ELSEVIER

Carbohydrate Research 297 (1997) 145–151

CARBOHYDRATE RESEARCH

^{1}H and ^{13}C NMR analysis of the pentasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)-[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc synthesized by the mid-chain β-(1 → 6)-D-*N*-acetylglucosaminyltransferase of rat serum

Hannu Maaheimo, Jarkko Räbinä, Ossi Renkonen *

Institute of Biotechnology and Division of Biochemistry, University of Helsinki, P.O. Box 56, FIN-00014 Helsinki, Finland

Received 24 June 1996; accepted 9 October 1996

Abstract

Chemical shifts and coupling constants of completely assigned ^{1}H and ^{13}C NMR spectra at 500 MHz, as well as ROESY and HMBC connectivities were used to establish the structure of the pentasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc, synthesized by the action of the mid-chain β-(1 → 6)-D-*N*-acetylglucosaminyltransferase of rat serum from UDP-GlcNAc and the linear tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc. © 1997 Elsevier Science Ltd.

Keywords: β-(1 → 6)-D-*N*-acetylglucosaminyltransferase (GlcNAc to Gal); Oligo-(*N*-acetyllactosamino)glycans; Mid-chain branching; NMR

1. Introduction

Blood serum of man and rat contains a β-D-*N*-acetylglucosaminyltransferase, which transfers β-D-GlcNAc branches to the inner galactose(s) of linear oligo-(*N*-acetyllactosamino)glycans [1–3]. This mid-chain transferase differs from a better known, distally acting β-(1 → 6)-D-*N*-acetylglucosaminyltransferase, which transfers the incoming GlcNAc to the penultimate galactose close to the non-reducing end of the acceptor [2,4–7]. The bond formed by the mid-chain transferase was identified as GlcNAcβ(1 → 6)Gal, but the possibility of GlcNAcβ(1 → 2)Gal-linkage formation was not rigidly excluded [1]. However, the knowledge of the exact structure of the branch is of major importance for the synthesis of multivalent polylactosaminoglycans that have proved to be superior inhibitors of sperm-to-egg binding as well as of selectin-mediated leukocyte to endothelium adhesion [8–12].

* Corresponding author. Tel: +358-9-708 59375; Fax: +358-9-708 59563; e-mail: ossi.renkonen@helsinki.fi.

0008-6215/97/$17.00 Copyright © 1997 Elsevier Science Ltd. All rights reserved.
PII S0008-6215(96)00259-5

To further establish the position and the type of the linkage created, we present here structural analysis by ^{1}H and ^{13}C NMR spectroscopy of the pentasaccharide, synthesized by the rat serum enzyme from UDP-GlcNAc and the tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc.

2. Results and discussion

For structural analysis, the ^{1}H and ^{13}C NMR spectra of both the tetrasaccharide acceptor and the pentasaccharide product of the mid-chain β-D-*N*-acetylglucosaminyltransferase reaction were assigned. Most of the proton signals were readily assigned from DQFCOSY and TOCSY spectra, using the structural reporter groups as starting points. The H-5, H-6, and H-6′ resonances of the two Gals were identified from their ROEs to the H-4s of the same residues (see below). After assignment of the proton signals, the corresponding ^{13}C resonances were located in the HMQC spectra. The ^{13}C assignments of Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc were identical to those published [13]. In the case of the pentasaccharide, the ^{1}H and ^{13}C assignments were also validated in the HMBC spectrum.

The small signals present in the proton spectra of both the tetra- and penta-saccharide (Fig. 1) arise from epimerized structures, where the reducing-end GlcNAc residue **1** (see Fig. 1 for numbering of the monosaccharide residues) has been converted to ManNAc, probably non-enzymatically by a base catalyzed mechanism [14]. Analogous ManNAc epimers are observed regularly in the samples of polylactosamines carrying GlcNAc at the reducing end [3,15]. In the present case about 10% of the samples appeared in the epimerized form.

