Available online at www.sciencedirect.com
SCIENCE DIRECT
Carbohydrate Research 341 (2006) 730–737
Carbohydrate RESEARCH

Lactones of disialyl lactose: characterisation by NMR and mass spectra

Geoffrey Ge Pan and Laurence D. Melton*

Department of Chemistry, The University of Auckland, Private Bag 92019, Auckland, New Zealand

Received 3 August 2005; received in revised form 7 December 2005; accepted 15 January 2006
Available online 3 February 2006

Abstract—The lactonisation of α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-D-Galp-(1→4)-D-Glc (disialyl lactose) was investigated. ^{1}H and ^{13}C NMR chemical shifts of disialyl lactose and α-Neu*p*5Ac-(2→8, 1→9)-α-Neu*p*5Ac-(2→3, 1→2)-β-D-Galp-(1→4)-D-Glc (disialyl lactose-dilactone) were assigned based on 1D and 2D NMR results, including edited HSQC, HSQC–TOSCY and HMBC. The time course of lactonisation was followed by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) with electrospray ionisation (ESI) mass spectrometry (MS) detection. The rate of lactonisation between α-^{8}Neu5Ac and α-^{3}Neu5Ac residues (lactonisation at the α-(2→8) linkage) was faster than that of lactonisation between α-^{3}Neu5Ac and Gal residues (lactonisation at the α-(2→3) linkage). The mass spectra of disialyl lactose, its lactones, α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac (α-(2→8) disialic acid) and α-Neu*p*5Ac-(2→3)-β-D-Galp-(1→4)-D-Glc-lactone (3′-sialyllactose-lactone) showed that the α-(2→8) linkage between Neu5Ac residues is difficult to cleave in the ESI-MS, compared with the α-(2→3) linkage between Neu5Ac and Gal residues.

Keywords: Disialyl lactose; Disialyl lactose-dilactone; Lactonisation; 2D NMR; HPLC–ESI-MS; Mass spectra

1. Introduction

α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (disialyl lactose) (Fig. 1A), a member of the sialyl oligosaccharide family, was first isolated from bovine colostrum some decades ago,[1–3] and more recently from buffalo colostrum.[4] The content of disialyl lactose in bovine colostrum is relatively low and few studies have been done on its characterisation, compared with the major sialyl oligosaccharide in bovine colostrum, α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-sialyllactose), which has been studied by many researchers, including one of the present authors.[5,6] Sialyl oligosaccharides contain sialic acids such as Neu5Ac. They occur as free oligosaccharides in body fluids and they are also important components of glycoproteins and gangliosides.[7,8] Mammalian milk sugars contain a highly complex mixture of sialyl oligosaccharides. They are of considerable interest because of their varied biological functions, such as cell surface receptors for pathogens.[9,10]

In sialyl oligosaccharides, lactonisation can occur between the carboxyl group of Neu5Ac and a hydroxyl group of an adjacent sugar residue. The lactonisation of 3′-sialyllactose has been studied[11–13] and used for its purification.[11,13] Lactonisation of α-(2→8) linked trisialic acid,[14] tetrasialic acid,[15] oligosialic acids[16,17] and polysialic acids[18,19] have also been reported. In addition, the sialic acid residues in gangliosides can undergo lactonisation,[20–23] and naturally occurring ganglioside lactones have also been identified.[24,25] However, the lactonisation of free disialyl lactose has not been reported.

In the present study, the disialyl lactose was extracted from bovine colostrum and the disialyl lactose was lactonised using glacial acetic acid. The formation of disialyl lactose-dilactone was followed by NMR and electrospray ionisation (ESI) mass spectrometry (MS). The decomposition of disialyl lactose in solution at room

