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Biosynthesis of lactosamine in bovine mammary gland

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Abstract

Lactosamine (β-D-Gal*p*-(1→4)-D-GlcN) was isolated from bovine milk sampled after intravenous infusion of glucosamine through the jugular vein of a lactating cow. The chemical structure was established by 2D NMR spectroscopy and electrospray ionisation mass spectrometry (ESIMS). Selected ion monitoring liquid chromatography-mass spectrometry (SIMLC-MS) of the perbenzoylated carbohydrate fraction showed the presence of the novel disaccharide in the milk sample after infusion, but not in the control bovine milk sample. The results showed the uptake of glucosamine in bovine mammary gland, and also indicated that a part of glucosamine was metabolised to the product lactosamine.

Keywords: Lactosamine; Glucosamine; Mammary gland; Glucose transporters; Uridine nucleotide cycle

1. Introduction

Mammalian milk generally contains lactose as a main soluble oligosaccharide. The mechanism of lactose biosynthesis is well established as the reaction of a 'uridine nucleotide cycle' that functionally links the cytosol and Golgi lumen compartments in the mammary gland of lactating mammals.[1,2] Lactose synthase (EC 2.4.1.22) located on the luminal face of the Golgi apparatus catalyses the transfer of D-galactose from UDP-galactose to the C-4 position of D-glucose transported from capillary blood in the final step of the cycle. Subsequently, lactose is secreted into milk by way of the Golgi system emptying into the lumen of the mammary gland.[3] Lactose synthase is composed of both UDP-galactose: β-*N*-acetyl-D-glucosamine β-(1→4)-galactosyltransferase (EC 2.4.1.38, β4Gal-T1), which itself catalyses the incorporation of D-galactose in β-(1→4) linkage to *N*-acetylglucosamine residues at the nonreducing termini of glycoproteins and the milk protein, α-lactalbumin which acts as a protein modifier of the galactosyltransferase and lowers the apparent K_m of D-glucose so that it becomes an effective substrate.[4] The latter is induced specifically in the mammary gland only during late pregnancy and lactation.[5,6]

In our previous study, the biosynthesis of β-(1→4)-galactopyranosyl-xylopyranoside in bovine milk was demonstrated by intravenous infusion of D-xylose as an exogenous acceptor substrate for lactose synthase in the mammary gland of lactating cow. The transportation of D-xylose via the glucose transporter (GLUT1) located on the mammary epithelial cell plasma membrane was followed by its penetration through Golgi membrane and the temporary utilisation of xylose in the uridine nucleotide cycle discussed.[7]

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Based on the possibility of the recognition by the transporter[8,9] and penetration through the Golgi membrane,[10–12] other possible acceptors for lactose synthase have been searched for. In this paper, we demonstrate the formation of glucosamine-containing disaccharide in the bovine mammary gland by infusion of D-glucosamine as an acceptor substrate for the enzyme.

2. Results and discussion

Preparation of bovine milk sample.—Ringer's solution containing GlcN hydrochloride (500 g) was infused through the jugular vein of a Holstein cow, and a total of 1750 mL of bovine milk was obtained 100 min after infusion. No unusual behaviour of the cow was observed during infusion or after milking. The carbohydrate fraction was obtained by our previous method.[7]

Chemical analysis of the carbohydrate fraction.—Selected ion monitoring liquid chromatography-mass spectrometry (SIMLC-MS) of the perbenzoylated carbohydrate fraction shows the molecular ions of $[M + Na]^+$ at m/z 1196 and 1197, corresponding to perbenzoylated lactosamine (retention time 9:56 and 10:53) and lactose (retention time 17:44 and 19:07), respectively (Fig. 1). The former ion