The structural analysis of the pentasaccharide was performed by observing the differences in the ^{1}H and ^{13}C chemical shifts of corresponding atoms of the tetra- and penta-saccharide, the type of interresidual ROE connectivities from the newly inserted GlcNAc residue, and the long-range interglycosidic $^{3}J_{C,H}$s from the HMBC spectrum.

The comparison of the one-dimensional (1D) proton spectrum of the pentasaccharide with that of the tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc (Fig. 1) reveals a new doublet, which has a typical chemical shift and coupling constant (4.585 ppm, 8.5 Hz) of a β-(1 → 6)-linked GlcNAc H-1 (GlcNAc to Gal) [7,16]. The H-1 and H-4 resonances of the Gal **2** have shifted to somewhat higher field, as is expected, if this Gal is at the branching position [16]. Table 1 reveals clearly that while the H-5, H-6, and H-6′ resonances of Gal **2** experience large changes due to the incoming of the GlcNAc, the H-2 signal remains uneffected. The signals of Gal **4** and the GlcNAc residues **1** and **3** are virtually unchanged.

When a glycosyl residue is bound to a certain carbon, it causes a major downfield shift (5–10 ppm) to the ^{13}C resonance of the carbon [17]. In the present case (Table 2), only the C-6 resonance of Gal **2** appeared to have experienced such a change (from 62.26 to 70.01 ppm). The CH_2 signal is easily recognized from its negative signal in the DEPT(135) spectrum (the DEPT spectrum is printed on the side of the HMQC spectrum in Fig. 2). While the C-5 resonance of Gal **2** has shifted slightly to the opposite direction, the other ^{13}C resonances of this residue, especially that of C-2, were not significantly affected by the incoming of the GlcNAc residue, and the chemical shifts of Gal **2** are typical of a 3,6-disubstituted Gal [16]. As in the proton spectrum, the transferase reaction caused only minor changes, if any, to the resonances of Gal **4** and GlcNAc residues **1** and **3**. Taken together, the ^{1}H and ^{13}C chemical shift data suggest that GlcNAc **5** is bound to position 6 of Gal **2**.

This result was confirmed in the ROESY spectrum (Fig. 3), in which cross-peaks were identified between H-1 of GlcNAc **5** and H-6 and H-6′ of Gal **2**. The galactose was identified as Gal **2**, because these protons had ROE connectivities also with H-4 of the same galactose at 4.149 ppm, which is a characteristic H-4 chemical shift of a galactose 3,6-disubstituted by β-GlcNAc residues [7]. Since the location of this H-4 signal at exceptionally low field is mainly caused by the free electron pairs of the GlcNAc bound at position 3 [18], a Gal substituted only at position 6 would have its H-4 resonance within the bulk region of the spectrum [19], excluding the possibility that GlcNAc **5** is bound to Gal **4**.

The most direct evidence of the (1 → 6) linkage was obtained from the HMBC spectrum (Fig. 2), in which long-range couplings from GlcNAc **5** C-1 to Gal **2** H-6 and H-6′ and from GlcNAc **5** H-1 to Gal **2** C-6 and the absence of a cross-peak between **5** H-1 and **2** C-2 reveal that the linkage between GlcNAc **5** and Gal **2** is (1 → 6). The Gal **2** C-6 is further coupled to **2** H-4, which has a chemical shift very characteristic of a 3,6-disubstituted Gal (see above) [20]. This H-4 is also coupled to **2** C-3, which has an interglycosidic coupling to H-1 of the GlcNAc **3**,

indicating that the Gal in question is in the middle of the chain. Interglycosidic cross-peaks were also found for the other glycosidic linkages of the pentasaccharide, further affirming its structure. As expected, no cross-peaks were found between GlcNAc **5** and Gal **4**.