* Corresponding author. Fax: +64 9 3737422; e-mail: l.melton@auckland.ac.nz

0008-6215/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2006.01.015

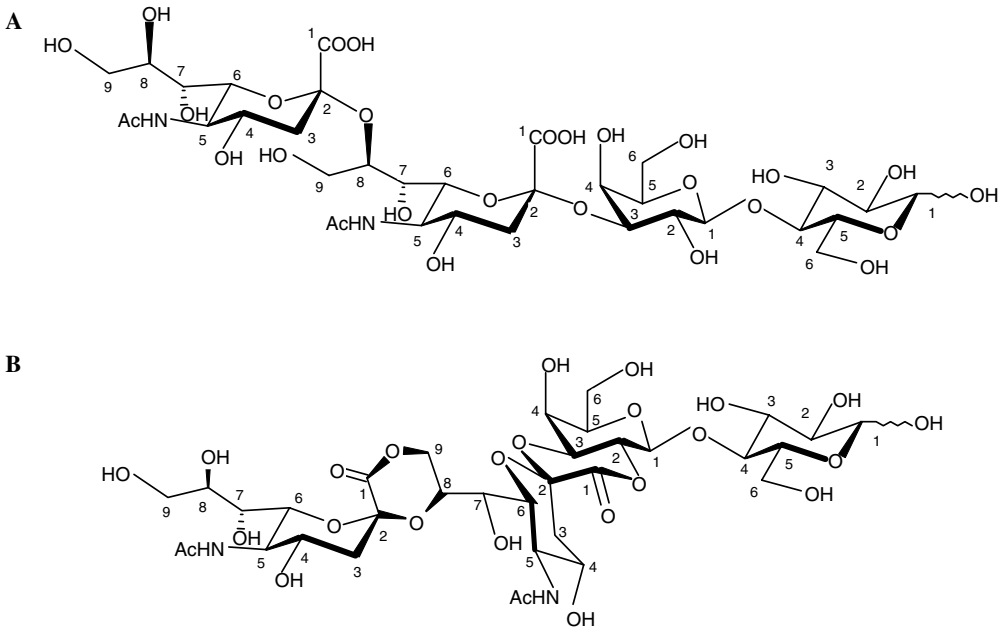

Figure 1. The structures of disialyl lactose and its dilactone: (A) α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc (disialyl lactose); (B) α-Neu*p*5Ac-(2→8, 1→9)-α-Neu*p*5Ac-(2→3, 1→2)-β-D-Gal-(1→4)-D-Glc (disialyl lactose-dilactone).

temperature was studied using HPLC–ESI-MS. In addition, the positive mode ESI mass spectra of disialyl lactose and its lactones were studied, with particular reference to the fragmentation of the α-(2→8) linkage between the two Neu5Ac residues.

2. Results and discussion

2.1. NMR spectra

In the present study, full ^{13}C and ^{1}H assignments of disialyl lactose and its dilactone were made. Some of the characteristic ^{13}C and ^{1}H resonances can be readily assigned with 1D NMR spectra. However, due to the complexity of the NMR spectra of the samples, the assignments of the resonances in the 'crowded region' (^{1}H: 69–81 ppm; ^{13}C: 3.2–5.0 ppm) were primarily based on the edited HSQC and HSQC–TOSCY results. The CH_2 containing groups were further identified by DEPT or edited HSQC with their phase-down peaks. In addition, some of the ^{1}H resonances, such as H-4 of α-^{3}Neu5Ac and α-^{8}Neu5Ac, were assigned according to HMBC results. HMBC and HSQC–TOSCY results were also used to confirm the assignments. The assignments of ^{1}H and ^{13}C of disialyl lactose and its dilactone are listed in Tables 1 and 2, respectively, and Figure 2 shows the core region of the HSQC spectrum of disialyl lactose-dilactone. Previous studies made full or partial assignments of ^{13}C spectrum,[4,26] and reporter groups of ^{1}H spectrum[4,13] of disialyl lactose. No study has been reported on disialyl lactose-dilactone.

There are three possible sites for the lactonisation of the carboxyl group at the α-(2→8) linkage (C-4, C-7 and C-9 of α-^{3}Neu5Ac), and two such sites at the α-(2→3) linkage (C-2 and C-4 of Gal residue). Our study found that C-9 (α-^{3}Neu5Ac) and C-2 (Gal) are the two lactonisation sites, with the resultant product: α-Neu*p*-5Ac-(2→8, 1→9)-α-Neu*p*5Ac-(2→3, 1→2)-β-D-Gal*p*-(1→4)-D-Glc (disialyl lactose-dilactone, Fig. 1B). This was based on the small changes in ^{13}C shifts of C-4 (0.0 ppm) and C-7 (−0.7 ppm) of α-^{3}Neu5Ac, as well as C-4 (−1.0 ppm) of Gal after the lactonisation, compared to the large changes for C-9 (+7.3 ppm) of α-^{3}Neu5Ac and C-2 (+4.9 ppm) of Gal (Table 2). The results agree with a previous study,[23] in which the disialyl lactose moiety was part of a ganglioside. Another study[12] also indicated that the major lactonisation site of 3′-sialyllactose is C-2 of Gal.

The C-9:H-9 signal (^{13}C: 71.5 ppm; ^{1}H: 4.75 ppm) of α-^{3}Neu5Ac residue of disialyl lactose-dilactone in the edited HSQC spectrum can be easily recognised, as it is a 'phase-down' peak (Fig. 2). Starting from this signal, C-8:H-8 (^{13}C: 73.1 ppm; ^{1}H: 4.33 ppm) and C-7:H-7 (^{13}C: 71.3 ppm; ^{1}H: 3.73 ppm) signals can be identified because: (1) in the HSQC–TOSCY spectrum, cross peaks were formed between each pair of them; (2) in the HMBC spectrum, C-7:H-9 and C-8:H-9 cross peaks can also be observed and the intensity of the former is stronger than the latter. The O-acetylation at C-9 of Neu5Ac can cause an upfield shift of 3.1 ppm for C-8.[27] In the present work, the unusual upfield shift of C-8 in α-^{3}Neu5Ac (7.5 ppm) agrees with a previous study[18] on the lactonisation of α-(2→8) linked sialic acid

