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Note

Complete assignments of ^{13}C NMR resonances to all the carbon atoms of the trimannosido-di-*N*-acetylchitobiosyl structure in a pentaantennary decasaccharide glycopeptide

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In our previous reports [1–3], we determined the structures of bulky *N*-linked glycan chains of hyosophorin molecules isolated from the eggs of different fish species. These glycan chains were revealed to have either tetra- or pentaantennary structure, depending on fish species. Our hypothesis is that such multiantennary-multibranched *N*-glycan structures constitute important structural units for hyosophorin to function during early embryogenesis [1–4].

Conformations of the trimannose structure in complex bi- and triantennary *N*-linked glycans [2,4-substituted α-mannose (α-Man) is attached to the 3 position of β-Man and 2-substituted α-Man attached to the 6 position of β-Man] have been well studied using ^{1}H NMR measurement [5–14]. However, the conformational elucidation of the trimannose structure in penta- and tetraantennary *N*-linked glycans which possess the 2,4,6-substituted α-Man or 2,6-substituted α-Man residue have not been so well studied. As the first step in elucidation of the conformations of multiantennary *N*-linked glycan chains, we have recently carried out the complete assignment of ^{1}H chemical shifts of

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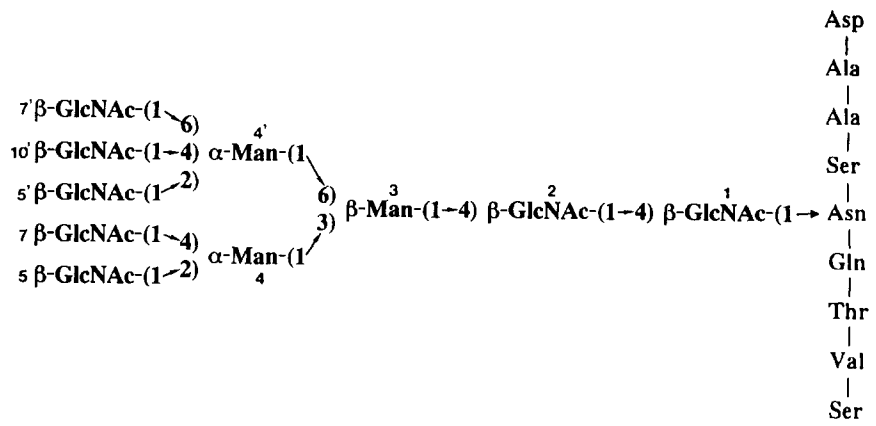

Fig. 1. Structure of pentaantennary-core glycopeptide used in this study. Residue numbers are included.

three Man residues and have also characterized the dihedral angle ω (O-6–C-6–C-5–H-5) of the α-Man-(1 → 6)-β-Man sequence in the pentaantennary-core glycopeptides prepared from *Oryzias latipes* hyosophorin molecule [15]. Measurements of proton–proton distances by nuclear Overhauser effect spectroscopy (NOESY) are necessary for determining the conformations. However, heavily overlapped chemical shifts of H-3–H-6,6′ (3.5–4.0 ppm region) disallowed assignments of a number of NOE signals. Recently, total correlation spectroscopy (TOCSY) and NOESY combined with $^{1}H-^{13}C$ heteronuclear multiple quantum coherence spectroscopy (HMQC) have been used to solve the chemical shift-overlapping problems [12,16,17]. We have started this line of experiments for conformational elucidation, and here we first report the complete ^{13}C chemical shift assignments of all carbons in the trimannose unit (Man-3, 4, and 4′ residues) of the pentaantennary-core glycopeptide (Fig. 1).

