ELSEVIER

www.elsevier.nl/locate/carres

Carbohydrate Research 329 (2000) 471–476

CARBOHYDRATE RESEARCH

Note

An NMR study of the lactonization of α-*N*-acetylneuraminyl-(2→3)-lactose

Tadashi Nakamura [a,*], William A. Bubb [b], Tadao Saito [c], Ikichi Arai [a], Tadasu Urashima [a]

[a] *Department of Bioresource Science, Obihiro University of Agriculture and Veterinary Medicine, Inada cho, Obihiro, Hokkaido 080-8555, Japan*

[b] *Department of Biochemistry, The University of Sydney, Sydney, NSW 2006, Australia*

[c] *Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi 981-8555, Japan*

Received 23 August 1999; received in revised form 22 May 2000; accepted 30 June 2000

Abstract

The composition of the products formed by treatment of commercial α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-sialyllactose) with glacial acetic acid was investigated by $^{1}H-^{13}C$ one- and two-dimensional NMR spectroscopy and fast atom bombardment-mass spectrometry. The data confirmed that the major product of the reaction was α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone, which reverted to the starting material on standing in aqueous solution at ambient temperature, but for which complete NMR assignments are reported. The NMR data led to the tentative conclusion that the reaction also yielded small amounts of lactose, and α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→4b)-lactone which was stable in aqueous solution.

Keywords: 3′-Sialyllactose lactonization; *N*-Acetylneuraminyllactose lactonization; GM3 oligosaccharide lactone; 3′-Sialyllactose lactones, NMR data

The milk or colostrum of many mammalian species, especially the colostrum at the initial stage of lactation, contains several kinds of sialyloligosaccharides [1,2]. Recently, we presented NMR evidence which suggested the presence of two different lactones of α-Neu5Gc-(2→3)-β-D-Gal*p*-(1→4)-D-Glc in ovine colostrum [3]. Although lactonization of sialic acid residues in gangliosides, including GM3, is well established [4,5], lactonization of the free GM3 oligosaccharide has only been reported in connection with a step in the isolation and purification of α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc (3′-sialyllactose) from bovine colostrum [6,7]. The conclusion that lactonization proceeded through OH-2 of the β-galactosyl residue was based on the chemical shifts of selected resonances in the ^{1}H NMR spectrum, whose assignments were determined from homo- and heteronuclear experiments, but no ^{13}C chemical shifts were presented.

In view of the continuing controversy as to the origins of ganglioside lactones [4], our NMR evidence for the natural occurrence of sialyl(Neu5Gc)-lactose lactones prompted us

* Corresponding author. Fax: +81-155-495577.
E-mail address: nakamura@obihiro.ac.jp (T. Nakamura).

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00202-0

to further investigate structural data for sialyllactose lactones derived from more readily available materials. NMR studies of 3′-sialyl-(Neu5Ac)-lactose carried out by different groups over more than two decades have led to an increasingly refined definition of its structure [8–19], but to our knowledge, the partial data cited above remains the only published evidence for the structure of a derived lactone. Here we present conclusive 2D NMR evidence to show that commercial 3′-sialyllactose when treated with glacial acetic acid gives α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone as the major product, together with much lower yields of two other products, tentatively identified as α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→4b)-lactone and lactose.

Characterization of 3′-sialyllactose-(1c→2b)-lactone.—Analysis by thin layer chromatography (TLC) showed that treatment of 3′-sialyllactose with glacial acetic acid gave one major product with $R_{3'\text{-SL}}$: 1.30. The FAB mass spectrum revealed prominent peaks at *m*/*z* 632 and *m*/*z* 614 consistent with $[M-1]^-$ ions for 3′-sialyllactose and a dehydrated product, respectively.

Following dissolution in ^2H_2O, the major product was almost fully hydrolyzed to 3′-sialyllactose after about 72 h at the probe temperature (20.1 °C) used for acquisition of NMR spectra. While decomposition may have been slowed at lower temperatures, the loss of resolution was considered unacceptable and the NMR assignments were therefore determined from spectra obtained for three different sample preparations. Resonances associated with the major product were readily distinguished from those of 3′-sialyllactose by their different intensities in NMR experiments conducted at different times after the sample had been dissolved.

