Carbohydrate Research, 100 (1982) 351–363
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

THE STRUCTURES OF SIX URINARY OLIGOSACCHARIDES THAT ARE CHARACTERISTIC FOR A PATIENT WITH MORQUIO SYNDROME TYPE B

JEAN-CLAUDE MICHALSKI, GÉRARD STRECKER,

Laboratoire de Chimie Biologique et Laboratoire Associé au C.N.R.S. No. 217, Université des Sciences et Techniques de Lille I, F-59655 Villeneuve d'Ascq Cedex (France)

HERMAN VAN HALBEEK, LAMBERTUS DORLAND, AND JOHANNES F. G. VLIEGENTHART

Department of Bio-Organic Chemistry, University of Utrecht, Croesestraat 79, NL-3522 AD Utrecht (The Netherlands)

(Received August 6th, 1981; accepted for publication, September 4th, 1981)

ABSTRACT

Morquio syndrome type B is an inherited, lysosomal storage disease characterised by a marked deficiency in acid β-D-galactosidase, while the 2-acetamido-2-deoxy-β-D-galactose 6-sulphate sulphatase activity is normal. Urinary oligosaccharides were studied in order to evaluate the effect of the diminished β-D-galactosidase activity on the catabolism of glycoconjugates and to compare their structures with those excreted by patients with GM_1-gangliosidosis. The following oligosaccharides were isolated: β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-D-Glc*p*NAc (**1**), β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-D-Glc*p*NAc (**2a**), β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-[α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-D-Glc*p*NAc (**2b**), β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-D-Glc*p*NAc (**3**), β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-{β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-α-D-Man*p*-(1→6)}-β-D-Man*p*-(1→4)-D-Glc*p*NAc (**4**), β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-[β-D-Glc*p*NAc-(1→4)]-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)]-β-D-Man*p*-(1→4)-D-Glc*p*NAc (**5**). Significant differences between Morquio syndrome type B and GM_1-gangliosidosis have been observed, with regard to the excretion rate and the specific structures of urinary oligosaccharides. Compounds **2a**, **2b**, and **5** are novel members of the series of oligosaccharides isolated from the urine of patients with inherited, lysosomal storage diseases.

INTRODUCTION

Morquio syndrome type B is an inborn error of metabolism, characterised by a marked diminution of acid β-D-galactosidase activity[1–3]. Patients suffering from this syndrome show growth retardation, dysostosis multiplex, cloudy corneas,

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odontoïd anomalies, and excessive excretion of keratan sulphate. Growth retardation and skeletal dysplasia are less severe than for Morquio syndrome type A, which is caused by the deficiency of 2-acetamido-2-deoxy-β-D-galactose 6-sulphate sulphatase[4-6]. The absence of any psychomotoric abnormalities and the presence of normal intelligence allow an easy differentiation from GM_1-gangliosidosis, which is caused by a virtually complete deficiency of β-D-galactosidase[7].

The lysosomal deficiency of β-D-galactosidase leads to the accumulation of oligosaccharides in the urine of patients with Morquio syndrome type B or GM_1-gangliosidosis. Such oligosaccharides have a D-galactosyl group at the non-reducing end. We now report on six oligosaccharides isolated from the urine of a patient with Morquio syndrome type B. The oligosaccharides were characterised in order to evaluate the effect of the diminished β-D-galactosidase activity on the catabolism of glycoconjugates, and to compare their structures with those known to be excreted by patients suffering from GM_1-gangliosidosis.

EXPERIMENTAL

Materials. — The urine sample was kindly provided by Dr. E. Paschke, and collected from patient R.E. of ref. 3.

Fractionation of urinary oligosaccharides. — Urine (5 L) was demineralised on a column (5 × 40 cm) of charcoal–Celite. After washing with water (5 L), the carbohydrate material was eluted with 50% ethanol (5 L). The ethanolic solution was evaporated to dryness under reduced pressure, and the residue dissolved in water (200 mL). Glycopeptides and sialyl-oligosaccharides were removed by using columns (2 × 20 cm) of Dowex 50-X2 (200–400 mesh; H^+) and Dowex 1-X2 (200–400 mesh; HCO_3^-) resins. The neutral effluent was then fractionated on a column (2 × 20 cm) of charcoal–Celite by elution with a discontinuous gradient of ethanol in water (1.5→18%). The oligosaccharides present in the various fractions were isolated by preparative paper chromatography, and analysed.

Analytical procedures. — Descending paper chromatography was performed on Whatman No. 3 paper with pyridine–ethyl acetate–acetic acid–water (5:5:1:3) and detection by the aniline oxalate reagent[8].

The molar ratios of hexoses and *N*-acetylhexosamines were determined by g.l.c. of the trifluoroacetyl derivatives, obtained[9] after methanolysis of oligosaccharides (methanol–0.5M HCl, 24 h, 80°).

Methylation analysis. — Oligosaccharides were reduced with $NaBD_4$, methylated[10], and methanolysed (methanol–0.5M HCl). The partially methylated methyl glycosides were acetylated[11] (pyridine–acetic anhydride, 1:1; 0.2 mL), and the products were analysed by g.l.c.–m.s. (Riber, model 10-10, Rueil-Malmaison, France), using a capillary column (0.35 mm × 60 m) coated with OV-101 or Carbowax 20-M (temperature programme, 100→220°, at 4°/min).

^1H-N.m.r. spectroscopy (500 MHz). — A Bruker WM-500 spectrometer, operating in the Fourier-transform mode, was used. Resolution enhancement of the

spectra was achieved by Lorentzian to Gaussian transformation from quadrature phase detection, according to Ernst[12]. Before analysis, the oligosaccharides were treated five times with D_2O with intermediate lyophilisation, finally using 99.96% D_2O (Aldrich). The probe temperature was 27°. The chemical shifts (δ) are expressed in p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate (DSS), but were actually measured by reference to internal acetone (δ 2.225) with an accuracy of 0.001 p.p.m.