HMQC and HMQC-TOCSY spectra ($\delta_{^1H}$, 3.0–5.5 ppm; $\delta_{^{13}C}$, 55–105 ppm) of the pentaantennary-core glycopeptide are shown in Figs 2 and 3, respectively. As shown in Table 1, the chemical shifts of anomeric carbon atoms of trimannosido-di-*N*-acetylchitobiose portion are unequivocally assigned in the HMQC spectrum (Fig. 2), using the anomeric proton resonances assigned previously [15]: Man-4, 5.13 ppm; Man-4′, 4.83 ppm; Man-3, 4.74 ppm; GlcNAc-1, 5.03 ppm; GlcNAc-2, 4.63 ppm. With respect to the five terminal GlcNAc residues (GlcNAc-5, 5′, 7, 7′, and 10′), we have determined from NOESY and TOCSY–NOESY experiments (unpublished results) that the H-1 of GlcNAc-5 resonates at 4.55 ppm, GlcNAc-5′ at 4.54 ppm, and GlcNAc-7, 7′, and 10′ at 4.51–4.53 ppm. From these results, combined with the information that the chemical shift of the anomeric carbon of the GlcNAc residue $\beta(1 \rightarrow 2)$ linked to the α-Man residue is shifted up-field compared to that of the GlcNAc residue $\beta(1 \rightarrow 4)$ or $\beta(1 \rightarrow 6)$ linked to the α-Man residue by about 2 ppm [14,18–20], two discrete groups of H-1–C-1 correlations A and B observed in the HMQC spectrum (Fig. 2) are assignable as follows: the cross peak A is composed of GlcNAc-5 and 5′ residues and B is composed of GlcNAc-7, 7′, and 10′ residues, respectively.

The assignments of C-2–C-6 atoms in the Man-4′ residue, which is $\alpha(1 \rightarrow 6)$ linked to the β-Man residue, is performed as follows. In the HMQC–TOCSY spectrum (Fig.

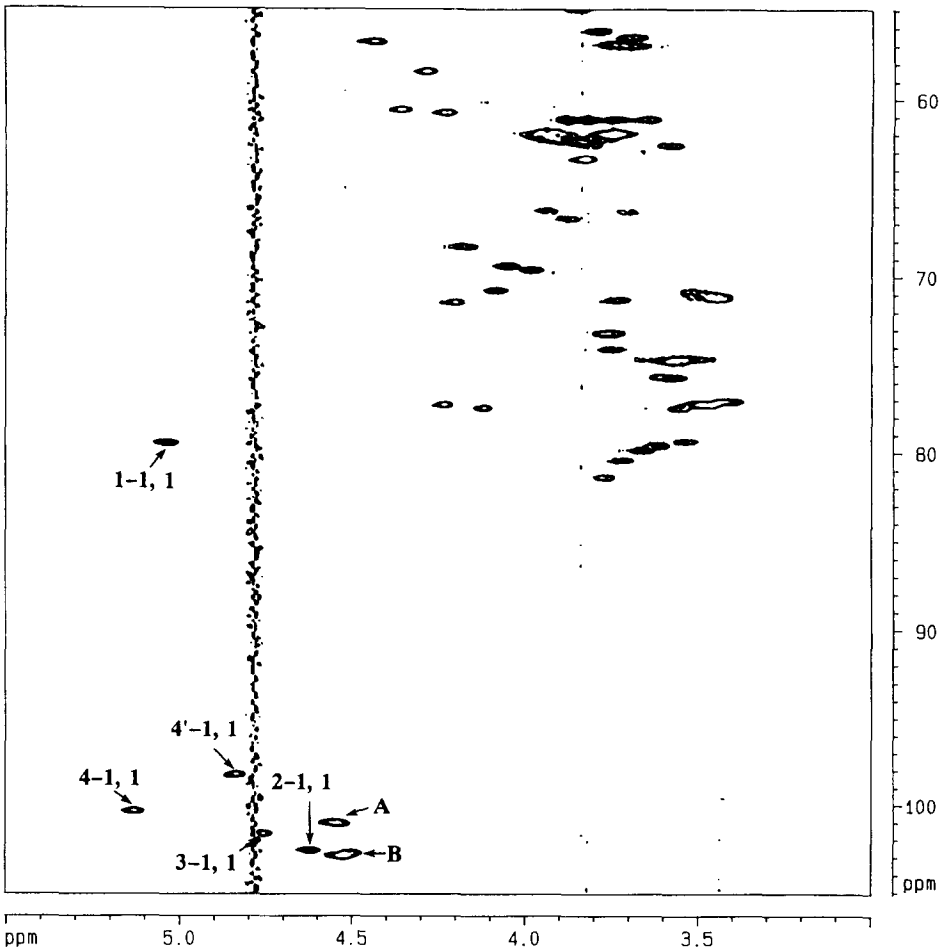

Fig. 2. $^{1}H-^{13}C$ HMQC spectrum ($\delta_{^{1}H}$, 3.0–5.5 ppm; $\delta_{^{13}C}$, 55–105 ppm) of pentaantennary-core glycopeptide in D_2O at 25°C. 4-1,1: cross peak between H-1 and C-1 of Man-4, etc.

