Carbohydrate Research, 145 (1986) 341–347
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Isolation and characterization of two sialyloligosaccharides containing *N*-acetyllactosamine from pregnancy urine

CHRISTIAN DERAPPE, CHANTAL BAUVY, MARGUERITE LEMONNIER,

INSERM U-180, UER Biomédicale des Saint-Pères, 45 rue des Saint-Pères, F-75270 Paris Cédex 06 (France)

MICHEL LHERMITTE,

INSERM U-16, Place de Verdun, F-59045 Lille Cédex (France)

AND NICOLE PLATZER

Laboratoire de Chimie Organique Structurale, CNRS ERA-557, Université Pierre et Marie Curie, 4 place Jussieu, F-75230 Paris Cédex 05 (France)

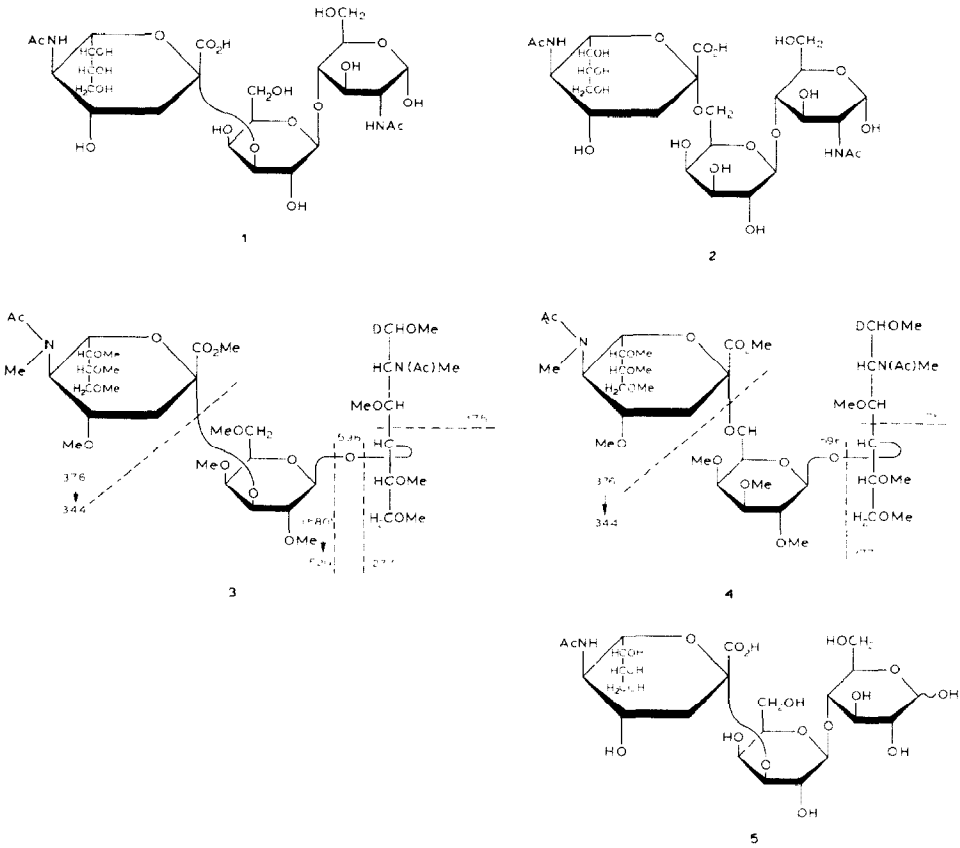
(Received February 5th, 1985; accepted for publication in revised form, May 29th, 1985)

Pregnancy and lactation urines are known to be rich sources of oligosaccharides[1–4] having at their reducing end either a D-glucose, a 2-acetamido-2-deoxy-D-glucose, or *myo*-inositol residue. In addition, these oligosaccharides generally contain L-fucosyl or sialyl, or both groups. Both 3′- (**1**) and 6′-*N*-acetylneuraminyllactosamines (**2**), described herein, were previously isolated from normal urine[5–7] and from bovine colostrum[8]. These oligosaccharides were characterized by chemical analysis and ^{13}C-n.m.r. spectrometry[7], but no information concerning their mass and ^{1}H-n.m.r. spectra has been available until now.