RESULTS

Isolation and purification of oligosaccharides. — In Fig. 1, the stained paper chromatogram of oligosaccharides eluted from the charcoal–Celite column by a discontinuous gradient of aqueous ethanol is depicted. Of the numerous oligosaccharides, **1–5** are characteristic for Morquio syndrome type B, whereas the other compounds are generally present in the urine of individuals with secretor phenotype[13]. Oligosaccharides **1–5** were isolated and purified by preparative paper chromatography.

Structure determination of **1–5**. — The main characteristics of **1–5** are given in Table I. The sugar compositions suggest that they are products of the catabolism

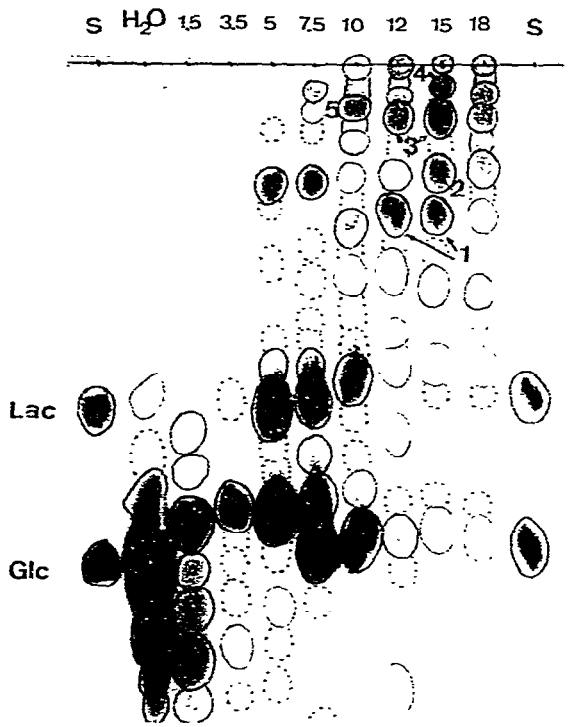
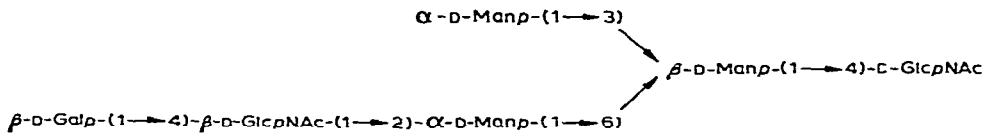


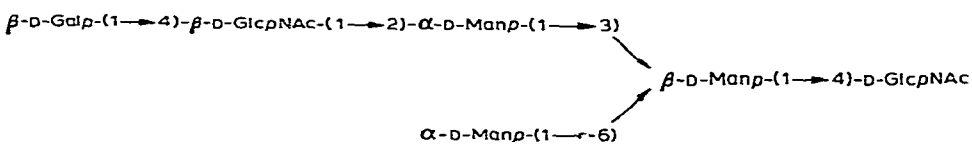
Fig. 1. Paper chromatogram of urinary oligosaccharides from a patient with Morquio syndrome type B, eluted from a charcoal–Celite column with a discontinuous gradient of aqueous ethanol (1.5 → 18%). Standards: Lac, lactose; Glc, D-glucose.


β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→6)-β-D-Manp-(1→4)-D-GlcpNAc

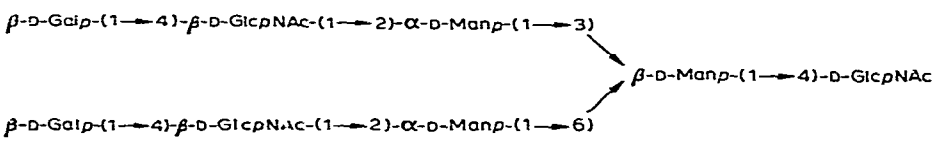
1


α-D-Manp-(1→3)
β-D-Manp-(1→4)-D-GlcpNAc
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→6)

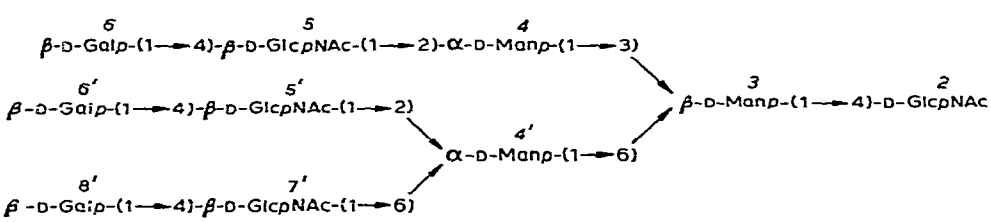
2a


β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→3)
β-D-Manp-(1→4)-D-GlcpNAc
α-D-Manp-(1→6)

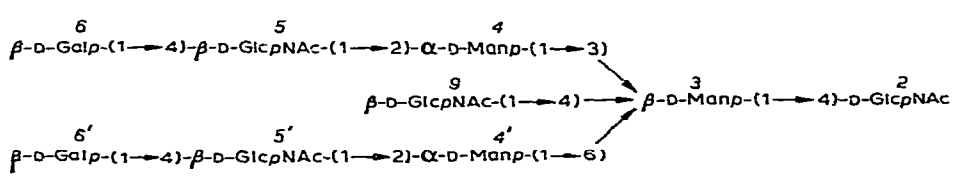
2b


β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→3)
β-D-Manp-(1→4)-D-GlcpNAc
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→6)

3


6
5
4
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→3)
6'
5'
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)
3
2
β-D-Manp-(1→4)-D-GlcpNAc
4'
α-D-Manp-(1→6)
8'
7'
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)

4


6
5
4
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→3)
9
β-D-GlcpNAc-(1→4)
3
2
β-D-Manp-(1→4)-D-GlcpNAc
6'
5'
4'
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→2)-α-D-Manp-(1→6)