Fig. 1 shows an HSQC spectrum acquired within 3 h of the lactonization product being dissolved in ^2H_2O. The assignments for the Glc, β-Gal (H-1–H-4) and Neu5Ac (H-3a,3e–H-6) residues were determined from COSY and TOCSY spectra and confirmed by identi-

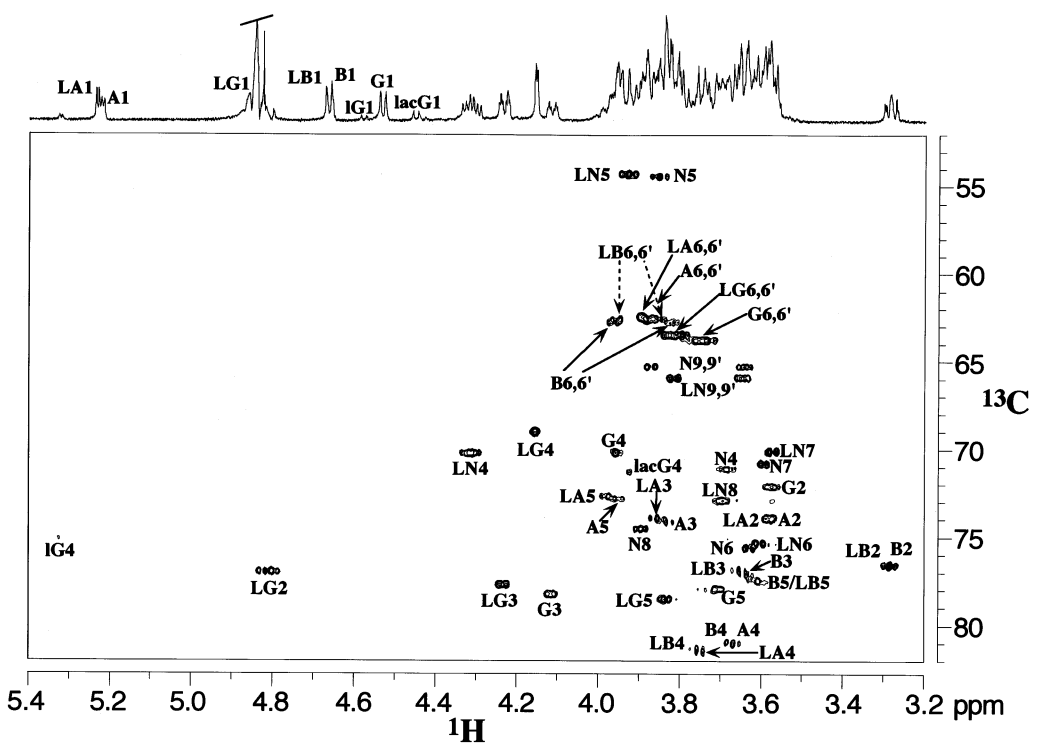

Fig. 1. Core region of the 600 MHz sensitivity-enhanced HSQC spectrum of the product obtained when 3′-sialyllactose was subject to a standard lactonization procedure, recorded in ^2H_2O at 20.1 °C. The spectrum is shown in phase-sensitive mode after processing with a $\pi/2$ shifted sine-bell in each dimension. Assignments: A, α-Glc; B, β-Glc; G, β-Gal; N, Neu5Ac; prefix L, (1c→2b)-lactone; prefix l, (1c→4b)-lactone; prefix lac, lactose. The ^1H 1D spectrum is shown above the contour plot with assignments for anomeric protons, whose 2D correlations are outside of the region depicted.