The *N*-acetylneuraminyllactosamines **1** and **2** were isolated from 15 liters of pregnancy urine according to the experimental procedure previously described[9]. The trisaccharide fraction was fractionated on DEAE Sephadex A-25 and elution was monitored by u.v. absorbance at 280 nm and colorimetric determination of neutral hexoses[10]. As shown in Fig. 1, several fractions were selected and analyzed for monosaccharide molar-ratios (Table I). Compounds **1** and **2** were further isolated from Fraction III by preparative paper chromatography in solvent system (*a*). From data included in Table I, the excretion rate of **1** and **2** was estimated to be 68.5 mg in Fraction II and 63 mg in Fraction III, *i.e.*, 8.7 mg/L of urine.

The trisaccharides **1** and **2** thus obtained appeared homogeneous in solvent systems (*a*)–(*d*). Their monosaccharide molar ratios, as well as the partially methylated alditol acetates obtained by methylation analysis, are reported in Table II. The 2-acetamido-2-deoxy-D-glucitol resulting from methylation analysis included a ^{2}H-1 atom, as shown by mass spectrometry, indicating a 2-acetamido-2-deoxy-D-glucose residue at the reducing end.

0008-6215/86/$ 03.50



Compounds **1** and **2** were labeled by reduction with sodium borotritide, according to Takasaki and Kobata[11], and submitted to enzymic treatment as previously reported[9]. Digestion with *Vibrio cholerae* sialidase completely converted them into neutral saccharides, exhibiting the same paper chromatographic mobility in solvent system (*d*) as 2-acetamido-2-deoxy-4-*O*-D-galactopyranosyl-D-glucitol. Treatment of the degraded products with Jack bean β-D-galactosidase gave D-galactose and 2-acetamido-2-deoxy-D-[^{2}H-1]glucitol, as shown by paper chromatography in solvent system (*d*).

No molecular ion appeared in the mass spectra of both permethylated, ^{2}H-labeled, reduced trisaccharides **3** and **4**. Fragments produced by loss of $-CH_2-O-CH_3$ (45) and $-CO_2CH_3$ (59) were present at respectively *m/z* 828 and 814; similar results were previously observed for 3′-*N*-acetylneuraminyllactose[12,13] (**5**), for which the ion *m/z* (M − 59) was the major ion in the high-mass region. Interestingly, no fragment at *m/z* (M − 32) (loss of methanol) was observed for any of the trisaccharides. Detection of strong ions at *m/z* 376 and 344 confirmed the presence of a terminal *N*-acetylneuraminyl residue[12] in both trisaccharides **1** and **2**, and existence of fragments at *m/z* 596 and 520 agrees with an *N*-acetylneuraminylhexose

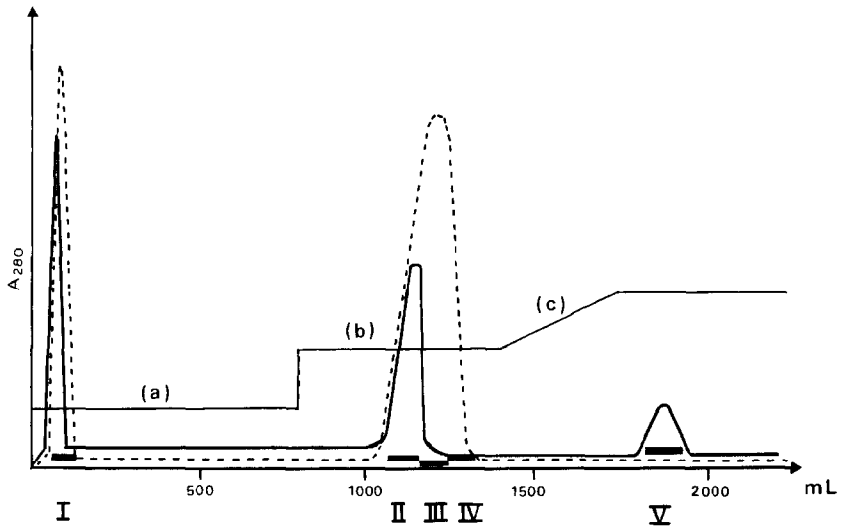


Fig. 1. Ion-exchange chromatography on DEAE Sephadex A-25 of the desalted oligosaccharide fraction. Elution was performed with pyridine acetate buffers: (a) 0.05M, pH 6.0; (b) 0.05M, pH 5.3; and (c) linear gradient from 0.05 to 0.5M, pH 5.3. Elution profiles were monitored by u.v. absorbance at 280 nm (——) and by colorimetric analysis for neutral hexoses (orcinol reagent) of each 2-mL fraction collected (------). Fractions I, II, III, IV, and V were pooled as indicated by bars and analyzed for monosaccharide molar ratios (Table I).