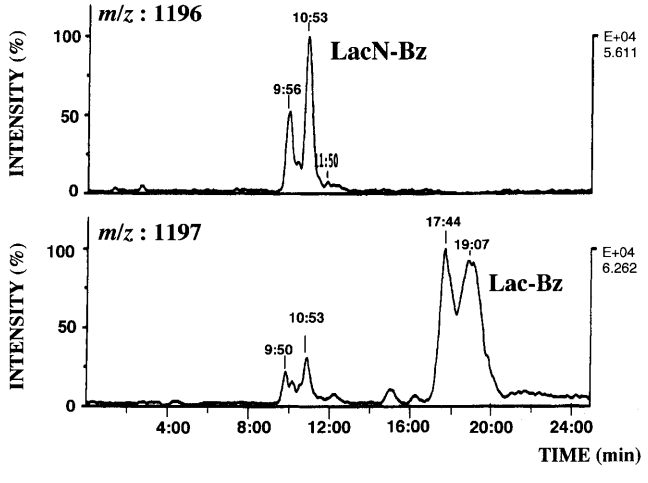

Fig. 1. Ion-monitored chromatograms of the perbenzoylated derivatives of lactosamine (LacN-Bz) and lactose (Lac-Bz) from the carbohydrates contained in bovine milk taken after infusion of glucosamine. Condition: column; C-18, mobile phase; $MeCN–H_2O = 80/20$, flow rate; 0.5 mL/min, temp; 40 °C, ionisation; positive ion ESI.

was not detected on analysis of the control milk sample.

Isolation and characterisation of the product fraction.—The prepared carbohydrate fraction gave two main spots (Spot A, R_f 0.50; lactose, 0.43) and two minor spots (Glc and Gal, R_f 0.58; Spot B, R_f 0.30) on TLC by treatment with diphenylamine–aniline–phosphoric acid reagent (Fig. 2). Spot A, which did not appear on analysis of the control milk, co-migrated with D-glucosamine hydrochloride. Spot B, which appeared on analysis of both the control and glucosamine-infused milk, did not show a red colour on treatment with ninhydrin reagent. After fractionation of the carbohydrates by silica gel column chromatography, two ninhydrin-positive fractions, Fraction A corresponding to Spot A, and Fraction C which appeared above Spot B on TLC (R_f 0.34 in Fig. 2) were obtained. Fraction A and C were further purified by cation exchange column chromatography to separate the positively charged amino sugars from uncharged neutral carbohydrates remaining in each fraction.

The ^1H NMR spectrum of the purified Fraction A (670 mg/L milk) was identical with that of the authentic glucosamine hydrochloride.

The ^1H NMR spectrum of the purified Fraction C (7 mg/L milk) showed three doublets in the anomeric region (4.4–5.4 ppm), corresponding to α (5.380 ppm) and β (4.799 ppm) anomers of the reducing end unit and β anomeric proton (4.451 and 4.445 ppm) at the linkage position, showing that the compound is a reducing type disaccharide (Fig. 3(A)). The coupling constant (3.3 Hz) at 3.65 and 3.92 ppm supported the presence of Gal residue due to axial–equatorial interaction between H-3′ and H-4′ protons, and all of the other Gal protons were assigned by use of ^1H-^1H COSY experiment. The ^1H chemical shifts of the Gal residue coincided approximately with those of the Gal residue of β-(1 → 4)-galactopyranosyl–xylopyranose obtained previously. A couple of the signals corresponding to Gal H-1′, 2′ and 3′ appeared owing to a slight difference in the chemical shifts of the α and β anomers. ESIMS of the purified Fraction C (Fig. 3(B)) gave a strong