Taken together, the NMR results establish that the linkage formed by the β-D-*N*-acetylgluco-

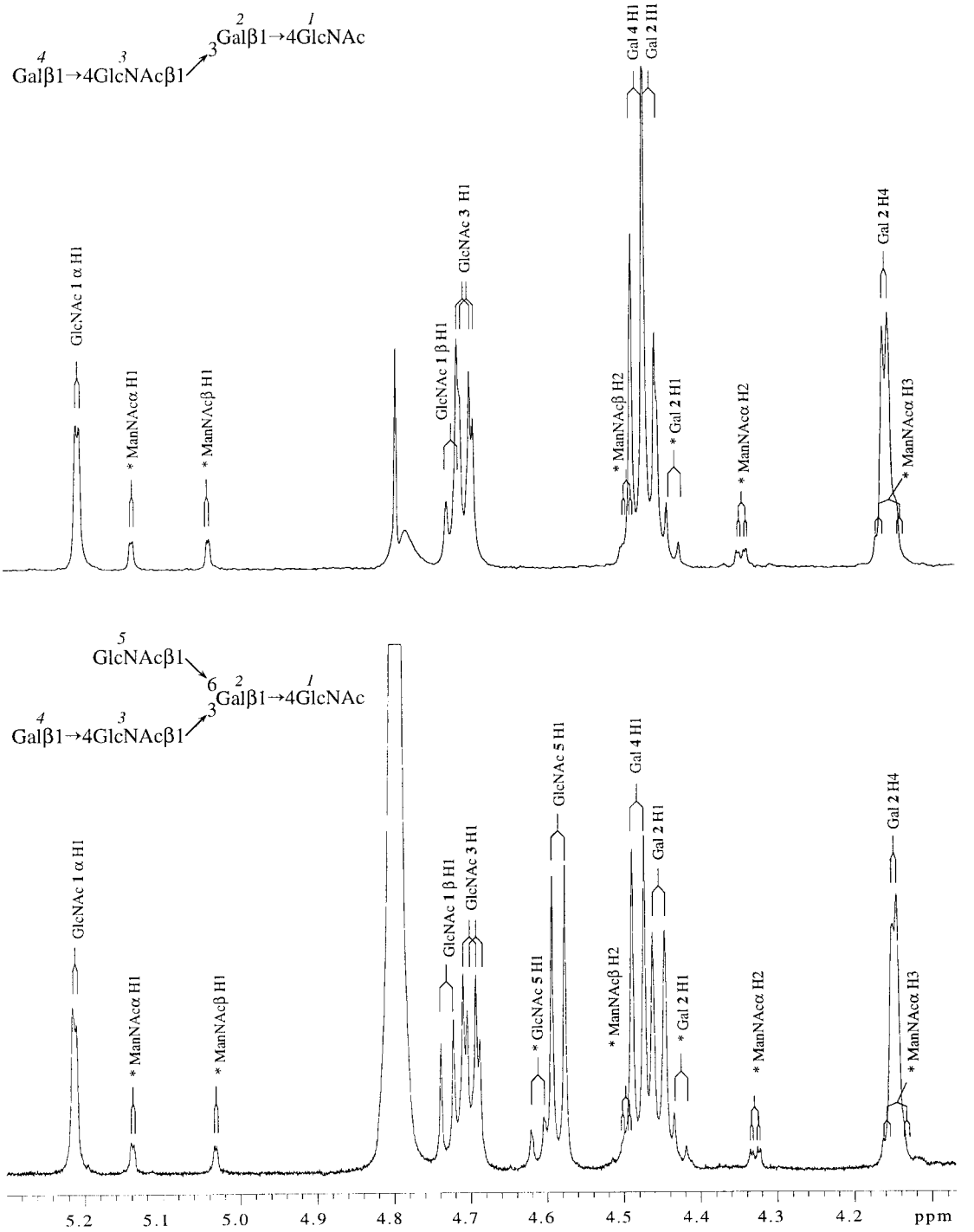

Fig. 1. Expansions of ^{1}H NMR spectra and denotation of the monosaccharide residues of the substrate tetrasaccharide (upper) and the product pentasaccharide (lower). The NMR signals marked by an asterisk (*) arise from the reducing-end ManNAc epimers Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)ManNAc and Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)ManNAc.