3), a cross peak at δ_H/δ_C 4.83/77.5 on the H-1 track through the anomeric $^{1}H-^{13}C$ correlation (δ_H/δ_C 4.83/98.3) and a cross peak at δ_H/δ_C 4.11/98.3 on the ^{13}C-1 track were observed. These two cross peaks lead to the assignment of the H-2/C-2 pair (δ_H/δ_C 4.11/77.5) of the Man-4′ residue. On the H-2 track through this H-2/C-2 cross peak (a) in Fig. 4, three relayed cross-peaks (b, δ_C 79.4; c, δ_C 71.2; d, δ_C 69.7) were observed. Inspection of the HMQC spectrum, combined with the chemical shift values of H-3, 4, and 5 of Man-4′ residue [15], identifies the corresponding $^{1}H-^{13}C$ correlations (e, H-3/C-3 3.97/69.7; f, H-4/C-4 3.54/79.4; g, H-5/C-5 3.74/71.2) on the respective ^{13}C tracks. A cross peak (h) on the H-3 track through the peak (e) and a peak (i) on the ^{13}C-3 track leads to assign the H-6–C-6 correlation (j, δ_H/δ_C 4.08/70.8) of this residue. All the ^{13}C chemical shift values of the Man-4′ residue are listed in Table 1.

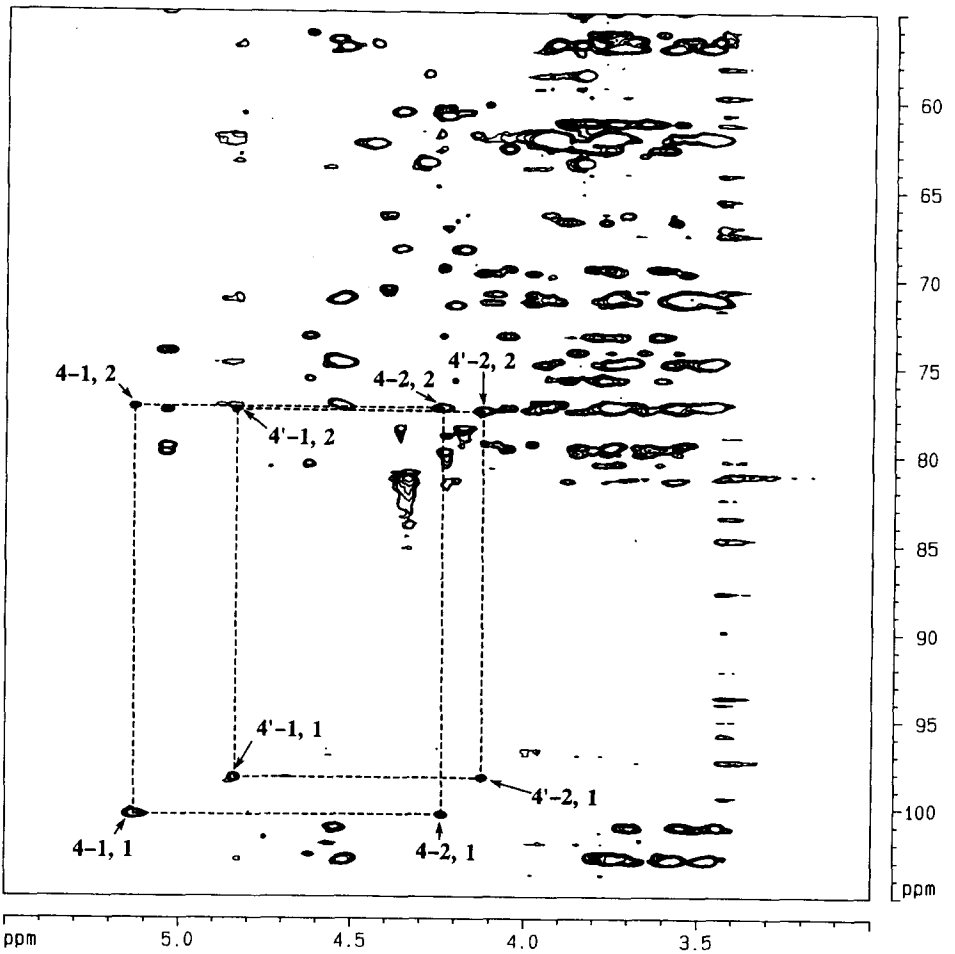

Fig. 3. $^{1}H-^{13}C$ HMQC–TOCSY spectrum (δ_{1_H}, 3.0–5.5 ppm; δ_{13_C}, 55–105 ppm) of pentaantennary-core glycopeptide in D_2O at 25°C. Broken lines are drawn to show some correlations present.