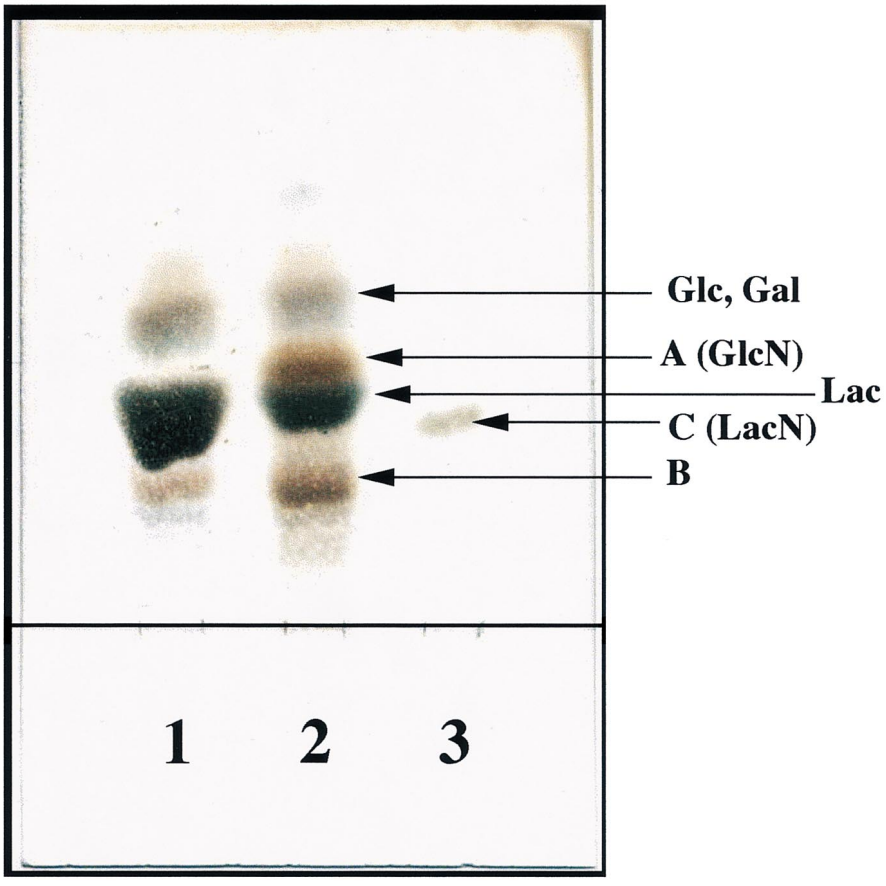

Fig. 2. TLC characterisation of carbohydrates in the milk samples, visualised by spraying 20 g/L diphenylamine, 20 g/L aniline, 220 g/L phosphoric acid in acetone and heating at 120 °C for 10 min. Lane 1: control sample milked just before starting the infusion, lane 2: milk sample milked 100 min after infusion, lane 3: lactosamine (Fraction C) separated by silica gel, and cation exchange column chromatography.

protonated molecular ion at m/z 342.0, followed by two molecular ions of $[M + Na]^+$ and $[M + K]^+$ at m/z 364.0 and 380.0, which is consistent with the molecular formula of $C_{12}H_{23}NO_{10}$ (m/z 341). Thus, it was deduced that another monosaccharide component (reducing-end unit) is hexosamine. The presence of the free amino group was also supported by the heavy ninhydrin positive reaction on TLC described above. Although the ^{13}C chemical shift values of the Gal residue of Fraction C matched those of lactose well, C-2 of the reducing-end unit was highly shielded (data not shown). The significant low-field shift of C-2 and smaller shift of C-1 and C-3 compared with the value for lactose is similar to the effect on the ^{13}C chemical shift caused by N-acetylation at C-2 of reducing Glc residue of lactose.[13] However, the ^1H NMR spectrum of Fraction C did not show any NAc signals. In addition, the hexosamine H-2 signals at 3.21 ppm (α anomer) and 2.87 ppm (β anomer) of Fraction C were highly shielded compared with the Glc H-2 signals of lactose (3.57 ppm for α anomer, 3.28 ppm for β anomer), in contrast to GlcNAc H-2 signals which are highly deshielded compared with Glc H-2 ($\delta\Delta \approx 0.4$), showing the presence of GlcN residue as the other monosaccharide component. The chemical shifts of the GlcN residue protons were assigned on the basis of ^1H-^1H COSY experiment. The coupling constant of βGlcN H-3 resonance which overlapped with Gal H-5′ resonance was established by decoupling difference experiment with irradiation at βGlcN H-2 (2.870 ppm). The GlcN H-4 signals of α and β anomers appeared as a triplet signal at 3.695 ppm ($J = 8.8$ Hz) on the ^1H NMR spectrum, since the chemical shifts of both anomers determined by NOE difference experiment with irradiation at GlcN H-2 coincided approxi-