Table 1. ^{1}H NMR chemical shifts (ppm) for disialyl lactose and its dilactone

Residue	^{1}H chemical shift (ppm)												
	H-1	H-2	H-3	H-3 ax	H-3 eq	H-4	H-5	H-6	H-6′	H-7	H-8	H-9	H-9′	CH_3 (CH_3CO)
Disialyl lactose-dilactone														
α-^{3}Neu5Ac				1.77	2.74	4.33	3.88	3.63		3.73	4.33	4.75		2.03
α-^{8}Neu5Ac				1.61	2.62	4.33	3.94	3.60		3.62	3.68	3.84	3.67	2.04
β-D-Gal	4.81	4.86	4.25			4.17	3.84	3.85						
α-D-Glc	5.24	3.59	3.86			3.77	3.97	3.90						
β-D-Glc	4.66	3.29	3.66			3.80	3.62	3.89	3.97					
Disialyl lactose														
α-^{3}Neu5Ac				1.73	2.78	3.69	3.81	3.62		3.58	4.14	4.18	3.74	2.04[a]
α-^{8}Neu5Ac				1.73	2.68	3.61	3.85	3.65		3.61	3.90	3.87	3.65	2.07[a]
β-D-Gal	4.52	3.59	4.12			3.97	3.71	3.75						
α-D-Glc	5.23	3.59	3.84			3.66	3.97	3.88						
β-D-Glc	4.66	3.28	3.64			3.68	3.63	3.88	3.98					

Chemical shifts in parts per million and acetone is used as the internal standard.

[a] The resonances could not be specifically assigned to α-^{3}Neu5Ac or α-^{8}Neu5Ac residue, thus the assignments are interchanged between the two residues.

Table 2. ^{13}C NMR chemical shifts (ppm) for disialyl lactose and its dilactone

Residue	^{13}C chemical shift (ppm)										
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CO (CH_3CO)	CH_3 (CH_3CO)
Disialyl lactose-dilactone											
α-^{3}Neu5Ac	169.8[a]	99.8[a]	42.1	70.5	54.8	75.4	71.3	73.1	71.5	177.7	24.3
α-^{8}Neu5Ac	168.5[a]	98.7[a]	42.0	70.5	54.4	73.7	70.9	73.2	66.0	177.7	24.3
β-D-Gal	102.2	76.8	77.9	69.1	78.2	63.6					
α-D-Glc	94.7	74.2	74.7	80.1	71.4	62.4					
β-D-Glc	99.0	76.9	77.0	80.5	77.6	62.4					
Disialyl lactose											
α-^{3}Neu5Ac	176.0[a]	102.9[a]	43.1	70.5	55.0	76.6	72.0	80.5	64.2	177.6	24.7
α-^{8}Neu5Ac	176.2[a]	103.2[a]	42.3	71.1	54.4	75.3	70.8	74.5	65.2	177.6	24.3
β-D-Gal	105.3	71.9	78.1	70.1	77.9	63.7					
α-D-Glc	94.5	73.8	74.0	80.6	72.7	62.5					
β-D-Glc	98.4	76.5	76.9	80.8	77.5	62.7					

Chemical shifts in parts per million and acetone is used as the internal standard.

[a] The resonances could not be specifically assigned to α-^{3}Neu5Ac or α-^{8}Neu5Ac residue, thus the assignments are interchanged between the two residues.

homopolymers, where the authors found the upfield shift of C-8 was 7.2 ppm. For a comparison, the ^{13}C upfield shift of C-3 in Gal residue is 0.2 ppm according to our present study (Table 2), and a previous study[12] found the corresponding shift was 0.5 ppm after lactonisation of 3′-sialyllactose. The unusually high upfield shift of C-8 was atttributed[18] to ring strain combined with lactonisation at C-9.

2.2. The time course of disialyl lactose lactonisation

Samples from the lactonisation of 3′-sialyllactose, disialyl lactose and α-Neu*p*5Ac-(2→8)-α-Neu*p*5Ac (α-(2→8) disialic acid) were withdrawn at different time intervals and analysed by TLC and HPLC. The TLC (Fig. 3) shows that two compounds (Spots B and C) were formed during the lactonisation of disialyl lactose (Spot A) in glacial acetic acid. Spot B disappeared when the mixture was treated with anion exchange resin, but spot C remained (results are not shown), indicating that B is an acidic compound and C is neutral. The intensity of spot B decreased between 12 and 72 h, while that of C increased. Both results suggest that C corresponds to disialyl lactose-dilactone, and B is a partially lactonised product: a disialyl lactose-monolactone. This was confirmed by HPLC. The peaks c and d (Fig. 4A) were identified as a disialyl lactose-monolactone and a disialyl lactose-dilactone, respectively, based on their pseudomolecular weights and mass spectra patterns (Fig. 5B and C, respectively). The mass spectrum of the disialyl lactose-monolactone (Fig. 5B) contained the fragment ion at *m*/*z* 727 (due to loss of the Glc residue from disialyl lactose-monolactone), and the fragment ion at *m*/*z* 565 could only be produced from the lactonisation at the α-(2→8) linkage. Therefore it is concluded that spot B in TLC and peak c in HPLC correspond to the