Table 1
^{1}H NMR chemical shifts for 3′-sialyllactose (3′-SL) and its (1c→2b)-lactone

Assignment	Residue and chemical shift (δ)							
	Neu5Ac		β-Gal		β-Glc		α-Glc	
	Lactone	3′-SL	Lactone	3′-SL	Lactone	3′-SL	Lactone	3′-SL
H-1			4.84	4.531	4.665	4.662	5.233	5.219
H-2			4.81	3.57	3.286	3.282	3.58	3.58
H-3			4.234	4.116	3.65	3.64	3.85	3.83
H-3a	1.766	1.800						
H-3e	2.648	2.758						
H-4	4.314	3.69	4.155	3.96	3.76	3.68	3.74	3.67
H-5	3.93	3.85	3.83	3.71	3.62	3.62	3.97	3.96
H-6	3.60	3.63	3.81	3.74	3.85	3.82	3.89	3.87
					3.96	3.97		
H-7	3.57	3.59						
H-8	3.69	3.90						
H-9	3.65	3.64						
H-9′	3.81	3.87						
CH_3	2.043	2.030						

fying all long-range connectivities in an HMBC experiment. The latter also led to the identification of the signals due to β-Gal (H-5 and H-6,6′) and, through correlations from both the Neu5Ac H-5 and CH_3 protons to each carbonyl carbon, assignment of the resonances for the acetyl groups. Further, in both starting material and product, the Neu5Ac H-3 equatorial and axial protons were each correlated with the quaternary carbon, C-2, but only the axial protons showed three-bond correlations to their respective C-1.

Resonances for the Neu5Ac CH_2OH are readily assigned from their distinctive ^{13}C chemical shift but the assignment of the remainder of the side-chain must rely on heteronuclear experiments since TOCSY transfer between the Neu5Ac ring (H-6) and side-chain (H-7) is hindered by the small homonuclear coupling ($J = 1.68$ Hz [14]). For both 3′-sialyllactose and the major product, a strong HMBC correlation between H-5 and the ^{13}C frequency of one of the unassigned resonances identified C-7 as there is no plausible heteronuclear coupling pathway to C-8 [20]. Heteronuclear RELAY [21] and HSQC-TOCSY [22] experiments confirmed the side-chain assignments.

Multiple-bond correlations confirmed the glycosidic linkage for the lactose moiety in both 3′-sialyllactose and the major product but the strong correlation between the β-Gal H-3 and Neu5Ac C-2 observed for 3′-sialyllactose was not evident for the product. A model of α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone showed that a dihedral angle of close to 90° might be expected for this pathway (and for the pathway between the β-Gal H-2 and Neu5Ac C-1) so that the failure to observe long-range C–H correlations, even with delays as long as 120 ms for evolution of the coupling, is not unreasonable [20]. Nevertheless, the significant high frequency shifts for β-Gal, H-1 and H-2, and Neu5Ac, H-4, compared with 3′-sialyllactose (Table 1) are similar to the changes observed on lactonization of gangliosides GM3 [23], and GM4 [24], while the low frequency shift for β-Gal C-2 (Table 2) is consistent with the change expected on esterification of a carboxylic acid [25]. Taken with the mass spectral evidence, the data support the conclusion that α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone is the major product of the reaction of 3′-sialyllactose with glacial acetic acid. There are some discrepancies (as great as

0.9 ppm for Neu5Ac, H-3_{eq}) between the data presented in Table 1 and published values obtained at 300 MHz for a ^2H_2O solution of the lactone [7]. Although these may in part be attributable to differences in experimental conditions, the compression of chemical shifts evident in the 600 MHz spectrum shown in Fig. 1 suggests that the greater dispersion and contemporary heteronuclear experiments employed in the present work may be necessary for the unambiguous assignment of the spectra of such compounds.