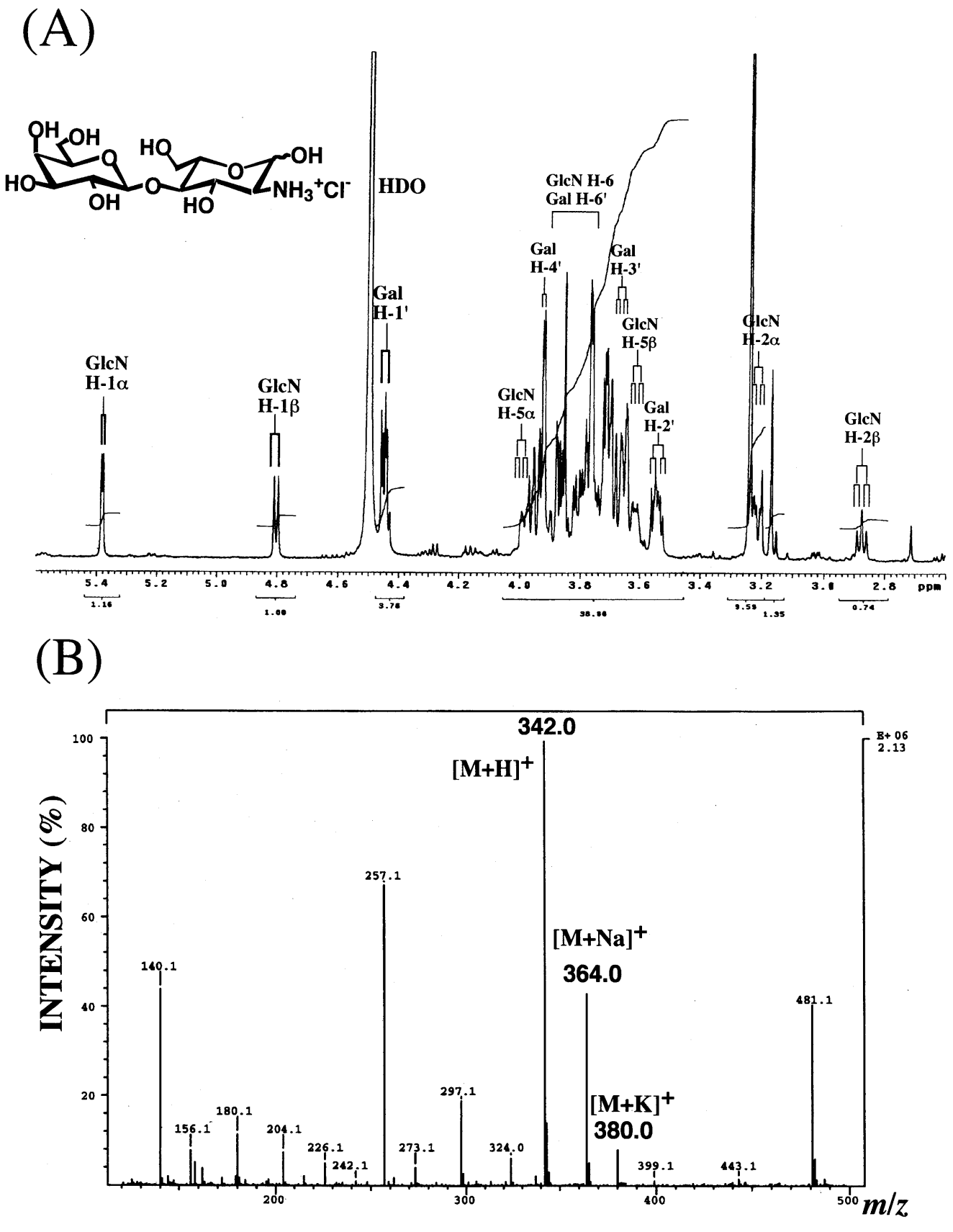

Fig. 3. (A) 600 MHz ^{1}H NMR spectrum of Fraction C (lactosamine hydrochloride) recorded in D_2O at 50 °C (*H*DO, $\delta = 4.50$). (B) Positive ion ESIMS of Fraction C.

mately with each other. The GlcN H-4 resonances of Fraction C were most highly deshielded ($\delta\Delta \approx 0.18$ ppm) of all the GlcN residue protons compared with those of authentic GlcN hydrochloride, indicating that the GlcN C-4 position is substituted by Gal. NOE difference experiments were used to further confirm the linkage and conformation. Irradiation at Gal H-1′ of Fraction C displayed a strong enhancement of GlcN H-4 resonance, and for other protons Gal H-3′ and Gal H-5′, whereas irradiation at GlcN H-1β only displayed enhancement of βGlcN H-3 and βGlcN H-5 resonances. Thus the cis-periplanar position of Gal H-1′ and GlcN H-4 was established. The linkage was also unambiguously established by observation of interglycosidic correlations in the HMBC spectrum. The evidence of the Galβ-(1→4)-GlcN linkage in Fraction C is demonstrated by cross-peaks correlating βGal H-1′ with GlcN C-4 and the corresponding βGal C-1′ with the GlcN H-4 resonance.