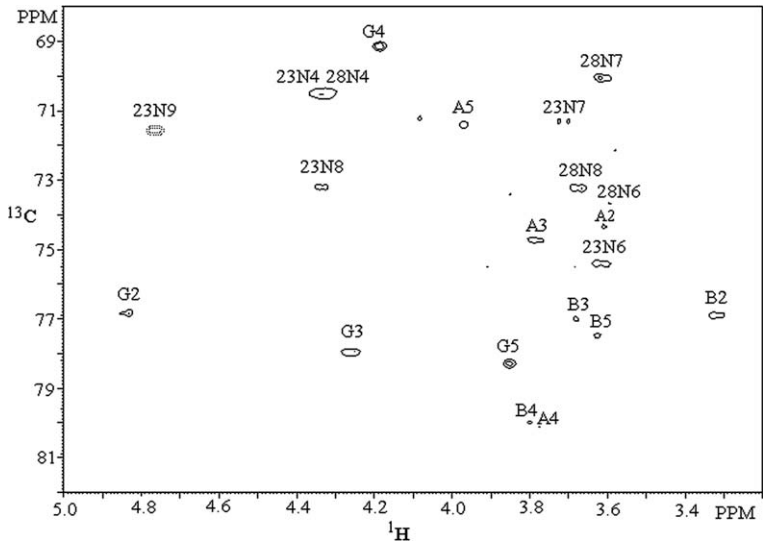

Figure 2. The core region of the edited HSQC spectrum of disialyl lactose-dilactone: (A) α-Glc; (B) β-Glc; (G) β-Gal; 23N, α-^{3}Neu5Ac; 28N, α-^{8}Neu5Ac. The dash line of 23N9 spot indicates that it is a CH_2 containing group.

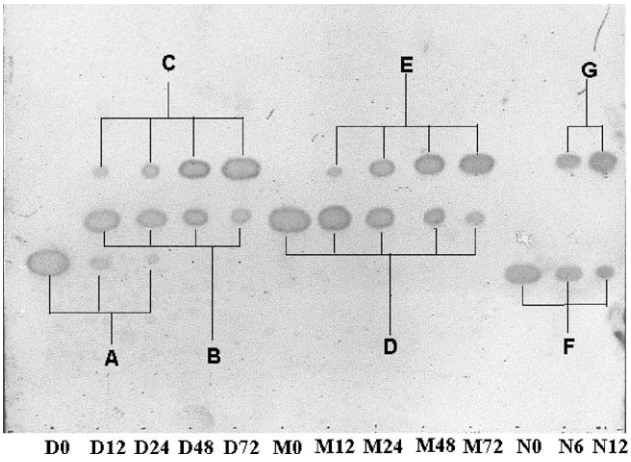

Figure 3. TLC chromatogram of sialyl oligosaccharides after lactonisation. D0–D72: lactonisation of disialyl lactose at 0, 12, 24, 48 and 72 h; M0–M72: lactonisation of 3′-sialyllactose at 0, 12, 24, 48, 72 h; N0–N12: lactonisation of α-(2→8) disialic acid at 0, 6, 12 h. (A) disialyl lactose; (B) disialyl lactose-(2→8, 1→9)-monolactone; (C) disialyl lactose-dilactone; (D) 3′-sialyllactose; (E) 3′-sialyllactose-lactone; (F) α-(2→8) disialic acid; (G) α-(2→8) disialic acid-lactone.

same compound: α-Neu*p*5Ac-(2→8, 1→9)-α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (disialyl lactose-(2→8, 1→9)-monolactone).

The results show that lactonisation at the α-(2→8) linkage occurs much faster than that at the α-(2→3) linkage. The same conclusion can be drawn, comparing the lactonisation rates of 3′-sialyllactose and α-(2→8) disialic acid. Figure 3 shows that only a small part of 3′-sialyllactose has been converted into its lactone form (spot E) after 12 h and there is still a significant amount of 3′-sialyllactose left even after 48 h. In contrast, the majority of α-(2→8) disialic acid has been converted to

