P.; Morgan, W. T. J.; Painter, T. J.; Watkins, W. M. *Nature* **1964**, *204*, 740–742.
29. Coxon, B.; Hough, L. *J. Chem. Soc.* **1961**, 1577–1579.
30. Angyal, S. J. *Top. Curr. Chem.* **2001**, *215*, 1–14.
31. Kainuma, K.; Tadokoro, K.; Suzuki, S. *Nougeikagakukaishi* **1968**, *42*, 173–177 (in Japanese).
32. Hadi, T.; Dahl, U.; Mayer, C.; Tanner, M. E. *Biochemistry* **2008**, *47*, 11547–11558.
33. Kikuchi, H.; Saito, Y.; Komiya, J.; Takaya, Y.; Honma, S.; Nakahata, N.; Ito, A.; Oshima, Y. *J. Org. Chem.* **2001**, *66*, 6982–6987.
34. Kitaoka, M.; Sasaki, T.; Taniguchi, H. *Denpun Kagaku* **1993**, *40*, 311–314.
35. Kitaoka, M.; Sasaki, T.; Taniguchi, H. *Arch. Biochem. Biophys.* **1993**, *304*, 508–514.
36. Nakajima, M.; Nishimoto, M.; Kitaoka, M. *J. Biol. Chem.* **2009**, *284*, 19220–19227.
37. Tariq, M. A.; Hayashi, K.; Tokuyasu, K.; Nagata, T. *Carbohydr. Res.* **1995**, *275*, 67–72.
38. Kitaoka, M.; Tian, J.; Nishimoto, M. *Appl. Environ. Microbiol.* **2005**, *71*, 3158–3162.