5

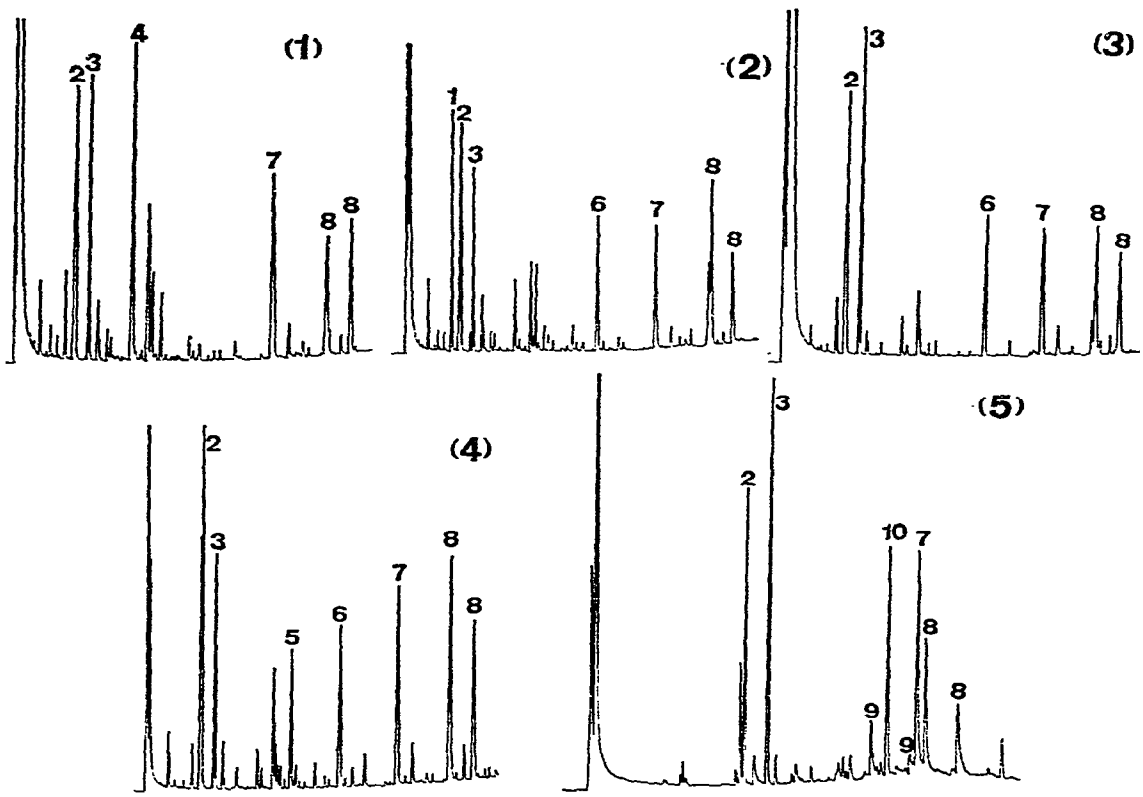


Fig. 2. G.l.c. analysis of partially methylated monosaccharide derivatives present in the methanolysates of the permethylated, reduced oligosaccharides 1–5: 1, 2,3,4,6-tetra-*O*-Me-Man; 2, 2,3,4,6-tetra-*O*-Me-Gal; 3, 3,4,6-tri-*O*-Me-Man; 4, 2,3,4-tri-*O*-Me-Man; 5, 3,4-di-*O*-Me-Man; 6, 2,4-di-*O*-Me-Man; 7, 1,3,5,6-tetra-*O*-Me-GlcN(Me)Ac-itol; 8, 3,6-di-*O*-Me-Glc(Me)Ac; 9, 3,4,6-tri-*O*-Me-GlcN(Me)Ac; 10, 2-*O*-Me-Man. For oligosaccharides 1–4: capillary column (0.35 mm × 60 m) coated with Carbowax 20-M; for 5: capillary column (0.35 mm × 60 m) coated with OV-101. Temperature 100 → 220°, at 4°/min.

TABLE I

ABUNDANCE AND MOLAR COMPOSITION OF THE OLIGOSACCHARIDES ISOLATED FROM THE URINE OF A PATIENT WITH MORQUIO SYNDROME TYPE B

Oligosaccharide	*Amount (mg/L)*	*Molar ratios*[a]		
		GlcNAc	*Gal*	*Man*
1	10	1.92	0.95	2.0
2	8	2.11	0.94	3.0
3	30	3.05	1.94	3.0
4	12	4.07	3.04	3.0
5	2.5	3.77	2.01	3.0

[a]Based upon 2 or 3 mol of mannose/mol of oligosaccharide.

TABLE II

MOLAR RATIOS OF PARTIALLY METHYLATED MONOSACCHARIDES PRESENT IN THE METHANOLYSATES OF THE PERMETHYLATED, REDUCED OLIGOSACCHARIDES FROM THE URINE OF A PATIENT WITH MORQUIO SYNDROME TYPE B

Partially methylated monosaccharide		*Oligosaccharide*				
		1	**2**	**3**	**4**	**5**
2,3,4,6-Tetra-*O*-Me-Man	(1)[a]	—	0.92	—	—	—
2,3,4,6-Tetra-*O*-Me-Gal	(2)	1.15	1.12	1.95	2.85	1.98
3,4,6-Tri-*O*-Me-Man	(3)	0.87	0.88	1.90	0.84	1.97
2,3,4-Tri-*O*-Me-Man	(4)	1.07	—	—	—	—
3,4-Di-*O*-Me-Man	(5)	—	—	—	0.82	—
2,4-Di-*O*-Me-Man	(6)	—	1.02	1.02	0.90	—
1,3,5,6-Tetra-*O*-Me-GlcN(Me)Ac-itol	(7)[b]	1.00	1.00	1.00	1.00	1.00
3,6-Di-*O*-Me-GlcN(Me)Ac	(8)	1.16	1.34	1.65	2.60	1.62
3,4,6-Tri-*O*-Me-GlcN(Me)Ac	(9)	—	—	—	—	0.71
2-*O*-Me-Man	(10)	—	—	—	—	1.05