TABLE I

ION-EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX A-25 OF THE DESALTED OLIGOSACCHARIDE FRACTION[a]

Sugar	*Fractions*				
	I	*II*	*III*	*IV*	*V*
Fucose	0.5				1.2
Mannose	0.5				
Galactose	1.0	1.0	1.0	1.0	1.0
Glucose	1.0	0.4	0.6	1.0	0.2
2-Acetamido-2-deoxygalactose	0.7			0.2	0.1
2-Acetamido-2-deoxyglucose	0.8	0.5	0.4	0.1	1.1
myo-Inositol		0.1	0.1	0.1	
N-Acetylneuraminic acid		1.0	1.0	1.0	
Weight (mg)	458	137	166	203	5

[a]Weights and monosaccharide molar ratios of the five pooled fractions (see Fig. 1). Molar ratios are given in relation to 1 mol of D-galactose.

TABLE II

CHARACTERIZATION OF COMPOUNDS **1** AND **2**[a]

Sugar	*Compound*	
	1	**2**
2-Acetamido-2-deoxyglucose	1.0	1.0
Galactose	1.0	1.0
N-Acetylneuraminic acid	0.9	1.0
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol	1.2	
1,5,6-Tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-galactitol		1.1
4-*O*-Acetyl-2-deoxy-1,3,5,6-tetra-*O*-methyl-2-(*N*-methylacetamido)-D-(1-^{2}H)glucitol	1.0	1.0

[a]The molar ratio of monosaccharides was determined by g.l.c. and is expressed relative to 1 mol of 2-acetamido-2-deoxyglucose, and that of partially methylated alditol acetates obtained by methylation analysis relative to 1 mol of 2-deoxy-2-(*N*-methylacetamido)-D-(1-^{2}H)glucitol.

sequence[13,14]. The ion at *m/z* 277 was characteristic of a monosubstituted 2-acetamido-2-deoxyhexitol residue having ^{2}H-1, and the ion at *m/z* 175 indicated substitution at O-4.

The ^{1}H-n.m.r. spectra of **1** and **2** were compared to that of **5** (Table III). For the *N*-acetylneuraminyl residue, H_2-3″ of both trisaccharides exhibited characteristic sets of signals[15]. Chemical shifts and coupling constants observed for H-1′ of the D-galactosyl residue indicated a β linkage for both trisaccharides[16]. The H-3′ signal of **1** was shifted to δ 4.12, as observed both here for **5**, and previously for α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-D-GalNAcol[17]. For **2**, the corresponding signal did not appear clearly since its resonance was in the bulk of ring-proton resonances. The chemical shift range of H-1 ($\delta \approx 5.2$), as well as its coupling constant ($J_{1,2} \approx 2$ Hz),

TABLE III

^{1}H-CHEMICAL SHIFTS (δ) OF STRUCTURAL-REPORTER-GROUP PROTONS OF COMPOUNDS **1**, **2**, AND **5**[a]

Protons	*Compound*		
	1	**2**	**5**
H-1 (α form)	5.20 ($J_{1,2}$ 2)	5.20 ($J_{1,2}$ 2)	
$COCH_3$-2	2.04	2.06	
H-1′	4.56 ($J_{1',2'}$ 7.6)	4.45 ($J_{1',2'}$ 7.8)	4.53 ($J_{1',2'}$ 7.9)
H-3′	4.12	[b]	4.12
H-3*a*″	1.80	1.72	1.81
H-3*e*″	2.76	2.68	2.76
$COCH_3$-4″	2.03	2.03	2.03