Using the same procedure, we can also assign all the carbon atoms of the Man-3 and Man-4 residues. Assignments of C-1 through C-5 of the GlcNAc-1 and GlcNAc-2 residues were made by comparison with the data reported in the literature [14,21], and the results are also included in Table 1. This represents the first complete assignments of the ^{13}C atoms in trimannosyl structure of multiantennary *N*-glycan chains.

The specific points noted for the Man-4′ residue are large down-field shifts of C-4 (79.4 ppm) and C-6 (70.8 ppm) compared with the corresponding values for the 2-substituted Man-4′ residue ($\delta_{C\text{-}4}$, 68.63 ppm; $\delta_{C\text{-}6}$, 63.14 ppm) in the triantennary *N*-linked glycan [14] and those for the 2-substituted Man residue ($\delta_{C\text{-}4}$, 68.2 ppm and $\delta_{C\text{-}6}$, 62.5 ppm) in the β-GlcNAc-(1 → 2)-α-Man-(1 → 6)-β-Glc-OR structure [22]. These large down-field shifts on C-4 and C-6 are considered to be due to the substitution effect of the 4 and 6 positions of Man-4′ by β-GlcNAc residues.

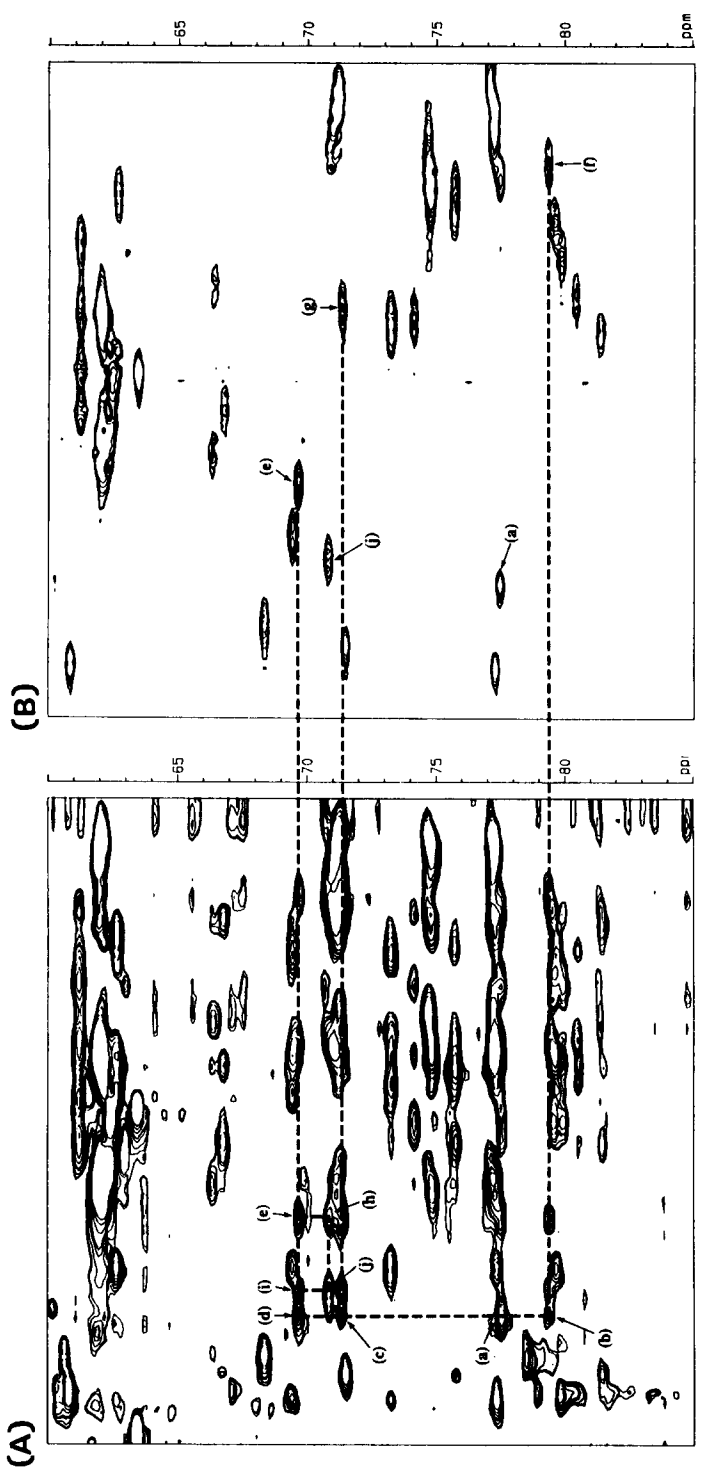

Fig. 4. (A) Part of the ^{1}H–^{13}C HMQC–TOCSY spectrum (δ_{H}, 3.4–4.3 ppm; $\delta_{^{13}C}$, 60–85 ppm) of pentaantennary-core glycopeptide in D_2O at 25°C and (B) a corresponding part of the HMQC spectrum. Broken lines are drawn to show some correlations present about Man-4′ residue.

Table 1
Assignments of carbon atoms of the trimannosido-di-*N*-acetyl-chitobiose structure in the pentaantennary-core glycopeptide [a]

	GlcNAc-1	GlcNAc-2	Man-3	Man-4	Man-4′
C-1	79.5	102.5	101.6	100.3	98.3
C-2	55.0	56.2	71.5	77.2	77.5
C-3	74.2	73.3	81.4	69.4	69.7
C-4	80.0	80.5	66.8	79.6	79.4
C-5	77.6	75.9	75.7	73.2	71.2
C-6	n.d.	n.d.	66.3	62.6	70.8

[a] Chemical shift values are expressed in ppm at 25°C.

Chemical shifts of C-3, 4, and 6 in the Man-3 residue are slightly different from those in the triantennary *N*-linked glycan ($\delta_{C\text{-}3}$, 81.63 ppm; $\delta_{C\text{-}4}$, 66.96 ppm; $\delta_{C\text{-}6}$, 66.96 ppm) [14]. This may represent the differences of conformation of the α-Man-(1 → 3)-β-Man → linkage between pentaantennary and triantennary glycans. The fact that dihedral angle ω (O-6–C-6–C-5–H-5) in the α-Man-(1 → 6)-β-Man → sequence of the pentaantennary glycan favours 180° (namely, *gauche–gauche* rotamer) [15] may also affect the carbon chemical shifts of the Man-3 residue (particularly on C-6).