[a]The numbers in brackets refer to the designation of the partially methylated monosaccharides in Fig. 2. [b]The molar ratios were determined on the basis of one residue of 1,3,5,6-tetra-*O*-Me-GlcN-(Me)Ac-itol.

of *N*-glycosylproteins. The results of qualitative g.l.c. analysis of the partially methylated methyl glycosides, obtained by methylation analysis of **1**–**5**, are shown in Fig. 2, and the molar ratios are summarised in Table II. The relevant ^{1}H-n.m.r. data, namely the chemical shifts of the structural reporter-groups[14] of the constituent monosaccharides, for **1**–**5** are compiled in Table III.

Oligosaccharide **1**. — The methylation studies (Table II) in combination with the molar carbohydrate composition (Table I) demonstrate that **1** contains a terminal Gal residue, whereas all of the other constituent monosaccharides are mono-substituted. Therefore, **1** is a linear pentasaccharide. The 500-MHz, ^{1}H-n.m.r. spectrum of **1** reveals the complete sequence of the monosaccharides and the configurations of their glycosidic linkages.

The spectrum shows the characteristic features of an *N*-acetyl-lactosaminic-type oligosaccharide, derived from a carbohydrate unit *N*-glycosylically linked to asparagine of a glycoprotein. As usual for reducing oligosaccharides of this type, terminating in a 4-substituted GlcNAc residue, denoted GlcNAc-*2* (for the numbering system of sugars residues, see ref. 15), the n.m.r. spectrum of **1** is a superposition of the subspectra of the two anomers containing the α and β forms of Glc*p*NAc-*2*[14,16]. In D_2O solution at room temperature, these anomers occur in a ratio of $\sim$2:1, as deduced from the intensity ratio of the GlcNAc-*2* H-1 signals at δ 5.216 and 4.726, and also from the *N*-acetyl signals of this residue at δ 2.063 and 2.059.

A β-Man residue (Man-*3*) is (1$\rightarrow$4)-linked to GlcNAc-*2*. Man-*3* is characterised by the chemical shifts (Table III) and the shapes of its H-1 and H-2 signals, together

TABLE III

^{1}H-CHEMICAL SHIFTS OF STRUCTURAL REPORTER-GROUPS OF CONSTITUENT MONOSACCHARIDES FOR SIX URINARY OLIGOSACCHARIDES, TYPICAL FOR A PATIENT WITH MORQUIO SYNDROME TYPE B.

Reporter group	*Residue*[a]	*Oligosaccharide anomer*	*Chemical shift in oligosaccharide*[a]					
			1	**2a**	**2b**	**3**	**4**	**5**
H-1 of	GlcNAc-*2*	α	5.216	5.213	5.213	5.211	5.206	5.204
		β	4.726	4.725	4.725	4.721	4.721	4.721
	Man-*3*	α	4.769	4.779	4.788	4.775	4.776	4.704
		β	4.760	4.768	4.779	4.765	4.770	4.686
	Man-*4*	α	—	5.106	5.122	5.122	5.133	5.059
		β	—	5.102		5.120	5.131	5.057
	Man-*4′*	α	4.920	4.925	4.920	4.926	4.874	5.008
		β	4.923	4.929	4.925	4.929		5.020
	GlcNAc-*5*	α,β	—	—	4.579	4.582	4.585	4.583
	GlcNAc-*5′*	α	4.582	4.583	—	4.582	4.592	4.579
		β						4.591
	Gal-*6*	α,β	—	—	4.467	4.467	4.468	4.468
	Gal-*6′*	α	4.470	4.470	—	4.470	4.468	4.473
		β	4.472	4.472	—	4.472	4.471	4.477
	GlcNAc-*7′*	α,β	—	—	—	—	4.555	—
	Gal-*8′*	α,β	—	—	—	—	4.480	—
	GlcNAc-*9*	α	—	—	—	—	—	4.466
		β	—	—	—	—	—	4.468
H-2 of	Man-*3*	α	4.089	4.263	4.263	4.259	4.260	4.190
		β	4.079	4.253	4.253	4.248	4.250	4.175
	Man-*4*	α,β	—	4.071	4.193	4.192	4.200	4.258
	Man-*4′*	α	4.104	4.112	<4.0	4.113	4.098	4.148
		β	4.107					4.142
NAc of	GlcNAc-*2*	α	2.063	2.061	2.058	2.060	2.059	2.064
		β	2.059	2.058	2.055	2.057	2.056	2.060
	GlcNAc-*5*	α,β	—	—	2.052	2.052	2.056	2.053
	GlcNAc-*5′*	α	2.048	2.048	—	2.048	2.043	2.044
		β	2.047	2.046	—	2.046	2.046	2.039
	GlcNAc-*7′*	α,β	—	—	—	—	2.039	—
	GlcNAc-*9*	α,β	—	—	—	—	—	2.064

[a]For complete structures and numbering of monosaccharide residues, see formulae.

with its $J_{1,2}$ value of 1.0 Hz[14,17]. Both signals are doubled, because **1** is a mixture of anomers as confirmed by the 2:1 intensity ratio for each pair of resonances. From the chemical shift of H-2 of Man-*3* (δ ~4.08), it can be concluded that this residue is mono-substituted at C-6 by an α-Man residue, denoted Man-*4′* (H-1, δ 4.92; $J_{1,2}$ 1.5 Hz)[14,18]. Man-*4′* bears a β-GlcNAc residue (H-1, δ 4.582; $J_{1,2}$ 7.9 Hz) at C-2, as can be inferred from the chemical shift of H-2 of Man-*4′* (δ 4.10)[18]. The terminal Gal residue (H-1, δ 4.47; $J_{1,2}$ 7.8 Hz) is β-(1→4)-linked to GlcNAc, completing the *N*-acetyl-lactosamine residue 5′–6′. The H-1 and H-2 signals of Man-*4′*,