Table 1
^{1}H Chemical shifts (ppm) of the saccharides at 23 °C

Residue		H-1	H-2	H-3	H-4	H-5	H-6	H-6′	NAc
		Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc							
GlcNAc	**1**α	5.205	3.896	3.897	3.723	3.971	3.878	n.d. [a]	2.040
GlcNAc	**1**β	4.720	3.724	3.71	3.725	3.598	3.962	3.818	2.040
Gal	**2**	4.464	3.595/3.581	3.732/3.726	4.158	3.72	3.77	3.77	–
GlcNAc	**3**	4.706/4.702	3.808	3.731	3.743	3.583	3.955	3.850	2.035
Gal	**4**	4.479	3.540	3.668	3.926	3.72	3.77	3.77	–
		Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc							
GlcNAc	**1**α	5.212	3.904	3.906	3.672	3.963	3.875	n.d.	2.056
GlcNAc	**1**β	4.731	3.750	3.69	3.666	3.599	3.956	3.819	2.071
Gal	**2**	4.454	3.580	3.711	4.149	3.823	3.992	3.834	–
GlcNAc	**3**	4.701/4.696	3.801	3.725	3.740	3.579	3.959	3.850	2.032
Gal	**4**	4.481	3.538	3.671	3.925	3.732	3.746	3.783	–
GlcNAc	**5**	4.585	3.688	3.542	3.427	3.452	3.917	3.739	2.051

[a] n.d., Not determined.

saminyltransferase present in rat serum is that of GlcNAcβ(1 → 6) to the mid-chain galactose of the acceptor Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc. This tetrasaccharide is the smallest acceptor for the mid-chain β-(1 → 6)-D-*N*-acetylglucosaminyltransferase of human [1] and rat serum ([2] and A. Leppänen et al., unpublished results). According to 1D ^{1}H NMR spectra, the same type of linkage is formed when the acceptor is a longer linear oligo-(*N*-acetyllactosamino)glycan, but in that case multiple β-(1 → 6)-GlcNAc branches are generated to the acceptor saccharide (A. Leppänen et al., unpublished results).

3. Experimental

Synthesis of the oligosaccharides.—The tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc was synthesized essentially as earlier described [21]. The NMR spectra were recorded from 1.8 μmol of the tetrasaccharide.

Table 2
^{13}C Chemical shifts (ppm) of the saccharides at 23 °C

Residue		C-1	C-2	C-3	C-4	C-5	C-6	CH_3
		Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc						
GlcNAc	**1** α	91.82	55.01	70.56	80.06	71.58	61.25	23.19
GlcNAc	**1** β	96.17	57.48	73.79	79.67	76.15	61.37	23.50
Gal	**2**	104.24 [a]	71.29	83.34	69.65	76.19	62.26	–
GlcNAc	**3**	104.07	56.51	73.50	79.46	75.87	61.17	23.50
Gal	**4**	104.07 [a]	72.28	73.81	69.86	76.66	62.35	–
		Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc						
GlcNAc	**1** α	91.80	55.07	70.51	80.65	71.48	61.3	23.21
GlcNAc	**1** β	96.11	57.48	75.00	80.30	76.07	61.3	23.51
Gal	**2**	104.33	71.14	83.10	69.80	74.92	70.01	–
GlcNAc	**3**	104.03	56.51	73.49	79.45	75.86	61.18	23.51
Gal	**4**	104.17	72.28	73.81	69.86	76.66	62.34	–
GlcNAc	**5**	102.40	56.81	75.09	71.20	77.16	61.99	23.79

[a] Assignments may have to be exchanged.