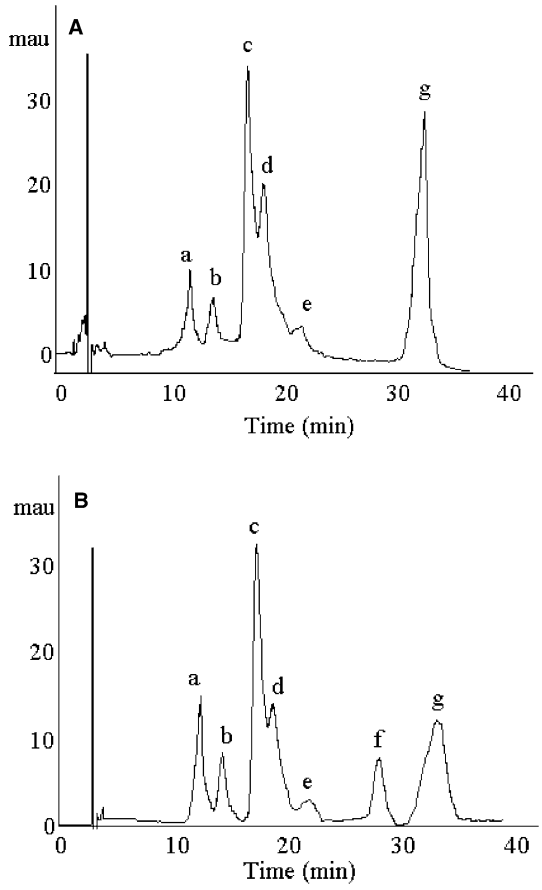

Figure 4. HPLC chromatograms of (A): disialyl lactose after lactonisation for 4 h in glacial acetic acid, and (B): disialyl lactose solution held at pH 3 for 2.5 days at 18 °C. For chromatographic conditions, refer to Section 3.5. Peak interpretation: (a) an unidentified peak; (b) 3′-sialyllactose; (c) disialyl lactose-(2→8, 1→9)-monolactone; (d) disialyl lactose-dilactone; (e) an un-identified disialyl lactose-monolactone; (f) α-(2→8) disialic acid; (g) disialyl lactose.

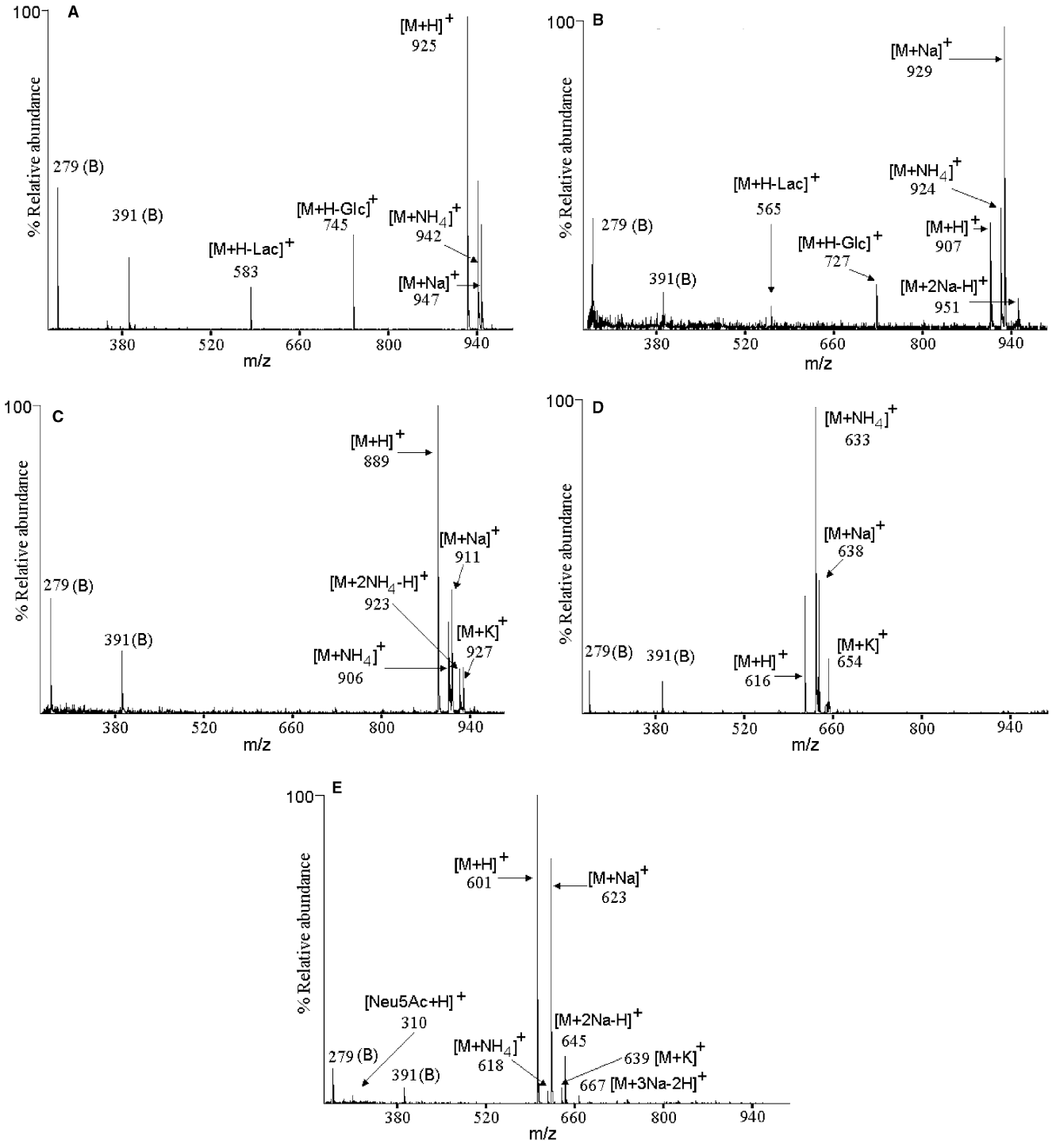

Figure 5. Positive electrospray mass spectra of sialyl oligosaccharides and their lactones. The spectra were obtained from their HPLC–ESI-MS results. M stands for molecular mass and B are residual background signals. (A) disialyl lactose; (B) disialyl lactose-(2→8, 1→9)-monolactone; (C) disialyl lactose-dilactone; (D) 3′-sialyllactose-lactone; (E) α-(2→8) disialic acid.