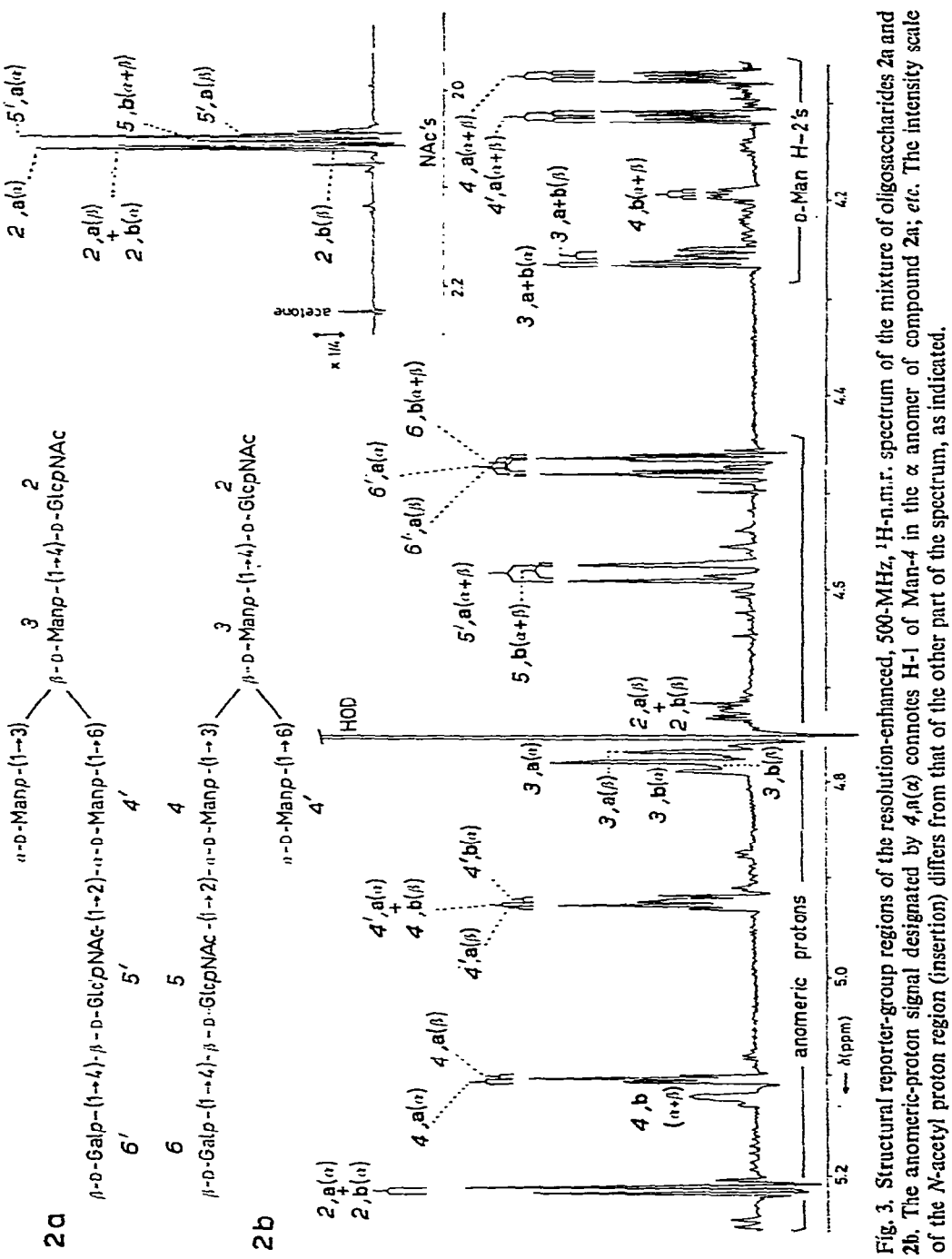


Fig. 3. Structural reporter-group regions of the resolution-enhanced, 500-MHz, ^{1}H-n.m.r. spectrum of the mixture of oligosaccharides 2a and 2b. The anomeric-proton signal designated by 4,a(α) connotes H-1 of Man-4 in the α anomer of compound 2a; *etc.* The intensity scale of the *N*-acetyl proton region (insertion) differs from that of the other part of the spectrum, as indicated.

the *N*-acetyl signal of GlcNAc-*5'*, and the H-1 doublet of Gal-*6'* are all doubled, in the anomeric ratio. This is in line with the earlier observation[14] that the influence of anomeric configuration is rather pronounced in the signals of the *4'–5'–6'* (lower) branch. Oligosaccharide **1** is therefore identified as a lower-branch, mono-antennary *N*-acetyl-lactosamine-type structure.

Oligosaccharide **2**. — The results of the methylation analysis of **2** (Table II) show that the Gal residues present occupy terminal positions. Further, the three Man derivatives indicate the presence of a Man residue which is substituted at C-3 and C-6, a terminal Man residue, and a Man residue substituted at C-2. The 500-MHz, ^{1}H-n.m.r. spectrum of **2** (Fig. 3) reveals that it is a mixture of two isomeric hexasaccharides, both ending in GlcNAc-*2*. These hexasaccharide components have in common the mannotriose branching-core in β-(1→4) linkage to GlcNAc-*2*, *i.e.*, a Man-*3* residue which is substituted at C-3 and C-6 by α-Man-*4* and α-Man-*4'*, respectively. This is evident from the chemical shift of H-2 of Man-*3* (Table III) in conjunction with the presence of Man-*4* and Man-*4'* structural reporter-group signals (Fig. 3)[14,17].

