Carbohydrate Research, 254 (1994) 289–294
Elsevier Science B.V., Amsterdam

Note

Reporter resonances in the NMR spectra of oligosaccharides containing sialic acid linked to galactopyranose rings

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(Received March 15th, 1993; accepted in revised form September 20th, 1993)

Oligosaccharides and glycoconjugates having sialic acid subunit(s) form a large group of naturally occurring compounds (see for example ref 1); *N*-acetyl-α-neuraminic acid (α-NeuAc) residues are often found in (2 → 3)- or (2 → 6)-linkage to a β-galactose (β-Gal) or to an *N*-acetyl-α-galactosamine (α-GalNAc) residue. One of the easiest and most reliable methods for determining whether the linkage is of the (2 → 3)- or (2 → 6)-type is through analysis of the ^{1}H NMR resonances of the H-3 protons of the α-NeuAc residues. According to Vliegenthart et al.[2], for N-linked sugars the chemical shift of H-3a and H-3e of the α-NeuAc residue – depending on the actual structure – is 1.70–1.72 ppm and ~ 2.67 ppm, respectively, for the (2 → 6)-linkage and ~ 1.80 ppm (H-3a) and 2.76 ppm (H-3e) for the (2 → 3)-linkage. These values can be also used as guides in the analysis of O-linked or free oligosaccharides. For some compounds, however, somewhat different values have been found (see Table I).

The foregoing structural problems may also be solved using NOE measurements, but for the analysis of these NOE spectra the full assignment of the ^{1}H NMR spectrum is obligatory; this can be a time-consuming process, especially for samples in small amounts.

We report herein the assignment of the ^{1}H and the ^{13}C NMR spectra of an α-NeuAc-containing glycopeptide (1 the structure is shown in Fig. 1) of the O-glycosidic type, which was isolated from the urine of patients with an hereditary deficiency in α-*N*-acetylgalactosaminidase activity[3,4]. The ^{1}H NMR chemical shifts of structural reporter groups of the corresponding oligosaccharide alditol were published earlier[5].

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SSDI 0008-6215(93)E0338-2

TABLE I

^{1}H NMR chemical shift values of H-3a and H-6 resonances (in ppm) of different sugars containing α-NeuAc(2 → 3/6)(β-Gal/α-GalNAc) subunits

Compound (see Fig. 1)	H-3a (2 → 3) [a]	H-3a (2 → 6)	H-6 (2 → 3)	H-6 (2 → 6)	Ref
1	1.81		3.65		
	1.79		3.64		
2	1.80	1.67	3.64	3.69	13
3	1.80		3.65		14
4		1.76		3.73	15
5	1.80		3.62		16
6		1.72		3.69	16
7		1.73		3.70	17
8	1.77		3.63		20
9	1.80		3.65		17
10	1.77		3.62		18
11		1.72		3.69	17
12	1.79		3.63		18
13		1.71		3.70	18
		1.72		3.70	
14	1.79		3.63		19
15		1.71		3.67	20
16	1.78		3.64		20
17	1.78		3.63		10
18	1.80		3.63		10
19	1.78		3.62		21 [b]
20	1.78		3.63		10
	1.79 ± 0.013 (n = 19)	1.72 ± 0.020 (n = 8)	3.63 ± 0.011 (n = 15)	3.70 ± 0.017 (n = 8)	

[a] Type of linkage. [b] An incorrect value is reported in ref 9.

The assignment of the ^{1}H and ^{13}C NMR spectra of the glycopeptide was achieved using the 2D COSY[6], 2D ROESY[7], 1D TOCSY[8], and HMQC[9] techniques.

The sample was a 3:2 mixture of **1** (major component) and another glycopeptide having the same oligosaccharide unit attached to threonine. The ^{1}H and ^{13}C NMR chemical shifts were determined for **1** and are listed in Tables II and III, respectively.

We collected ^{1}H NMR data for 19 other compounds (see Fig. 1) containing α-NeuAc(2 → 3/6)(β-Gal/α-GalNAc) subunits and found that besides the chemical-shift values of the H-3 protons of the α-NeuAc, the chemical shift of the H-6 of the NeuAc is also characteristic for the type of the substitution, that is to say whether the linkage is (2 → 3) or (2 → 6) (see Table I). All these spectra were measured under similar conditions, i.e., neutral pH, at room temperature, in D_2O. The highest value measured for the (2 → 3)-linkage is 3.65 ppm, the lowest for the (2 → 6)-linkage is 3.67 ppm. For the (2 → 6)-linkage this value is higher than for the (2 → 3)-linkage with a mean and standard deviation of 3.70 ± 0.017 (n = 8) and 3.63 ± 0.011 (n = 15) ppm, respectively. It is clear from the data in Table I that the