its lactone form (spot G) after 12 h. This observation agrees with a ^{1}H NMR study of lactonisation of disialyl lactose chain in gangliosides.[23]

During the lactonisation of disialyl lactose, a small amount of 3′-sialyllactose (peak b) resulted from the hydrolysis of disialyl lactose (Fig. 4A). Peak e, another minor component (Fig. 4A), had a *m/z* value of 907, corresponding to the pseudomolecular weight of disialyl lactose-monolactose $[M+H]^{+}$, and thus was a different disialyl lactose-monolactose.

2.3. ESI-MS

The ESI mass spectra of disialyl lactose, disialyl lactose-monolactone, disialyl lactose-dilactone, α-(2→8) disialic acid and 3′-sialyllactose-lactone are shown in Figure 5.

In a recent study[28] on sialyl oligosaccharides, including 3′-sialyllactose, α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-D-Glc (6′-sialyllactose), α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-GlcNAc (3′-sialyl-*N*-acetyllactosamine), α-Neu*p*5Ac-(2→6)-β-D-Gal*p*-(1→4)-D-GlcNAc (6′-sialyl-*N*-acetyllactosamine), α-Neu*p*5Ac-(2→3)-β-D-Gal*p*-(1→4)-α-L-Fuc*p*-(1→3)-D-Glc (sialyl fucosyllactose) and disialyl lactose, we found that under positive ionisation mode ESI-MS, mass spectral patterns of α-(2→3) and α-(2→6) linkages between the Neu5Ac and Gal residues are different, and the former produces a prominent fragment ion, which is due to the cleavage of the glycosidic linkage between the Gal and Glc (or GlcNAc, or Fuc in sialyl fucosyllactose) residues, and a minor fragment ion due to the cleavage between Gal and Neu5Ac residues. Those fragment ions were either absent from the spectra of α-(2→6) linked sialyl oligosaccharides, or in much lower abundance. An explanation is that the steric hindrance effect of the α-(2→3) linkage is relatively large because the two sugar residues linking to Gal are on the same side of the pyranose ring of Gal. In the high voltage field, this steric hindrance causes the removal of sugar unit on either side of Gal. In contrast, the steric hindrance effect of α-(2→6) linked sugars is reduced because the C-6 is not in the pyranose ring and has larger freedom of rotation. In the present study, the mass spectrum of disialyl lactose-(2→8, 1→9)-monolactone, shows two fragment ions at *m*/*z* 727 and 565, which are due to the cleavage between Gal and Glc, and between α-^{3}Neu5Ac and Gal, respectively. This is further support for the observation that sialyl oligosaccharides with the α-(2→3) linkage are more prone to produce fragment ions.

In contrast to disialyl lactose and 3′-sialyllactose, no corresponding fragment ion was found in the mass spectra of disialyl lactose-dilactone (Fig. 5C) and 3′-sialyllactose-lactone (Fig. 5D). The implication is that the lactonisation of hydroxyl group of C-2 in Gal residue stabilises the β-(1→4) linkage between Gal and Glc residues under the positive mode ESI-MS conditions.

The α-(2→8) linkage between the two Neu5Ac residues commonly exists in the naturally occurring polysialic acids, sialyl oligosaccharides and sugar chains of glycoproteins or gangliosides. It is interesting to know if the α-(2→8) linkage is easy to cleave in the ESI-MS. The mass spectra of disialyl lactose (Fig. 5A) shows that it contains two relatively abundant fragments: *m*/*z* 583 and 745, which are due to the cleavage between the α-^{3}Neu5Ac and Gal residues, as well as between Gal and Glc residues, and no fragment ion was found corresponding to the cleavage of α-(2→8) linkage. For the mass spectrum of α-(2→8) disialic acid (Fig. 5E), a tiny peak at *m*/*z* 310 ($[Neu5Ac+H]^{+}$) was detected with an abundance much less than that of the pseudomolecular ion peaks. Hence, both the spectra show that α-(2→8) linked sialyl oligosaccharides are difficult to cleave in the ESI-MS. This may also be explained by the steric hindrance effect, as mentioned above.

2.4. The instability of disialyl lactose at low pH

The glycosidic linkage between Neu5Ac and other monosaccharides is susceptible to hydrolysis at low pH.[29] To study its stability, disialyl lactose was incubated in pH 3, 5 and 7 solutions for 2.5 days, which were then analysed by HPLC. At pH 3, a range of products, including 3′-sialyllactose, α-(2→8) disialic acid, disialyl lactose-(2→8, 1→9)-monolactone and disialyl lactose-dilactone were detected by UV (Fig. 4B). At pH 5, no lactonisation product was detected but very small amounts of 3′-sialyllactose and α-(2→8) disialic acid were detected at pH 5, by ESI-MS (result is not shown). No products were detected by the less sensitive UV detector. At pH 7, neither hydrolysis nor lactonisation products could be detected by UV or MS. The above results show that disialyl lactose is unstable under acidic conditions and the lactonisation of disialyl lactose is sensitive to pH.