All of these data (Table 1) allowed us to assign the structure of Fraction C as lactosamine hydrochloride.

Possible mechanism of the lactosamine occurrence.—The recovery of the infused glucosamine from the bovine milk sample shows that the substrate, as well as D-xylose, was transported from capillary blood across the mammary epithelial cell plasma membrane, mediated by the facilitative glucose transporter 1(GLUT1) which is predominantly expressed in the lactating mammary gland.[14,15] The result may not be unexpected since the conformation of D-glucosamine exactly resembles that of D-glucose at C-1 and C-3 position, the oxygen atoms at the sites proposed to act as H bond acceptors for the active centre of the outward facing binding site of GLUT1,[16] supported by the fact that glucosamine inhibited the uptake of 2-deoxyglucose into rat mammary acini.[8] As glucosamine was not detected on the TLC analysis of the control milk sample, and the presence of glucosamine in bovine milk has not been reported, it is confirmed that the monosaccharide identified in the bovine milk after infusion did not occur endogenously.

D-Glucosamine is known as an acceptor substrate for both β4Gal-T1[17] and β-galactosidase.[18] In our previous study, lactose synthase-catalysed galactosylation of the exogenous D-xylose was proved by isolating both β-(1→4)- and β-(1↔1)-β-isomers,[7] the latter of which can not be formed by β-galactosidase catalysis.[19] Therefore, the occurrence of lactosamine in bovine milk should be ascribed to lactose synthase-catalysis, providing that glucosamine is taken into the same pathway as that of xylose. In support of this, no

Table 1
NMR data for lactosamine hydrochloride (D_2O, 50 °C)

Quantity	Residue	Designated proton/carbon and value						
		1	2	3	4	5	6	6′
δ (^{1}H) [a]	β-Gal	4.451, 4.445	3.545, 3.537	3.653, 3.650	3.923	3.713	3.791	3.753
	α-GlcN	5.380	3.207	3.951	3.695	3.984	3.888	3.853
	β-GlcN	4.799	2.870	3.708	3.695	3.612	3.948	3.807
δ (^{13}C) [b]	β-Gal	105.8	73.7	75.3	71.3	78.1	63.7	
	α-GlcN	92.7	57.0	72.0	81.2	73.1	62.6	
	β-GlcN	97.2	59.5	75.1	81.4	77.7	62.8	
$^3J_{HH}$		$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$
	β-Gal	7.8, 7.7	9.9, 9.9	3.3	ND [c]	7.9	4.2	12.4
	α-GlcN	3.6	10.5	9.0	10.0	2.6	4.5	12.2
	β-GlcN	8.3	9.7	8.4	8.9	2.2	5.2	12.4

[a] Chemical shifts were expressed relative to HDO signal at δ 4.50.
[b] Chemical shifts were expressed relative to internal acetone (CH_3CO, δ 32.91).
[c] ND: the coupling constant was not determined.

regio-isomer of the reducing disaccharide, which is generally formed by β-galactosidase catalysis, was obtained in the product fraction. Since β4Gal-T1 utilise glucosamine both in the presence and absence of α-lactalbumin,[20,21] it is likely that the product lactosamine can be formed in the cells of other tissues, such as hepatocytes, where α-lactalbumin is not induced and glucosamine is transported via GLUT2.[22]

Lactosamine sequence exists as the core structure in many glycoproteins and glycolipids, normally in the form of *N*-acetyllactosamine. Although *N*-acetyllactosamine itself has been isolated from bovine colostrum,[13] and known as a growth factor for *Lactobacillus bifidus* var. *pennsylvanicus*,[23] the disaccharide with a free amino group has not been identified in vivo. The present study showed that mammary cells take up glucosamine in vivo, and also indicated that the product lactosamine might be formed by lactose synthase catalysis in the uridine nucleotide cycle.