1 α-NeuAc-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 6)[α-NeuAc-(2 → 3)-β-Gal-(1 → 3)]α-GalNAc-Ser
2 α-NeuAc-(2 → 3)-β-Gal-(1 → 3)[α-NeuAc-(2 → 6)]α-GalNAc-Thr
3 α-NeuAc-(2 → 3)-β-Gal-(1 → 4)-Glc
4 α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-Glc
5 α-NeuAc-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-Asn
6 α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-Asn
7 α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 3)[β-Gal-(1 → 4)[α-Fuc-(1 → 3)]β-GlcNAc-(1 → 6)]β-Gal-(1 → 4)-Glc
8 α-NeuAc-(2 → 3)-β-Gal-(1 → 3)[α-Fuc-(1 → 4)]β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc
9 α-NeuAc-(2 → 3)-β-Gal-(1 → 4)[α-Fuc-(1 → 3)]Glc
10 α-NeuAc-(2 → 3)-β-Gal-(1 → 3)[α-Fuc-(1 → 4)][α-NeuAc-(2 → 6)]β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc
11 α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 3)[α-Fuc-(1 → 2)-β-Gal-(1 → 4)[α-Fuc-(1 → 3)]β-GlcNAc-(1 → 6)]β-Gal-(1 → 4)-Glc
12 α-NeuAc-(2 → 3)-β-Gal-(1 → 3)[α-NeuAc-(2 → 6)]β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)[α-Fuc-(1 → 3)]-Glc
13 α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 3)[α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-β-(1 → 6)]β-Gal-(1 → 4)-Glc
14 α-NeuAc-(2 → 3)-β-Gal-(1 → 3)-α-GalNAc[Ala]SerProAlaGlu
15 α-NeuAc-(2 → 6)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc
16 α-NeuAc-(2 → 3)-β-Gal-(1 → 3)[α-NeuAc-(2 → 6)]β-GlcNAc-(1 → 3)-β-Gal-(1 → 4)-Glc
17 β-Gal-(1 → 4)-β-GlcNAc-(1 → 6)[α-NeuAc-(2 → 3)-β-Gal-(1 → 3)]α-GalNAc-(1 → 3)-GalNAc-ol
18 α-NeuAc-(2 → 3)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 6)[β-Gal-(1 → 3)]α-GalNAc-(1 → 3)-GalNAc-ol
19 β-Gal-(1 → 4)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 6)[α-NeuAc-(2 → 3)-β-Gal-(1 → 3)]α-GalNAc-(1 → 3)-GalNAc-ol
20 α-Gal-(1 → 4)-β-Gal-(1 → 4)-β-Gal-(1 → 4)-β-GlcNAc-(1 → 6)[α-NeuAc-(2 → 3)-β-Gal-(1 → 3)]α-GalNAc-(1 → 3)-GalNAc-ol

Fig. 1. Structures of the compounds screened.

average difference in chemical shifts for the H-6 protons in (2 → 3)- and (2 → 6)-linked compounds is statistically different ($p < 0.01$), comparable to the differences seen for one of the previously identified reporter groups, H-3a. This observation can be very useful in solving structural problems of the above type. It is important to note that for branched sugars the above rule is not valid if the monosaccharide in the branching position is in the alditol form (see data of ref 10).

For fast screening we suggest the use of the 1D TOCSY experiment. The experiment can easily be performed as the resonances belonging to H-3a protons

TABLE II

^{1}H NMR chemical-shift values of **1** (in ppm)

Residue	H-1	H-2	H-3	H-4	H-5	H-6A	H-6B		
GalNAc	4.894	4.346	4.089	4.225	4.19	4.10	3.73		
Gal(1 → 4)	4.564	3.589	4.128	3.975	3.74	n.d. [a]	n.d. [a]		
Gal(1 → 3)	4.542	3.541	4.076	3.947	3.65	3.74	3.74		
GlcNAc	4.582	3.761	3.71	3.73	3.61	4.01	3.86		
NeuAc	H-3ax	H-3eq	H-4	H-5	H-6	H-7	H-8	H-9A	H-9B
−[Gal(1 → 4)]	1.810	2.779	3.71 [b]	3.856 [c]	3.652 [d]	3.61	3.91	3.87	3.66
−[Gal(1 → 3)]	1.793	2.770	3.70 [b]	3.851 [c]	3.643 [d]	3.61	3.91	3.87	3.66

[a] Not determined. [b,c,d] Interchangable assignments.