3. Experimental

3.1. Materials

Bovine colostrum (the first milking) was collected from Friesian cows at a local dairy farm. 3′-Sialyllactose (sodium salt) and disialyl lactose (sodium salt) standards were purchased from Dextra Laboratories (Reading, UK). α-(2→8) Disialic acid (sodium salt) was from Nacalai Tesque (Kyoto, Japan). Dowex resin (1-X4, 200-400 mesh) was from Supelco (Bellefonte, USA). Sephadex G-25 (fine) resin was from Pharmacia (Uppsala, Sweden).

3.2. Isolation of disialyl lactose and 3′-sialyllactose

Sialyl oligosaccharide mixture was extracted from bovine colostrum using a modification of the method of Schauer and Kamerling.[8] In brief, the colostrum sample (500 mL) was centrifuged at 4 °C to remove the fat, then an equal volume of MeOH was added and the sample was centrifuged at 4 °C. The supernatant was applied to a column (40 × 2.8 cm) of Dowex 1-X4 (OH^{-}). The column was washed with water to remove lactose, and then eluted with 0.1 and 0.2 M equimolar pyridine/acetic acid for 3′-sialyllactose, and disialyl lactose, respectively. Fractions (5 mL) were collected and

5 μL of each fraction was spotted on a GF_{254} TLC plate, which was developed with ethanol–*n*-butanol–pyridine–water–acetic acid (100:10:10:30:3, v/v/v/v/v) and visualised with the orcinol–H_2SO_4 spray reagent.[2] Sialyl oligosaccharide-containing fractions were subjected to HPLC. Fractions containing 3′-sialyllactose were combined, as were those containing diasialyl lactose. The combined fractions were lyophilised, dissolved in water and then desalted using a Sephadex G-25 column. The fractions containing 3′-sialyllactose or disialyl lactose were collected, combined, lyophilised and then re-chromatographed on Dowex 1-X4 and Sephadex G-25 columns, as described above.

3.3. Lactonisation

Disialyl lactose (50 mg) was added in 2 mL of glacial acetic acid and the solution was stirred for 3 days at room temperature. Samples were withdrawn at different time intervals and analysed by TLC and HPLC–ESI-MS. At the end of the reaction, the solution was immediately added to a beaker containing a slurry of Dowex 1-X4 resin (OH^-). After stirring briefly, the disialyl lactose-dilactone-containing supernatant was decanted, lyophilised and analysed by TLC, NMR and ESI-MS. The 3′-sialyllactose and α-(2→8) disialic acid were treated in the same manner to obtain their respective lactone products.

3.4. Stability of disialyl lactose at different pH values

Solutions of pH 3 and 5 were made by adding glacial acetic acid dropwise to water. The pH 7 solution was made by adding 1 M NaOH dropwise to water. Disialyl lactose (2 mg) was dissolved in 2 mL of the above solutions and kept at room temperature for 2.5 days.

3.5. HPLC and ESI-MS

HPLC analysis was performed using a HP1100 (Agilent Technologies, Wilmington, USA). A Phenosphere NH_2 column (250 × 4.60 mm, particle size: 5 μm; from Phenomenex, CA) was used. The mobile phase was 0.005 M ammonium bicarbonate aqueous solution/acetonitrile (20/80, v/v) at a flow rate of 1 mL min^{-1} and detection was at 195 nm.

For the MS analysis, a Mariner Mass spectrometer (PerSeptive Biosystems, Framingham, USA) was used with an electrospray source (positive mode) and a time-of-flight mass analyser. The above HPLC was also connected by an on-line splitter (splitting ratio 20:1) to the MS. The operating parameters were: spray tip potential 3804 V; spray chamber temperature 0 °C; acceleration potential 4000 V; scan range *m*/*z* 99–1200; seconds per spectrum 1.00.

3.6. NMR spectroscopy

Samples were dissolved in D_2O (99.9%). A trace amount of acetone (^1H, 2.225 ppm; ^{13}C, 32.9 ppm) was added as internal standard. ^1H, ^{13}C, DEPT, edited HSQC, HSQC–TOSCY and HMBC were performed on disialyl lactose and its dilactone, using a Bruker Avance DRX 400 NMR spectrometer (400 MHz ^1H and 100 MHz ^{13}C). The probe temperature was set to 300.0 K; the mixing time for HSQC–TOSCY was 75 ms; the delay time for HMBC was 60 ms.

Acknowledgements

We thank Michael Walker for his expert assistance with NMR. This project was supported by Fonterra Co-operative Group Ltd and New Zealand Foundation for Research, Science and Technology.