The conclusion that a mixture of two oligosaccharides is involved is primarily based on the occurrence of a pair of well-separated signals for H-1 of Man-*4* and for H-2 of Man-*4*, with relative intensities of 3:1 within each pair (Fig. 3). The main component (**2a**, 75%) contains a terminal Man-*4*. This can be deduced from the chemical shifts of H-1 and H-2 of this residue (δ 5.105 and 4.071, respectively). These values are identical with those for structurally related oligosaccharides containing a terminal Man-*4* residue, namely, α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-Glc*p*NAc[16] and α-D-NeuAc*p*-(2→6)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-[α-D-Man*p*-(1→3)]-β-D-Man*p*-(1→4)-D-Glc*p*NAc[19]. The relatively narrow lines of the H-1 signal for Man-*4* at δ 5.105 reflect the relatively large flexibility of Man-*4* with respect to the linkage conformation, inherent in a terminal sugar residue[17]. The Man-*4'* residue in **2a** bears a β-linked *N*-acetyl-lactosaminyl group at O-2, as is evident from the chemical shift for H-2 of Man-*4'* (δ 4.112) (*cf.* **1**, Table III). The n.m.r. features of GlcNAc-*5'* and Gal-*6'* in **2a** are in full agreement with those described for **1** (see Table III).

The set of signals with relatively low intensity (Fig. 3) is attributed to the minor component (**2b**, 25%) of the mixture. Compounds **2a** and **2b** differ in the location of the *N*-acetyl-lactosaminyl group. Compound **2b** contains a terminal Man-*4'*, since the H-2 signal of Man-*4'* is hidden in the bulk resonance of sugar skeleton protons ($3.9 < \delta$ H-2 < 4.0) (not shown in Fig. 3). This criterion has been applied in the structural characterisation of various oligosaccharides and glycopeptides containing a terminal Man-*4'* residue[14,16,18–20]. The substitution of Man-*4* in **2b** at O-2 by a β-linked *N*-acetyl-lactosaminyl group is reflected in the downfield shifts for H-1 ($\Delta\delta$ 0.018) and H-2 ($\Delta\delta$ 0.122) of Man-*4*, as compared to **2a** (Table III). Furthermore, the chemical shifts of the structural reporter-groups of GlcNAc-*5* and Gal-*6* in **2b** are slightly, but significantly, different from those of GlcNAc-*5'* and Gal-*6'* in **2a**, respectively. This is in line with earlier, 500-MHz, ^{1}H-n.m.r. observations on

related structures containing the *5–6* as well as the *5′–6′* *N*-acetyl-lactosaminyl group[14,17]. Finally, it is worth mentioning that the relatively small line-width of the H-1 signal of the terminal Man-*4* in **2a** renders possible the recognition of the influences of anomerisation upon the chemical shift of this structural reporter-group, whereas, for the substituted Man-*4* in **2b**, these are masked by considerable line-broadening.

Oligosaccharide **3**. — The results of sugar and methylation analyses (Table I and II) of **3** point to a doubly branched structure, terminated in both branches by a Gal residue, and possessing a 3,6-substituted Man residue as the branch point. The ^{1}H-n.m.r. data (Table III) indicate that **3** is a di-antennary oligosaccharide of the *N*-acetyl-lactosamine type, with a GlcNAc-*2* terminus. The presence of the mannotriose branching-core is inferred from comparison with the data for **2a** and **2b**. Both Man-*4* (H-2, δ 4.192) and Man-*4′* (H-2, δ 4.113) bear an *N*-acetyl-lactosaminyl group at O-2. The spectral features of **3** accord with those of the asialo di-antennary oligosaccharide isolated from the urine of patients with GM_1-gangliosidosis[14].

Oligosaccharide **4**. — The methylation studies of **4** (Table II) demonstrate that all three Gal residues (Table I) occupy terminal positions. Comparison with the corresponding data for **3** indicates that one of the two α-Man residues is substituted at C-2 and C-6, indicating the presence of an additional substituent on this Man residue. The 500-MHz, ^{1}H-n.m.r. data for **4** suggest the presence of a tri-branched structure, containing three *N*-acetyl-lactosaminyl groups linked to the mannotriose branching-core. This is indicated by the occurrence of three Gal H-1 doublets (one of which is doubled in the anomeric, intensity ratio) at $4.4 < \delta < 4.5$ and three H-1 doublets belonging to non-reducing GlcNAc residues at $4.5 < \delta < 4.6$, together with the presence of all of the Man-*3*, Man-*4*, and Man-*4′* structural reporter-group signals (Table III). Both Man-*4* and Man-*4′* bear an *N*-acetyl-lactosaminyl group at O-2 (Table II).

The assignment of the position of attachment of the third *N*-acetyl-lactosaminyl group to O-6 of Man-*4′* (and not Man-*4*) is based primarily on the shift decrements for H-1 and H-2 of Man-*4′* in comparison to **3** (Table III), whereas the chemical shifts for H-1 and H-2 of Man-*4* remain unaltered. The shift decrements are similar to those observed for these protons as a result of the introduction of the β-(1→6)-linked *N*-acetyl-lactosaminyl group in the step from tri- to tetra-antennary glycopeptides of the *N*-acetyl-lactosamine type[14,17]. Moreover, the chemical shifts for H-1 of the additional Gal (δ 4.481) and H-1 of the additional GlcNAc (δ 4.555) closely resemble those of the *N*-acetyl-lactosaminyl group *7′–8′*, β-(1→6)-linked to Man-*4′* in tetra-antennary structures[17]. The small downfield-shift for H-1 of GlcNAc-*5′* ($\Delta\delta$ 0.010, Table III) with respect to **3**, concomitant with a considerable line-broadening of this resonance due to the attachment of the third unit, corroborates the tri′-antennary* *N*-acetyl-lactosamine-type structure for oligosaccharide **4**.