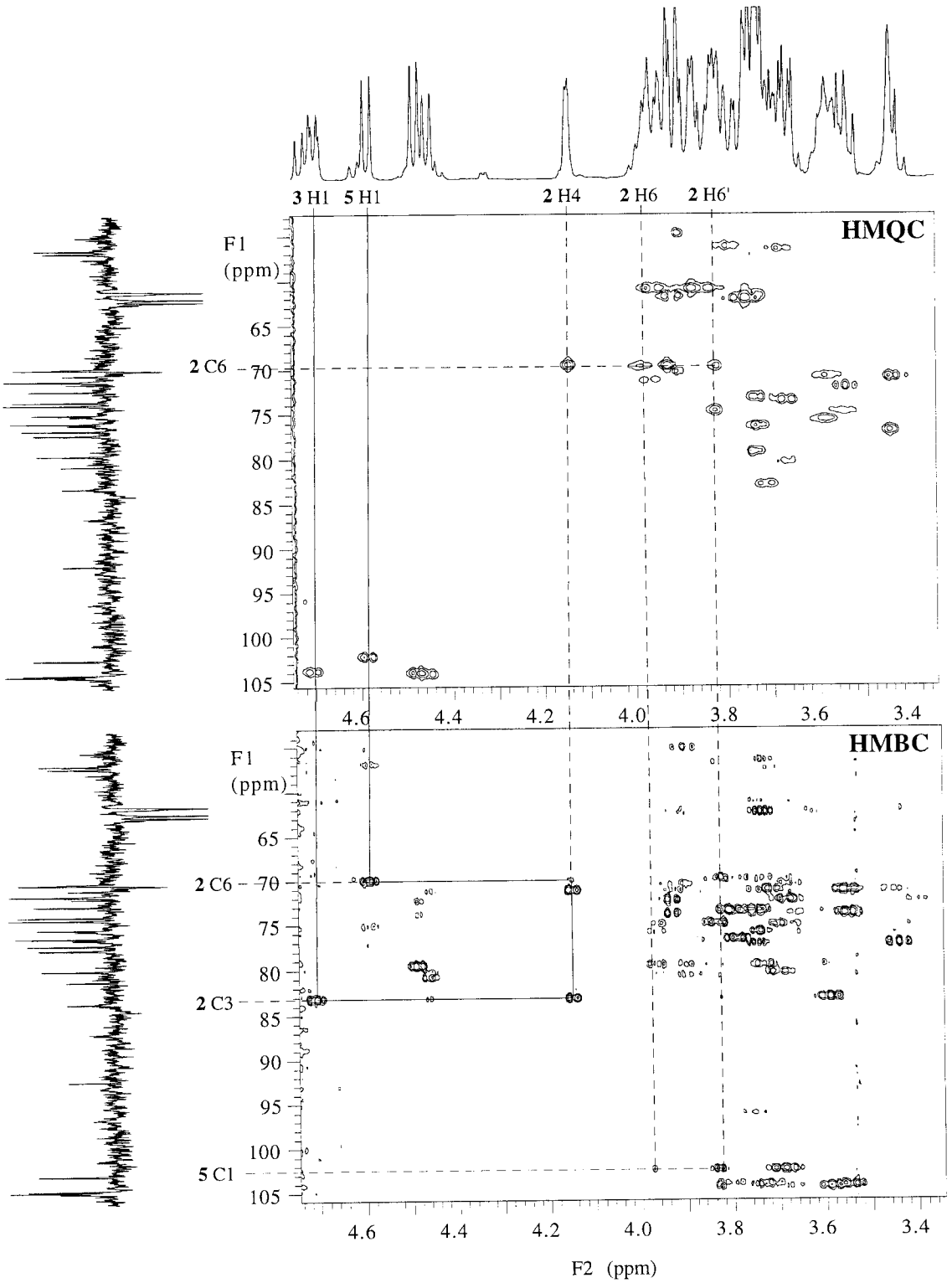

Fig. 2. HMQC and HMBC spectra of the pentasaccharide product with ^{1}H and ^{13}C DEPT(135) spectra printed on the sides of the 2D spectra. The series of $^{3}J_{C,H}$s **5** H-1 – **2** C-6 – **2** H-4 – **2** C-3 – **3** H-1 (solid line) establishes the insertion site to be the C-6 of Gal **2**. Another interglycosidic $^{3}J_{C,H}$ over the newly generated linkage is also evident between **5** C-1 and **2** H-6 and **2** H-6′.