3. Experimental

Materials.—Ringer's soln was purchased from Nihon Zenyaku Kogyo, Japan; cation exchange resin (AG 50W-X2, 100–200 mesh, H^+ form) was from Bio-Rad Laboratories, USA; Silica Gel 60 (particle size 63–200 μm) for column chromatography and TLC plates (Silica Gel 60 precoated on aluminium sheets, 20 × 20 cm) were from E. Merck, Germany; deuterium oxide (99.97%D) was purchased from Euriso-Top, France; all other chemicals were from Nacalai Tesque, Japan.

Animal.—A Holstein cow, at 24 days post partum, aged 2 years, reared at the Kawatabi Farm of Tohoku University was used. The animal-use protocols were approved by the Animal Experimentation Ethics Committee in Tohoku University, and the infusion experiment was performed in accordance with the Guidelines for Animal Experimentation.

Preparation of bovine milk sample.—The Holstein cow was milked out in order to empty the udder and the bovine milk was utilised as a control. Subsequently 4000 mL of Ringer's soln containing 125 g/L D-glucosamine (GlcN) hydrochloride (125 mg/mL in 9 g/L NaCl, 0.3 g/L KCl, 0.3 g/L $CaCl_2$, 0.1 g/L thiamine chloride) was infused at 13.3 mL/min for 5 h through a polyethylene cannula inserted into the right jugular vein. During infusion, the cow had free access to food and water. Bovine milk was obtained by milking 100 min after injection. The precipitation of proteins by addition of MeOH, crystallisation of lactose, and removal of lipids by centrifugation were performed as described previously.[7]

SIMLC-MS of the carbohydrate fraction.—The prepared carbohydrate fractions were dried in vacuo over phosphorus pentoxide. To each sample (5 mg), 5.0 mL perbenzoylation reagent (50 mg/mL benzoic anhydride and 25 mg/ml 4-dimethylaminopyridine in dry Py) was added, and the mixture was stirred for 30 h at 37 °C. Water (50 mL) was added to each sample and resulting soln was extracted with three 50 mL portions of $CHCl_3$, followed by evaporation of the organic layer under reduced pressure. The resulting perbenzoylated oligosaccharides were dissolved in 6 mL MeCN, and 4 μL injected for LCMS analysis. Analyses were performed on a Hitachi L-6200 intelligent pump equipped with a LiChroCART Super spher RP-18 column (4 × 125 mm) and FLOM 502 column oven coupled to a Finnigan MAT TSQ 700 mass-selective detector. The perbenzoylated oligosaccharides were resolved by reversed-phase HPLC using a solvent of (4:1 v/v) MeCN–H_2O at a flow rate of 0.5 mL/min. The column temperature was 40 °C. SIMLCMS was used to detect perbenzoylated lactosamine ($[M + Na]^+ = 1196$) and lactose ($[M + Na]^+ = 1197$).

TLC.—TLC was conducted using an ascending system (2:1:1 v/v) EtOAc–HOAc–H_2O with two developments. Each spot on the plate was visualised by spraying a soln of 20 g/L diphenylamine, 20 g/L aniline and 220 g/L phosphoric acid in acetone and then heating at 120 °C for 10 min. Selective characterisation of amino sugars was performed by spraying 5 g/L ninhydrin in EtOH, and heating at 120 °C for 5 min.

Isolation of amino sugars.—The oligosaccharides prepared from bovine milk taken after infusion of GlcN hydrochloride were

fractionated by silica gel column chromatography with a solvent system (3:2:1:1 v/v) *n*-BuOH–HOAc–Et_2O–H_2O. The separated amino sugar fractions were loaded onto a cation exchange resin column (4 mL) respectively, and neutral carbohydrates (lactose, Glc, and Gal) were eluted with 0.03 M HCl. After the column was washed with distilled water, the remaining amino sugars were eluted with 0.6 M NH_3. The elution was monitored by TLC. The eluates were neutralised by 0.1 M HCl to keep the amino sugars as hydrochloride salt, followed by lyophilisation.

Spectroscopy.—Electrospray ionisation mass spectrometry (ESIMS) was performed on a Finnigan MAT TSQ 700 instrument. ^1H and ^{13}C NMR spectra were recorded at 50 °C in D_2O with a Varian Unity INOVA 600 spectrometer. Chemical shifts for ^1H and ^{13}C NMR spectra are expressed relative to internal deuterium oxide (*H*DO, $\delta = 4.50$) and acetone (CH_3CO^-, $\delta = 32.908$), respectively.

Acknowledgements

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