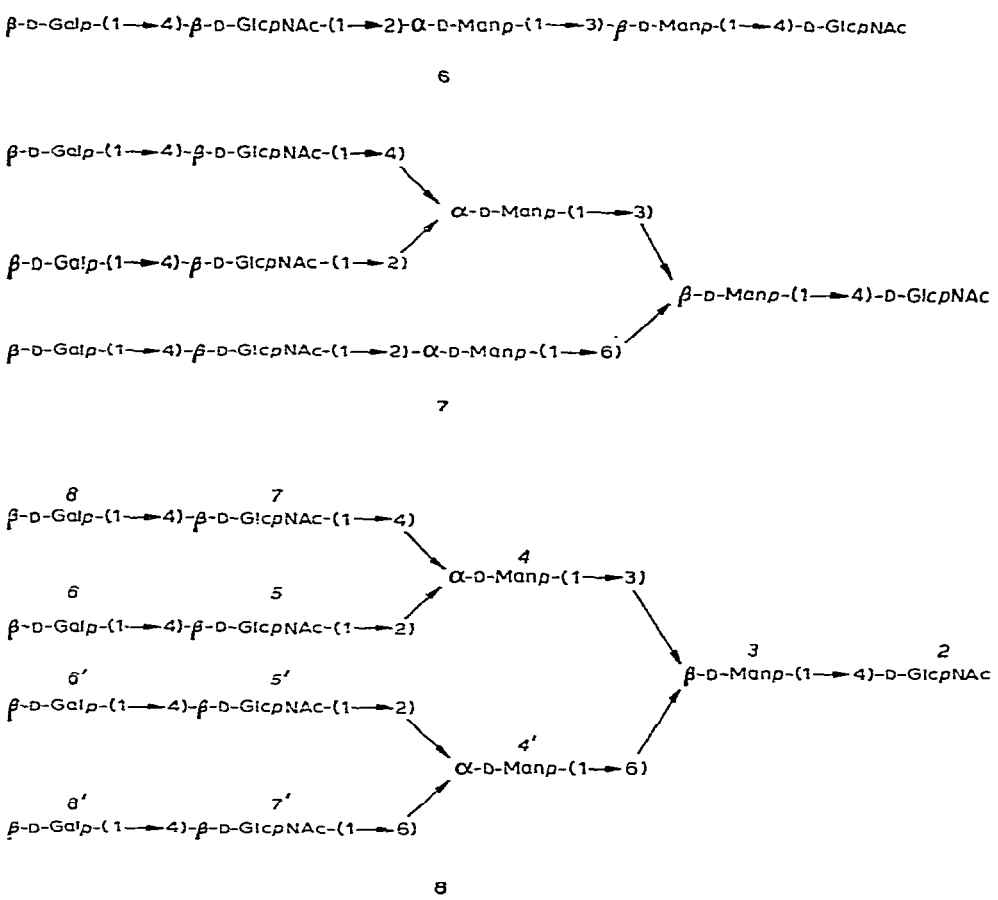
*It is necessary to introduce this designation provisionally, in order to discriminate between the tri-antennary structure[17] having the third *N*-acetyl-lactosaminyl group linked to O-4 of Man-*4*, and the structure of **4** presented here.

Oligosaccharide **5**. — The methylation analysis of **5** (Table II) affords, *inter alia*, 2-*O*-methylmannoside, implicating the occurrence of a 3,4,6-substituted Man residue in the structure. Comparison of the results of sugar and methylation analyses for **5** and **3** (Tables I and II) leads to the hypothesis that **5** is a di-antennary *N*-acetyl-lactosamine-type oligosaccharide, having an additional GlcNAc residue linked to O-4 of Man-*3*. This assumption is confirmed by the ^{1}H-n.m.r. data for **5** (Table III) which reveal the presence of an additional GlcNAc residue, when compared to **3**; the chemical shifts for H-1 (δ 4.47) and the *N*-acetyl group (δ 2.064) of this residue, together with the $J_{1,2}$ value (8.4 Hz), provide strong evidence for its location in β-(1→4)-linkage to Man-*3*. Similar values have been reported for the chemical shifts of the structural reporter-groups of this intersecting GlcNAc residue (designated GlcNAc-*9*), which occurs in a heptasaccharide isolated from the urine of a patient having Sandhoff's disease (GM_2-gangliosidosis, variant O)[21], and in the carbohydrate chain of chicken ovotransferrin[22]. The profound, characteristic influences of the attachment of GlcNAc-*9* on the chemical shifts of the signals for H-1 and H-2 of Man-*3*, Man-*4*, and Man-*4'* (*cf.* the data for **3** and **5** in Table III) are completely analogous to those described earlier[21,22]. Therefore, the structure of oligosaccharide **5** is identified.

DISCUSSION

The structures of six urinary oligosaccharides that are typical for a patient with Morquio syndrome type B were elucidated by 500-MHz, ^{1}H-n.m.r. spectroscopy in combination with methylation analysis. Oligosaccharides **1**–**5** belong to the family of *N*-acetyl-lactosamine-type carbohydrate chains, which probably have been split off from the GlcNAc residue, *N*-glycosylically linked to asparagine of a glycoprotein, by the action of an endo-β-D-hexosaminidase[15]. A striking, common feature of the structures is the preponderance of lower branch *N*-acetyl-lactosaminyl groups β-(1→2)- or β-(1→6)-linked to Man-*4'*. In this respect, there is a clear difference between the structures of urinary oligosaccharides from patients with GM_1-gangliosidosis and those with Morquio syndrome type B. Oligosaccharides **1**, **3**, and **4** have been reported to be excreted also by patients suffering from GM_1-gangliosidosis[15], but, in addition, oligosaccharides **6**–**8** have been found in the urine of these patients[15]. Oligosaccharides **2a**, **2b** and **5** are novel constituents in the series of oligosaccharides excreted by patients with lysosomal storage diseases.