The tetrasaccharide was converted to the pentasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc by a reaction with rat serum β-(1 → 6)-D-*N*-acetylglucosaminyltransferase as described earlier [3]. The NMR spectra were recorded from 2.7 μmol of the pentasaccharide.

NMR spectroscopy.—Prior to NMR experiments the saccharides were lyophilized twice from D_2O and then dissolved in 600 μL of D_2O (99.996 atom%, Cambridge Isotope Laboratories, Woburn, MA, USA). The NMR experiments were carried out on a Varian Unity 500 spectrometer at 23 °C. In recording 1D proton spectra a modification of the WEFT sequence [22] was used.

For the DEPT(135) [23] spectrum, 90,000 points were recorded with a spectral width of 15,000 Hz.

For the DQFCOSY [24] and TOCSY [25] experiments (16 scans per t_1 value), matrices of 2k × 256 and 4k × 512 points were collected and zero-filled to 2k × 512 and 4k × 1k, respectively. A 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations. In TOCSY, spin-lock times of 80 and 200 ms (MLEV-17) were used.

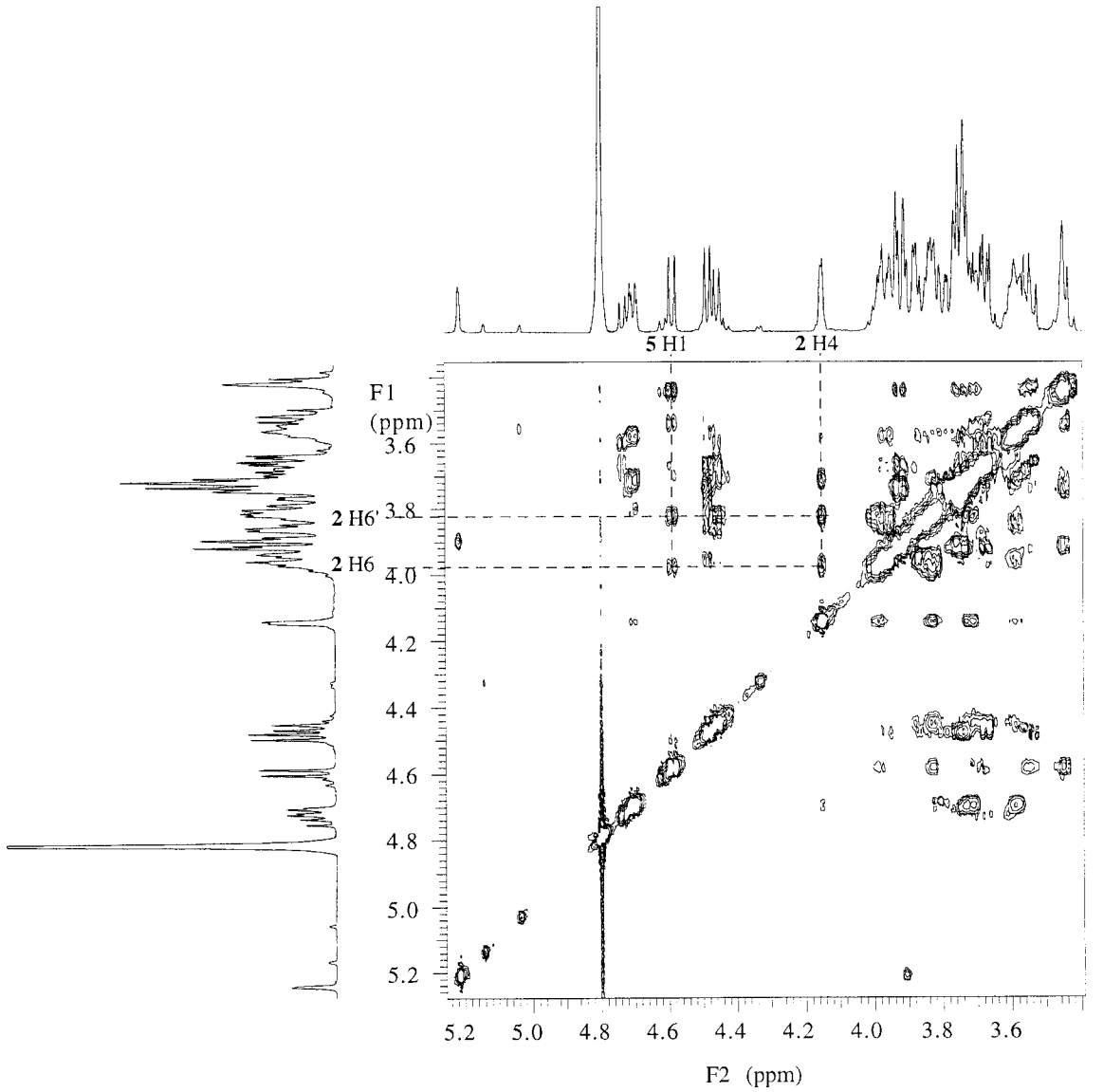

Fig. 3. ROESY spectrum of the pentasaccharide product of the mid-chain β-(1 → 6)-D-*N*-acetylglucosaminyltransferase reaction. The dashed lines indicate ROE connectivities between H-1 of the newly inserted GlcNAc residue (**5** H-1) and H-6 and H-6′ of a Gal residue (**2** H-6 and **2** H-6′). The ROE further to H-4 of the same Gal residue indicates that this is Gal **2** rather than **4**.

In recording the ROESY spectrum [26,27] (48 scans per t_1 value) of the deoxygenated sample, the transmitter was placed outside the signal area at 5.750 ppm and a continuous-wave spin-lock with spin-lock time of 300 ms was employed. A matrix of $2k \times 256$ points was collected with a spectral width of 3000 Hz, leaving the signals of *N*-acetyl groups as fold-back peaks at 8.03–8.05 ppm. The matrix was zero-filled to $2k \times 512$ points and a 90° shifted sine-bell weighting function was used in both directions.

For the HMQC [28] and HMBC [29] spectra (128 and 256 scans per t_1 value, respectively), matrices of $1k \times 256$ points were recorded and zero-filled to $1k \times 512$ points and a shifted sine-bell weighting function was used. The average $^{1}H-^{13}C$ coupling constant was estimated to be 150 Hz and Δ_2 was 60 ms.

The ^{1}H and ^{13}C chemical shifts were referenced to internal acetone, 2.225 and 31.55 ppm, respectively.

Acknowledgements

We thank Dr. Anita Teleman for a critical review of the manuscript. This work was supported in part by grants from the Technology Development Centre TEKES, the Academy of Finland, and the University of Helsinki.