References

1. Kuhn, R.; Gauhe, A. *Chem. Ber.* **1965**, *98*, 395–413.
2. Veh, R. W.; Michalski, J. C.; Corfield, A. P.; Sander-Wewer, M.; Gies, D.; Schauer, R. *J. Chromatogr.* **1981**, *212*, 313–322.
3. Parkkinen, J.; Finne, J. *Methods Enzymol.* **1987**, *138*, 289–300.
4. Aparna, H. S.; Salimath, P. V. *Carbohydr. Res.* **1995**, *268*, 313–318.
5. Melton, L. D.; Morris, E. R.; Rees, D. A.; Thom, D. *J. Chem. Soc., Perkin Trans. 2* **1979**, 10–17.
6. Melton, L. D.; Morris, E. R.; Rees, D. A.; Thom, D.; Bociek, S. M. *Carbohydr. Res.* **1980**, *81*, 295–303.
7. Corfield, A. P.; Schauer, R. Occurrence of Sialic Acids. In *Sialic acids: Chemistry, Metabolism and Function*; Schauer, R., Ed.; Springer: Wien, 1982; pp 5–50.
8. Schauer, R.; Kamerling, J. P. Chemistry, Biochemistry and Biology of Sialic Acids. In *Glycoproteins II*; Montreuil, J., Vliegenthart, J. F. G., Schachter, H., Eds.; Elsevier: Amsterdam, 1997; pp 243–402.
9. Matrosovich, M. N.; Gambaryan, A. S.; Teneberg, S.; Piskarev, V. E.; Yamnikova, S. S.; Lvov, D. K.; Robertson, J. S.; Karlsson, K. A. *Virology* **1997**, *233*, 224–234.
10. Murray, P. A.; Levine, M. J.; Tabak, L. A.; Reddy, M. S. *Biochem. Biophys. Res. Commun.* **1982**, *106*, 390–396.
11. Roy, R.; Laferrière, C. A.; Dettman, H. *Carbohydr. Res.* **1989**, *186*, C1–C5.
12. Nakamura, T.; Bubb, W. A.; Saito, T.; Arai, I.; Urashima, T. *Carbohydr. Res.* **2000**, *329*, 471–476.
13. Laferrière, C. A.; Roy, R. *Methods Enzymol.* **1994**, *242*, 102–108.
14. Cheng, M.-C.; Lin, C.-H.; Khoo, K.-H.; Wu, S.-H. *Angew. Chem., Int. Ed.* **1999**, *38*, 686–689.
15. Cheng, M.-C.; Lin, C.-H.; Wang, H.-Y.; Lin, H.-R.; Wu, S.-H. *Angew. Chem., Int. Ed.* **2000**, *39*, 772–776.
16. Yu, Y.-P.; Cheng, M.-C.; Lin, H.-R.; Lin, C.-H.; Wu, S.-H. *J. Org. Chem.* **2001**, *66*, 5248–5251.

17. Zhang, Y.; Lee, Y.-C. *J. Biol. Chem.* **1999**, *274*, 6183–6189.
18. Lifely, M. R.; Gilbert, A. S.; Moreno, C. *Carbohydr. Res.* **1981**, *94*, 193–203.
19. Lifely, M. R.; Gilbert, A. S.; Moreno, C. *Carbohydr. Res.* **1984**, *134*, 229–243.
20. Bassi, R.; Riboni, L.; Sonnino, S.; Tettamanti, G. *Carbohydr. Res.* **1989**, *193*, 141–146.
21. Fronza, G.; Krischner, G.; Acquotti, D.; Sonnino, S. *Carbohydr. Res.* **1989**, *195*, 51–58.
22. Fronza, G.; Krischner, G.; Acquotti, D.; Bassi, R.; Tagliavacca, L.; Sonnino, S. *Carbohydr. Res.* **1988**, *182*, 31–40.
23. Ando, S.; Yu, R. K.; Scarsdale, J. N.; Kusunoki, S.; Prestegard, J. H. *J. Biol. Chem.* **1989**, *264*, 3478–3483.
24. Riboni, L.; Sonnino, S.; Acquotti, D.; Malesci, A.; Ghidoni, R.; Egge, H.; Mingrino, S.; Tettamanti, G. *J. Biol. Chem.* **1986**, *261*, 8514–8519.
25. Terabayashi, T.; Kawanishi, Y. *Carbohydr. Res.* **1998**, *307*, 281–290.
26. Berman, E. *Biochemistry* **1984**, *23*, 3754–3759.
27. Vliegenthart, J. F. G.; Dorland, L.; van Halbeek, H. NMR Spectroscopy of Sialic Acids. In *Sialic Acids: Chemistry, Metabolism and Function*; Schauer, R., Ed.; Springer: Wien, 1982; pp 127–172.
28. Pan, Ge G.; Melton, L. D. *J. Chromatogr. A* **2005**, *1077*, 136–142.
29. Schauer, R. Isolation and Purification of Sialic Acids. In *Sialic Acids: Chemistry, Metabolism and Function*; Schauer, R., Ed.; Springer: Wien, 1982; pp 51–57.