The relationship of β-D-galactosidase deficiency in GM_1-gangliosidosis and in Morquio syndrome type B is not clear. The structure of the excreted oligosaccharides points to an impaired degradation of glycoconjugates in both disorders. The lower rate of excretion (10-fold less than in GM_1-gangliosidosis) and the absence of some oligosaccharide structures of the more-complete type (**6**–**8**) may be related to the presence of residual, acid β-D-galactosidase activity in Morquio syndrome type B. The mutant β-D-galactosidase in Morquio syndrome type B fibroblasts retains a residual activity of 5–10% of controls[3] towards various natural substrates, including



asialofetuin, keratan sulphate, GM_1-ganglioside, and lactose. The mutant enzyme is mainly present in a polymeric form, whereas the monomeric form prevails in control fibroblasts[23]. The Michaelis–Menten constant of the mutant enzyme towards *p*-nitrophenyl β-D-galactoside is increased[3] more than 10-fold. By the use of the oligosaccharides described here, which possess galactose residues at various non-reducing positions, as substrates for the residual β-D-galactosidase activity in Morquio syndrome type B, further information on the pathogenesis of the impaired catabolism of glycoconjugates may be obtainable.

ACKNOWLEDGMENTS

We thank Dr. E. Paschke (Münster, West Germany) for the generous gift of urine samples from a patient with Morquio syndrome type B, Professor K. von Figura (Münster) for his keen interest, Dr. W. E. Hull (Bruker Analytische Messtechnik, Rheinstetten, West Germany) for recording the 500-MHz, ^{1}H-n.m.r. spectra, and Mr. Y. Leroy, C.N.R.S. technician, for recording the mass spectra and for his expert

technical assistance with g.l.c. This investigation was supported by C.N.R.S. (Laboratoire Associé No. 217; Director: Professor J. Montreuil) I.N.S.E.R.M. (Contract No. 78.1.0523), the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organisation for the Advancement of Pure Research (Z.W.O.), and by grant UUKC.OC 79.13 from the Netherlands Foundation for Cancer Research (K.W.F.).

REFERENCES

1 J. S. O'Brien, E. Gogler, A. Giedon, U. Weissmann, N. Herschkowitz, C. Mier, and J. Leroy, *Clin. Genet.*, 9 (1976) 495–504.
2 A. I. Arbisser, K. A. Donelly, C. I. Scott, N. Di Ferrante, J. Singh, R. E. Stevenson, A. S. Aylsworth, and R. R. Howell, *Am. J. Med. Genet.*, 1 (1977) 195–205.
3 H. Groebe, M. Krins, H. Schmidberger, K. von Figura, K. Harzer, H. Kresse, E. Paschke, A. Sewell, and K. Ullrich, *Am. J. Hum. Genet.*, 32 (1980) 258–272.
4 R. Matalon, B. Argobast, and A. Dorfman, *Biochem. Biophys. Res. Commun.*, 61 (1974) 759–765.
5 J. Singh, N. Di Ferrante, P. Niebes, and D. Tavella, *J. Clin. Invest.*, 57 (1976) 1036–1040.
6 A. L. Horwitz and A. Dorfman, *Biochem. Biophys. Res. Commun.*, 80 (1978) 819–825.
7 S. Okada and J. S. O'Brien, *Science*, 160 (1968) 1002–1004.
8 S. M. Partridge, *Biochem. Soc. Symp.*, 3 (1950) 52–61.
9 J. P. Zanetta, W. C. Breckenridge, and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291–301.
10 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
11 B. Fournet, G. Strecker, Y. Leroy, and J. Montreuil, *Anal. Biochem.*, 116 (1981) 489–502.
12 R. R. Ernst, *Adv. Magn. Reson.*, 2 (1966) 1–135.
13 G. Strecker, C. Trentesaux-Chauvet, A. Poitau, and J. Montreuil, *Biochimie*, 58 (1976) 805–814.
14 J. F. G. Vliegenthart, H. van Halbeek, and L. Dorland, *Pure Appl. Chem.*, 53 (1981) 45–77.
15 J. Montreuil, *Adv. Carbohydr. Chem. Biochem.*, 37 (1980) 157–223.
16 H. van Halbeek, L. Dorland, G. A. Veldink, J. F. G. Vliegenthart, G. Strecker, J.-C. Michalski, J. Montreuil, and W. E. Hull, *FEBS Lett.*, 121 (1980) 71–77.
17 H. van Halbeek, L. Dorland, J. F. G. Vliegenthart, K. Schmid, J. Montreuil, B. Fournet, and W. E. Hull, *FEBS Lett.*, 114 (1980) 11–16.
18 G. Strecker, B. Fournet, J. Montreuil, L. Dorland, J. Haverkamp, J. F. G. Vliegenthart, and D. Dubesset, *Biochimie*, 60 (1978) 725–734.
19 M. C. Herlant-Peers, J. Montreuil, G. Strecker, L. Dorland, H. van Halbeek, G. A. Veldink, and J. F. G. Vliegenthart, *Eur. J. Biochem.*, 117 (1981) 291–300.
20 H. van Halbeek, L. Dorland, G. A. Veldink, J. F. G. Vliegenthart, J. C. Michalski, J. Montreuil, G. Strecker, and W. E. Hull, *FEBS Lett.*, 121 (1980) 65–70.
21 G. Strecker, M. C. Herlant-Peers, B. Fournet, J. Montreuil, L. Dorland, J. Haverkamp, J. F. G. Vliegenthart, and J. P. Farriaux, *Eur. J. Biochem.*, 81 (1977) 165–171.
22 L. Dorland, J. Haverkamp, J. F. G. Vliegenthart, G. Spik, B. Fournet, and J. Montreuil, *Eur. J. Biochem.*, 100 (1979) 569–574.
23 E. Paschke, K. Ullrich, K. von Figura, and H. Kresse, in R. Schauer, P. Boer, E. Buddecke, M. F. Kramer, J. F. G. Vliegenthart, and H. Wiegandt (Eds.), *Proc. Int. Symp. Glycoconjugates, 5th, Kiel*, Thieme, Stuttgart, 1979, pp. 404–405.