References

[1] A. Leppänen, L. Penttilä, R. Niemelä, J. Helin, A. Seppo, S. Lusa, and O. Renkonen, *Biochem.*, 30 (1991) 9287–9296.
[2] J. Gu, A. Nishikawa, S. Fujii, S. Gasa, and N. Taniguchi, *J. Biol. Chem.*, 267 (1992) 2994–2999.
[3] R. Niemelä, J. Räbinä, A. Leppänen, H. Maaheimo, C.E. Costello, and O. Renkonen, *Carbohydr. Res.*, 279 (1995) 331–338.
[4] F. Piller, J.-P. Cartron, A. Maranduba, A. Veyrières, Y. Leroy, and B. Fournet, *J. Biol. Chem.*, 259 (1984) 13385–13390.
[5] I. Brockhausen, K.L. Matta, J. Orr, H. Schachter, A.H.L. Koenderman, and D.H. van den Eijnden, *Eur. J. Biochem.*, 157 (1986) 463–474.
[6] P. Ropp, M.R. Little, and P.-W. Cheng, *J. Biol. Chem.*, 266 (1991) 23863–23871.
[7] A.H.L. Koenderman, P.L. Koppen, and D.H. van den Eijnden, *Eur. J. Biochem.*, 166 (1987) 199–208.
[8] S.A. DeFrees, W. Kosch, W. Way, J.C. Paulson, S. Sabesan, R.L. Halcomb, D.-H. Huang, Y. Ichikawa, and C.-H. Wong, *J. Am. Chem. Soc.*, 117 (1995) 66–79.
[9] E.S. Litscher, K. Juntunen, A. Seppo, L. Penttilä, R. Niemelä, O. Renkonen, and P.M. Wassarman, *Biochemistry*, 34 (1995) 4662–4669.
[10] H. Maaheimo, R. Renkonen, J.P. Turunen, L. Penttilä, and O. Renkonen, *Eur. J. Biochem.*, 234 (1995) 616–625.
[11] J.P. Turunen, M.-L. Majuri, A. Seppo, S. Tiisala, T. Paavonen, M. Miyasaka, K. Lemström, L. Penttilä, O. Renkonen, and R. Renkonen, *J. Exp. Med.*, 182 (1995) 1133–1142.
[12] A. Seppo, J.P. Turunen, L. Penttilä, A. Keane, O. Renkonen, and R. Renkonen, *Glycobiology*, 6 (1996) 65–71.
[13] H.A. Nunez and R. Barker, *Biochemistry*, 19 (1980) 489–495.
[14] J.C. Speck, *Adv. Carbohydr. Chem.*, 13 (1958) 63–103.
[15] R. Niemelä, L. Penttilä, A. Seppo, J. Helin, A. Leppänen, J. Räbinä, L. Uusitalo, H. Maaheimo, J. Taskinen, C.E. Costello, and O. Renkonen, *FEBS Lett.*, 367 (1995) 67–72.
[16] D.M. Whitfield, H. Pang, J.P. Carver, and J.J. Krepinsky, *Can. J. Chem.*, 68 (1990) 942–952.
[17] K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
[18] J. Dabrowski, P. Hanfland, and H. Egge, *Biochem.*, 19 (1980) 5652–5658.
[19] H. Maaheimo, L. Penttilä, and O. Renkonen, *FEBS Lett.*, 349 (1994) 55–59.
[20] J.F.G. Vliegenthart, L. Dorland, and H. van Halbeek, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
[21] O. Renkonen, L. Penttilä, R. Niemelä, and A. Leppänen, *Glycoconj. J.*, 8 (1991) 376–380.
[22] K. Hård, G. van Zadelhoff, P. Moonen, J.P. Kamerling, and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 209 (1992) 895–915.
[23] D. Doddrell, D. Pegg, and M. Bendall, *J. Magn. Reson.*, 48 (1982) 323–327.
[24] D. Marion and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 117 (1985) 967–974.
[25] A. Bax and D.G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
[26] A.A. Bothner-By, R.L. Stephens, J. Lee, C.D. Warren, and R.W. Jeanloz, *J. Am. Chem. Soc.*, 106 (1984) 811–813.
[27] A. Bax and D.G. Davis, *J. Magn. Reson.*, 63 (1985) 207–213.
[28] M.F. Summers, L.G. Marzilli, and A. Bax, *J. Am. Chem. Soc.*, 108 (1986) 4285–4294.
[29] A. Bax and M.F. Summers, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.