[a]Recorded on the same 250 MHz spectrometer. Coupling constants (in Hz) for H-1 and -2 observed for the D-galactose and 2-acetamido-2-deoxy-D-glucose residues are indicated. [b]Not assigned.

suggested that in both **1** and **2** this residue is present in the α form[18]. From a ^{13}C-n.m.r. study previously performed on these two trisaccharides[6], it was concluded that chemical shifts observed could result from different intramolecular interactions between the acidic *N*-acetylneuraminyl group and the 2-acetamido-2-deoxy-D-glucopyranosyl residue. In the present ^1H-n.m.r. study, the chemical shift and coupling constant values observed, for both **1** and **2**, for the H-1 signal suggest a preponderance of the α form, which agrees with the existence of such intramolecular interactions. Several other single peaks produced by contaminating products also appeared on the spectra at δ 0.21, 1.94, and 2.37.

The presence of structures corresponding to **1** and **2** in various glycoproteins[19,20] might imply that, as for other *N*-acetylglucosamine-containing oligosaccharides found in pathological urine[4,21], **1** and **2** reported herein have a catabolic origin. Unfortunately, no endo-*N*-acetyl-β-D-glucosaminidase having such specificities has ever been reported. Furthermore, comparison of the excretion rates of **1** and **2** in the case of normal and pregnancy urine show that their excretion is pregnancy-dependent. From Berman's results[6], an excretion rate of 3 mg/L for both trisaccharides was calculated for normal urine, and from Parkkinen and Finne's results[7] an excretion rate of 1.8 mg/L for **1** and 0.9 mg/L for **2**. In the present work, a total excretion rate of 8.7 mg/L was observed for both trisaccharides, about three times that found for normal urine. This finding agrees with previous results observed[22] for **5**. Thus, this pregnancy dependence observed for the excretion of **1** and **2** supports the hypothesis that, like the lactose-containing oligosaccharides from human milk and pregnancy urine, both **1** and **2** originate from a biosynthetic process.

EXPERIMENTAL

Third-term pregnancy urine was collected from a non-secretor woman (blood-group A) not subject to dietary restrictions. Urine was kept frozen at −18° until use.

The homogeneity of the oligosaccharides was monitored by analytical paper chromatography (Schleicher and Schüll 2043-b paper) in the following solvent systems: (*a*) 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water, (*b*) 2:1:2 (upper phase, v/v) ethyl acetate–pyridine–water, (*c*) 3:1:1 (v/v) ethyl acetate–acetic acid–water, and (*d*) 10:4:3 (v/v) ethyl acetate–pyridine–water. Papers were stained with the $AgNO_3$ reagent[23]. The molar ratios of monosaccharides were determined by g.l.c. of the per-*O*-(trimethylsilyl)ated derivatives of methyl glycosides formed by methanolysis[24] in methanol–1.5M HCl for 18 h at 85°. The oligosaccharides were permethylated according to Stellner *et al.*[25], by use of potassium *tert*-butoxide as a base[26]. The permethylated derivatives were treated and analyzed as previously reported[27].

The sequence analysis of the permethylated, deuterium-labeled, reduced trisaccharides was carried out by e.i.–m.s. (Riber 10-10 apparatus) with a solid-probe

inlet; ionization current, 200 μA; ionization energy, 70 eV; accelerating voltage, 8.2 kV; and ion-source temperature, 130°.

The 250.13 MHz, ^{1}H-n.m.r. spectra were recorded with a Bruker SY.250 spectrometer operating in the F.t. mode at 298 K. The trisaccharides were dissolved in $(^2H)_2O$ at a concentration of 1 mg/mL. Chemical shifts were calculated in relation to the signal of sodium 4,4-dimethyl-4-silapentane-1-sulfonate by indirect reference to acetone (δ 2.225). The acquisition parameters were: pulse width, 3μs (corresponding to a flip angle of ~30°); spectra width, 2500 Hz; and data points, 32 k. Chemical shifts and coupling constants were determined with an accuracy of ±0.15 Hz.*

ACKNOWLEDGMENTS

This work was supported by grants from CNRS ERA-557 and LA-293, and from INSERM U-16 and U-180. The authors thank Professors Y. Goussault and P. Roussel for helpful discussions. They are also grateful to Drs. C. Lange and P. Richardin for valuable assistance in mass spectrometry determination.