TABLE III

^{13}C NMR chemical-shift values of 1 (in ppm)

Residue	C-1	C-2	C-3	C-4	C-5	C-6		
GalNAc	100.95	51.15	79.51	71.82	72.22	72.99		
Gal(1 → 4)	105.48	72.23	78.35 [a]	70.35	78.03	63.86 [b]		
Gal(1 → 3)	107.27	71.85	78.49 [a]	70.21	77.62	63.83 [b]		
GlcNAc	103.97	57.88	75.17	81.26	77.64 or 75.62	62.94		
NeuAc	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
	102.68 and 103.28	42.65 and 43.05	71.16 and 71.20	54.55 and 54.58	75.75 and 75.65	71.00 and 71.00	74.60 and n.d.	65.47 and 65.42

[a,b] Interchangable assignments.

of the α-NeuAc appear in a well-resolved region of the ^1H NMR spectrum. During the 1D TOCSY experiment the selective pulse is applied to the H-3a proton of the α-NeuAc and the experiment results in a subspectrum containing H-3a, H-3e, H-4,. H-5, and H-6 of the α-NeuAc (see Fig. 2). Because of the small $^3J_{H\text{-}6,H\text{-}7}$ coupling constant, the magnetization transfer stops at H-6. The assignment of

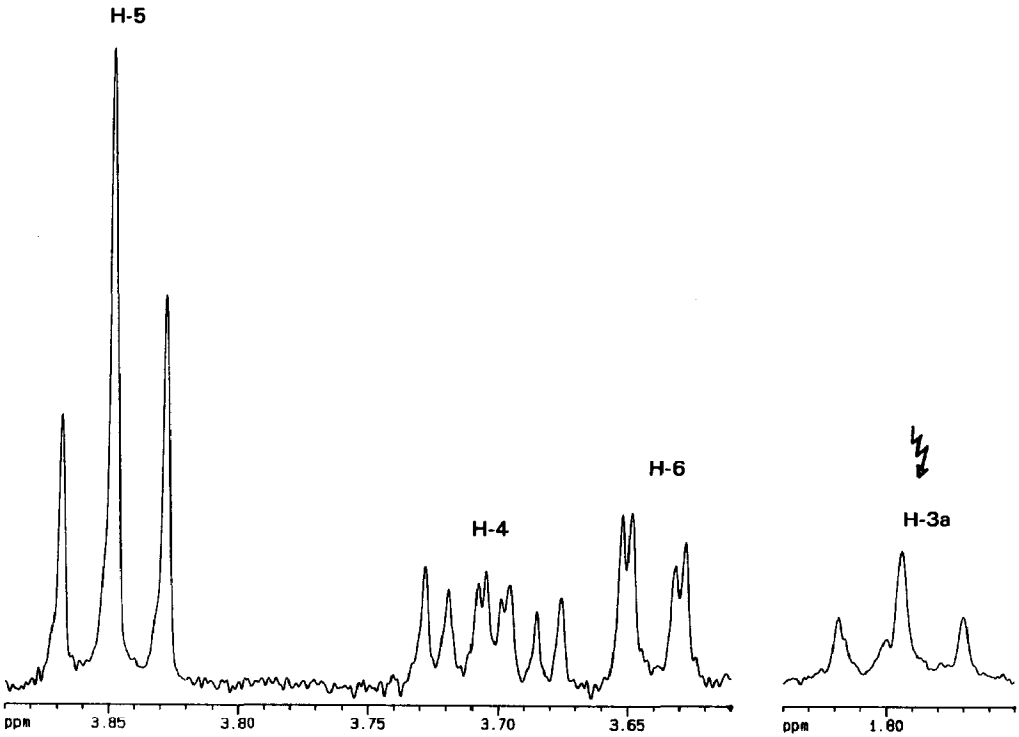

Fig. 2. 500-MHz 1D TOCSY spectrum. Selective irradiation at H-3a (H-3e is not shown).

these protons in the subspectrum is straightforward because of the different coupling pattern for these protons, so that the chemical shift of the H-6 proton(s) is easily obtained. This method gives relatively fast results even for samples in small amounts.

EXPERIMENTAL

Compound 1 was isolated and purified as described earlier[3]. The sample was repeatedly exchanged with D_2O and lyophilized prior to making the measurements. The NMR spectra were measured in 9:1 D_2O–acetone-d_6 at 303 K using a Bruker AMX-500 spectrometer. TSP was used as an internal standard.

For the 2D COSY, 1D TOCSY, and the HMQC experiments standard Bruker software was used (cosy45, selmlzf, invbtp). The 1D TOCSY experiments were performed using 90° half-Gaussian pulses of 120–160 ms length for the selective excitation[5]; for z-filter averaging ten different delay values were co-added; the mixing time was varied between 100 and 250 ms.

The ROESY experiment was performed using a z-filter[11]; spin lock was achieved with a train of pulses of small flip-angles[12]; the strength of the spin-lock field was 1800 Hz; during spin-lock the spectral offset was moved to the edge of the spectral window and the mixing time was 200 ms; the spectrum was measured in phase-sensitive mode using TPPI.

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