Minor products.—The 1D spectrum in Fig. 1 shows resonances of considerably lower relative intensity at 4.451, 4.579, and 5.324 ppm. Through COSY, TOCSY and HSQC experiments on a sample which had been subject to the lactonization conditions but in which only traces of α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone remained, the peaks at 4.579 and 5.324 ppm were shown to be linked as H-1 and H-4, respectively, of the same molecule. The appreciable high-frequency shift of H-4 (Table 3) is comparable

Table 2
^{13}C NMR chemical shifts for 3′-sialyllactose (3′-SL) and its (1c→2b)-lactone

Assignment	Residue and chemical shift (δ)							
	Neu5Ac		β-Gal		β-Glc		α-Glc	
	Lactone	3′-SL	Lactone	3′-SL	Lactonc	3′-SL	Lactone	3′-SL
C-1	168.17	176.30	102.64	105.29	98.54	98.47	94.55	94.52
C-2	99.92	102.31	76.94	72.07	76.56	76.49	73.83	73.83
C-3	42.17	42.23	77.63	78.15	76.75	77.03	73.91	74.09
C-4	70.20	70.96	68.97	70.17	81.19	80.79	81.45	80.94
C-5	54.20	54.35	78.45	77.84	77.31	77.50	72.62	72.78
C-6	75.27	75.60	63.44	63.72	62.50	62.73	62.38	62.59
C-7	70.08	70.77						
C-8	72.87	74.35						
C-9	65.87	65.30						
$CH_3CO(CH_3)$	24.73	24.71						
$CH_3CO(CO)$	177.72	177.68						

Table 3
NMR data for minor products

Assignment	3′-Sialyllactose (1c→4b)-lactone [a]				Lactose [a]	
	Neu5Ac		β-Gal		β-Gal	
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C
CH-1		168.8	4.579	104.6	4.451	105.6
CH-2		98.3	3.59		3.54	73.7
CH-3			4.34	76.2	3.66	75.2
CH-3a	1.82	42.1				
CH-3e	2.604	42.1				
CH-4	4.31	70.3	5.324	75.1	3.93	71.2
CH-5	3.91	54.3				
CH-6	3.57	75.4				
CH-7	3.57	70.0				
CH-8						
CH-9	3.61	65.9				
CH-9′	3.81	65.9				

[a] Structures consistent with the partial data available but necessarily tentative.

with chemical shifts observed for the β-Gal H-4 of the Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-(1b→4a)-lactone unit of GM4 [24] and for related synthetic intermediates [26,27]. By similar arguments to those advanced above, the majority of the ^{1}H chemical shifts of a minor Neu5Ac residue could be determined and were found to differ from those of 3′-sialyllactose in the same way as the Neu5Ac chemical shifts of the GM4-(1b→4a)-lactone differed from those of GM4 [24]. Although the relatively low concentration did not permit its spectrum to be fully assigned, the close similarity with literature data suggests that the product giving rise to the ^{1}H resonances at 4.579 and 5.324 ppm should be tentatively designated as α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→4b)-lactone.

From the same spectra used for characterization of the (1c→4b)-lactone, the ^{1}H resonance at δ 4.451 was correlated with chemical shifts (Table 3) which are in good agreement with ^{1}H [15] and ^{13}C [28] literature data for the β-Gal residue of lactose. Overlaying an HSQC spectrum of this sample with that of an authentic sample of lactose revealed that all other correlations due to lactose were coincident with those of 3′-sialyllactose.

Stability of sialyllactose lactones.—Whereas the major product, α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone, was hydrolysed in ^2H_2O at 20 °C to give 3′-sialyllactose, the putative α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→4b)-lactone appeared to be stable under the same conditions. In the previous application [6,7] of the lactonization as a step in the purification of 3′-sialyllactose, the product was obtained by hydrolysis of the intermediate α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone with 0.1 M sodium hydroxide but the susceptibility of the lactone to hydrolysis when dissolved in ^2H_2O was not specifically noted. Nakahara and co-workers [26] described an α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-(1b→4a)-lactone which was stable when heated in ^2H_2O at 80 °C for 12 h but a similar compound [29] decomposed fully in water at 25 °C in 2 h. Accordingly, it appears that the susceptibility of sialyllactose lactones to hydrolysis may be very dependent on the presence of specific substituents. Although the mild conditions used for the lactonization would not normally be associated with sialic acid release [30], the production of lactose did not appear to increase with decomposition of α-Neu5Ac-(2→3)-β-D-Gal*p*-(1→4)-D-Glc-(1c→2b)-lactone and we assume that lactose was produced by degradation of 3′-sialyllactose directly.