REFERENCES

1 J. W. Date, *Scand. J. Clin. Lab. Invest.*, 16 (1964) 604–613
2 M. Lemonnier and R. Bourrillon, *Biomedicine*, 24 (1976) 253–258.
3 P. Hallgren, B. S. Lindberg, and A. Lundblad, *J. Biol. Chem.*, 252 (1977) 1034–1040.
4 G. Strecker, T. Hondi-Assah, B. Fournet, G Spik, J. Montreuil, P. Maroteaux, P. Durand, and J. P. Farriaux, *Biochim. Biophys. Acta*, 444 (1976) 349–358.
5 J K Huttunen, *Ann Med Exp. Biol. Fenniae Suppl.*, 44 (12) (1966) 1–60
6 E. Berman, *Carbohydr. Res.*, 118 (1983) 9–20
7 J Parkkinen and J. Finne, *Eur. J. Biochem.*, 136 (1983) 355–361.
8 R. Kuhn and A. Gauhe, *Chem. Ber.*, 98 (1965) 395–413.
9 C. Derappe, C. Bauvy, D. M. Meyer, M. Lemonnier, M. Lhermitte and N. Platzer, *Carbohydr. Res.*, 115 (1983) 221–229.
10 E. Vasseur, *Acta Chem. Scand.*, 2 (1948) 693–701.
11 S. Takasaki and A. Kobata, *J. Biochem. (Tokyo)*, 76 (1974) 783–789.
12 M. Renlund, M. A. Chester, A. Lundblad, P Aula, K. O. Raivio, S. Autio, and S L. Koskela, *Eur. J Biochem.*, 101 (1979) 245–250
13 J. P Kamerling, L. Dorland, H. van Halbeek, J. F G. Vliegenthart, M. Messer, and R. Schauer, *Carbohydr. Res.*, 100 (1982) 331–340.
14 T. Saito, I. Takatoshi, S. Adachi, T Suzuki, and T. Usui, *Biochim. Biophys. Acta*, 678 (1981) 257–267.
15 J. F. G. Vliegenthart, H. van Halbeek, and L. Dorland, *Adv. Carbohydr. Chem. Biochem*, 41 (1983) 209–374
16 R. U. Lemieux, K. Bock, L. T. J Delbaere, S. Koto, and V S. Rao, *Can. J. Chem*, 58 (1980) 631–653.
17 H. van Halbeek, L. Dorland, J. F. G. Vliegenthart, A. M Fiat, and P. Jolles, *Biochim. Biophys. Acta*, 623 (1980) 295–300
18 S. J. Perkins, L. N. Johnson, D. C. Phillips, and R A. Dwek, *Carbohydr. Res.*, 59 (1977) 19–34
19 G. Strecker, B. Fournet, J. Montreuil, L. Dorland, J. Haverkamp, J. F. G. Vliegenthart, and D Dubesset, *Biochimie*, 60 (1978) 725–734.

*Copies of the mass and ^{1}H-n.m.r. spectra are available from the authors upon request.

20 K. YAMASHITA, C. L. LIANG, S. FUNAKOSHI, AND A. KOBATA, *J. Biol. Chem.*, 256 (1981) 1283–1289.
21 G. C. TSAY, G. DAWSON, AND S. S. SUNG, *J. Biol. Chem.*, 251 (1976) 5852–5859.
22 P. MAURY, *J. Biol. Chem.*, 247 (1972) 3153–3158.
23 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)* 166 (1950) 444–445.
24 R. E. CHAMBERS AND J. R. CLAMP, *Biochem. J.*, 125 (1971) 1009–1018.
25 K. STELLNER, H. SAITO, AND S. I. HAKOMORI, *Arch. Biochem. Biophys.*, 155 (1973) 464–472.
26 J. FINNE, T. KRUSIUS, AND H. RAUVALA, *Carbohydr. Res.*, 80 (1980) 336–339.
27 D. LECAT, M. LEMONNIER, C. DERAPPE, M. LHERMITTE, H. VAN HALBEEK, L. DORLAND, AND J. F. G. VLIEGENTHART, *Eur. J. Biochem.*, 140 (1984) 415–420.