Although $\delta_{H\text{-}3}$ of the Man-3 residue and $\delta_{H\text{-}5}$ of the Man-4 residue were found to be so close to each other in our recent investigation [15], the ^{13}C chemical shift of C-3 (81.4 ppm) of the Man-3 residue was observed as much as ca. 8 ppm from that of C-5 (73.2 ppm) of the Man-4 residue. This observation may provide key information [8] on linkage conformational analysis for the α-Man-(1 → 3)-β-Man → sequence of the pentaantennary *N*-glycan chain. Studies aimed at elucidating this linkage conformation by NOESY and HMQC–NOESY experiments are currently being further explored in our laboratory.

1. Experimental

The pentaantennary-core glycopeptide was prepared as described earlier [3,15]. All the NMR spectra were recorded with a Bruker AMX-500 spectrometer at 25°C. ^1H chemical shifts were expressed relative to the methyl proton signal of sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 set equal to 0.00 ppm and ^{13}C chemical shifts relative to 1,4-dioxane at 67.9 ppm. The 2D HMQC experiment [23] was carried out with a delay time of 2.5 ms. The spectral width for ^1H was 2400 Hz and that for ^{13}C was 8000 Hz. The GARP1 pulse sequence [24] was used for ^{13}C decoupling during the acquisition. Free induction decays (64 scans each) of 2K real data points in the t_2 domain were collected for 512 data points in the t_1 domain. By zero-filling in the t_1 domain, spectra of 2K × 1K data points were obtained. 2D HMQC–TOCSY experiment was performed with modification in the HMQC part for the suppression of ^{12}C magnetization [25]. The spectral width for ^1H was 4000 Hz and that for ^{13}C was 8000 Hz. Isotropic mixing was induced by an MLEV17 pulse train [26] of 70 ms with preceding trim pulse. The value

of delay time ($1/4J_{CH}$) was set to 1.7 ms. Free induction decays (192 scans each) of 1K real data points in the t_2 domain were collected for 512 data points in the t_1 domain. By zero-filling in the t_1 domain, spectra of 1K × 1K data points were obtained.

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