1. Experimental

Lactonization of 3′-sialyllactose.—3′-Sialyllactose (1 mg, Dextra Laboratories Ltd, Reading, UK) was allowed to incubate in glacial AcOH (500 μL) for 3 days at 20 °C [7].

Thin-layer chromatography (TLC).—A Silica Gel 60 plate (Art. 5553, E. Merck, Germany) was used with 4:1 (v/v) *n*-propanol–water [7], and detection effected by spraying with 5% H_2SO_4–MeOH and heating at 150 °C for 5 min [31].

Fast-atom bombardment mass spectrometry (FABMS).—FABMS was performed on a JEOL MS700 instrument in the negative ion mode. The sample dissolved in a glycerol matrix was loaded on the stainless steel target and bombarded at a kinetic energy of 8 keV.

NMR experiments.—Samples were dissolved directly in ^2H_2O (99.96 atom % ^{2}H) containing a small amount of acetone as chemical shift reference (^{1}H, δ 2.225; ^{13}C, δ 32.9) and all spectra were recorded with the probe temperature set to 20.1 °C. The 1D ^{13}C spectrum was recorded on a Varian Unity Plus-500 spectrometer and all other spectra on a Bruker DRX-600 spectrometer with a 5 mm triple-resonance inverse-detection *xyz*-gradient probe, employing standard Bruker pulse sequences (XWIN-NMR version 2.5 or 2.6 [22]), with no solvent presaturation, and coherence selection using *z* axis gradients, except for COSY experiments for which magic-angle gradients [32] were employed to enhance the suppression of the solvent resonance. All chemical shifts of adequately resolved signals were determined from 1D spectra and all other chemical shifts from a gradient-selected sensitivity-enhanced HSQC spectrum [33], optimised for $^1J_{CH}$ of 145 Hz, with WURST-40 decoupling [34] employing a 112-step phase

cycle [35] during acquisition. TOCSY spectra were recorded with mixing times of 28–150 ms with the spin-lock field strength adjusted for a 90° pulse-length of 29–30 μs, the HSQC-TOCSY experiment with a mixing time of 28 ms, and HMBC spectra with delays of 80 and 120 ms for evolution of long-range $^{13}C-^{1}H$ couplings. Except for the HMBC and HMQC-RELAY experiments, all spectra were recorded in phase-sensitive mode.

Acknowledgements

We thank Professor Takatoshi Itoh of Tohoku University for FABMS and ^{1}H NMR measurements. This work was partially supported by a Grant-in-Aid for Encouragement of Young Scientists in Japan.

References

[1] A. Kobata, in M.I. Horowitz, W. Pigman (Eds.), *The Glycoconjugates*, Vol. 1, Academic, New York, 1977, pp. 423–440.
[2] D.S. Newburg, S.H. Neubayer, in R.G. Jensen (Ed.), *Handbook of Milk Composition*, Academic, New York, 1995, pp. 273–349.
[3] T. Nakamura, T. Urashima, M. Nakagawa, T. Saito, *Biochim. Biophys. Acta*, 1381 (1998) 286–292.
[4] T. Terabayashi, Y. Kawanishi, *Carbohydr. Res.*, 307 (1998) 281–290.
[5] T. Sato, M. Ishii, T. Terabayashi, Y. Kawanishi, Y. Okahata, *Chem. Lett.*, (1997) 669–670.
[6] R. Roy, C.A. Laferrière, H. Dettman, *Carbohydr. Res.*, 186 (1989) c1–c5.
[7] C.A. Laferrière, R. Roy, *Methods Enzymol.*, 242 (1994) 102–108.
[8] V. Eschenfelder, R. Brossmer, H. Friebolin, *Tetrahedron Lett.*, 16 (1975) 3069–3072.
[9] M.F. Czarniecki, E.R. Thornton, *J. Am. Chem. Soc.*, 99 (1977) 8279–8282.
[10] L.W. Jaques, S. Glant, W. Weltner, Jr., *Carbohydr. Res.*, 80 (1980) 207–211.
[11] J. Haverkamp, H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, R. Pfeil, R. Schauer, *Eur. J. Biochem.*, 122 (1982) 305–311.
[12] E. Berman, *Carbohydr. Res.*, 118 (1983) 9–20.
[13] S. Sabesan, J.C. Paulson, *J. Am. Chem. Soc.*, 108 (1986) 2068–2080.
[14] L. Lerner, A. Bax, *Carbohydr. Res.*, 166 (1987) 35–46.
[15] N. Platzer, D. Davoust, M. Lhermitte, C. Bauvy, D.M. Meyer, C. Derappe, *Carbohydr. Res.*, 191 (1989) 191–207.
[16] L. Poppe, H. van Halbeek, *J. Am. Chem. Soc.*, 113 (1991) 363–365.
[17] H.-C. Siebert, G. Reuter, R. Schauer, C.-W. von der Lieth, J. Dabrowski, *Biochemistry*, 31 (1992) 6962–6971.
[18] G.R. Kiddle, S.W. Homans, *FEBS Lett.*, 436 (1998) 128–130.
[19] M.J. Milton, R. Harris, M.A. Probert, R.A. Field, S.W. Homans, *Glycobiology*, 8 (1998) 147–153.
[20] I. Tvaroska, F.R. Taravel, *Adv. Carbohydr. Chem. Biochem.*, 51 (1995) 15–61.
[21] L. Lerner, A. Bax, *J. Magn. Reson.*, 69 (1986) 375–380.
[22] W. Willker, D. Leibfritz, R. Kerssebaum, W. Bermel, *Magn. Reson. Chem.*, 31 (1993) 287–292.
[23] R.K. Yu, T.A.W. Koerner, S. Ando, H.C. Yohe, J.H. Prestegard, *J. Biochem.*, 98 (1985) 1367–1373.
[24] T. Terabayashi, T. Ogawa, Y. Kawanishi, *J. Biochem.*, 107 (1990) 868–871.
[25] H.-O. Kalinowski, S. Berger, S. Braun, *Carbon-13 NMR Spectroscopy*, Wiley, Chichester, 1994, pp. 213–214.
[26] Y. Nakahara, H. Iijima, T. Ogawa, *Tetrahedron Lett.*, 35 (1994) 3321–3324.
[27] L. Singh, Y. Nakahara, Y. Ito, Y. Nakahara, *Carbohydr. Res.*, 325 (2000) 132–142.
[28] H.A. Nunez, R. Barker, *Biochemistry*, 19 (1980) 489–495.
[29] K.C. Nicolaou, C.W. Hummel, Y. Iwabuchi, *J. Am. Chem. Soc.*, 114 (1992) 3126–3128.
[30] A. Varki, S. Diaz, *Anal. Biochem.*, 137 (1984) 236–247.
[31] G.W. Hay, B.A. Lewis, F. Smith, *J. Chromatogr.*, 11 (1963) 479–486.
[32] P.C.M. van Zijl, M. O'Neil-Johnson, S. Mori, R.E. Hurd, *J. Magn. Reson. A*, 113 (1995) 265–270.
[33] J. Schleucher, M. Schwendinger, M. Sattler, P. Schmidt, O. Schedletzky, S.J. Glaser, O.W. Sørensen, C. Griesinger, *J. Biomol. NMR*, 4 (1994) 301–306.
[34] E. Kupće, R. Freeman, *J. Magn. Reson. A*, 115 (1995) 273–276.
[35] T.E. Skinner, M.R. Bendall, *J. Magn. Reson.*, 124 (1997) 474–478.