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2D Selective-TOCSY–DQFCOSY and HSQC–TOCSY NMR experiments for assignment of a homogeneous asparagine-linked triantennary complex type undecasaccharide

Hajime Sato,[a,*] Kazuhiro Fukae[b] and Yasuhiro Kajihara[c]

[a]*Bruker Biospin K.K., 3-21-5 Ninomiya Tsukuba, Ibaraki 305-0051, Japan*
[b]*Research and Technical Center, Otsuka Chemical Co. Ltd, 463 Kagasuno Kawauchi, Tokushima 771-0193, Japan*
[c]*International Graduate School of Arts and Sciences, Yokohama City University, 22-2 Seto Kanazawa-ku, Yokohama 236-0027, Japan*

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Abstract—The assignment of ^{1}H and ^{13}C NMR signals of a complex type triantennary asialooligosaccharide was examined using 2D selective-TOCSY–DQFCOSY and HSQC–TOCSY experiments. The 2D selective-TOCSY–DQFCOSY experiment exhibits a 2D DQFCOSY spectrum of an individual monosaccharide in the undecasaccharide, although the NMR signals of several monosaccharides in the triantennary undecasaccharide are heavily overlapped. Selective excitation of each anomeric proton signal and subsequent TOCSY experiment afforded transverse magnetization corresponding to all of the proton signals of the monosaccharide. This magnetization was then developed with the corresponding DQFCOSY pulse sequence to afford the DQFCOSY spectrum of the individual monosugars. In this case, four GlcNAc-b, -e, -j, and -h residues were excited as a mixture. In order to assign ^{13}C signals, a conventional 2D HSQC–TOCSY spectrum was examined and compared with an unambiguous assignment of 2D selective-TOCSY–DQFCOSY thus obtained. This systematic analysis made it possible to obtain an assignment of the ^{1}H and ^{13}C NMR signals of the triantennary undecasaccharide. In addition, these experiments also revealed all of the glycosyl positions in the triantennary undecasaccharide.

Keywords: Triantennary undecasaccharide; 2D Selective-TOCSY–DQFCOSY; HSQC–TOCSY; Complete assignment; Glycosyl position

1. Introduction

An asparagine-linked oligosaccharide (N-linked oligosaccharide) consists of 5–7 monosaccharides and exhibits several antennary structures. The N-linked oligosaccharide is known to be the essential molecule for protein function and is divided into three groups, the high mannose, complex, and hybrid type. High mannose oligosaccharides have been shown to be involved in protein quality control in the endoplasmic reticulum, while complex type oligosaccharides seem to be essential for regulating protein half life in blood, antigenicity, and protein conformational properties.[1] In order to investigate such oligosaccharide functions, oligosaccharides have been prepared from a natural source or have been synthesized. Recently, synthetic technology has been refined, and high-antennary oligosaccharides can now be synthesized.[2] In addition, trNOE,[3] STD,[4] and intermolecular NOE experiments[5] have also been conducted using such oligosaccharides in order to elucidate oligosaccharide and protein interactions.

For these experiments, unambiguous assignment of the ^{1}H and ^{13}C signals of the oligosaccharide is essential. However, ^{1}H signals are observed in a very narrow region of the ^{1}H NMR spectrum, and this has been a hindrance making it difficult to assign all of the ^{1}H of the oligosaccharides. TOCSY-based experiments, such as 1D selective-TOCSY, 2D HSQC–TOCSY, and TOCSY–HSQC experiments, are useful methods for an analysis of the individual sugar components in oligosaccharides.[6–16] The proton signals which are remotely

* Corresponding author. Fax: +81 29 858 0322; e-mail: hajime.sato@bruker-biospin.jp

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positioned from the anomeric proton (H-1), such as the 6-position (H-6), can be empirically assigned by employing various isotropic-mixing times.

A spectral resolution has improved by a band-selective homonuclear-decoupled 2D NMR experiments.[17] This experiment enabled us to resolve signals, which overlap in normal 2D spectra. And recently, an H2BC experiment has been developed to solve such problems.[18–20] This experiment enabled us to sequentially assign ^{1}H and ^{13}C signals of oligosaccharide by connecting the 1-bond correlation by the relationship of H-1 to C-1 (H1/C1) to the 2-bond correlation (H1/C2), then H1/C2 to H2/C2 and all the way to H6/C6 on the overlaid HSQC and H2BC spectra. We have also examined the possibility of establishing a convenient ^{1}H and ^{13}C signal assignment method for large oligosaccharides by use of the TOCSY-based experiments. In our experiments, a selective excitation pulse such as RE-BURP[21] was used for excitation of the anomeric proton signal of individual monosugar residues, and then this magnetization was applied to the TOCSY pulse sequence to obtain a signal set of corresponding monosugars in the oligosaccharide.

Using this simple method, we have developed 2D selective-TOCSY–DQFCOSY[22–24] and the 2D selective-TOCSY–HSQC[24] experiments to assign ^{1}H and ^{13}C signals of a biantennary asparagine-linked complex type undecasaccharide. Assignment of the ^{1}H and ^{13}C signals of the biantennary undecasaccharide was feasible. The critical point in this system is that the extracted transverse magnetization of a desired monosugar in the biantennary undecasaccharide by the selective-TOCSY experiment can be used for making DQFCOSY and HSQC spectra corresponding to the desired monosugar by means of the DQFCOSY or HSQC pulse sequences. This makes it feasible to assign these signal patterns such as monosugars without requiring a lot of empirical knowledge. In addition, the information thus obtained from the assignment of DQFCOSY supports the assignment of carbon signals in the selective-TOCSY–HSQC spectrum, which also displays a simplified HSQC spectrum corre-

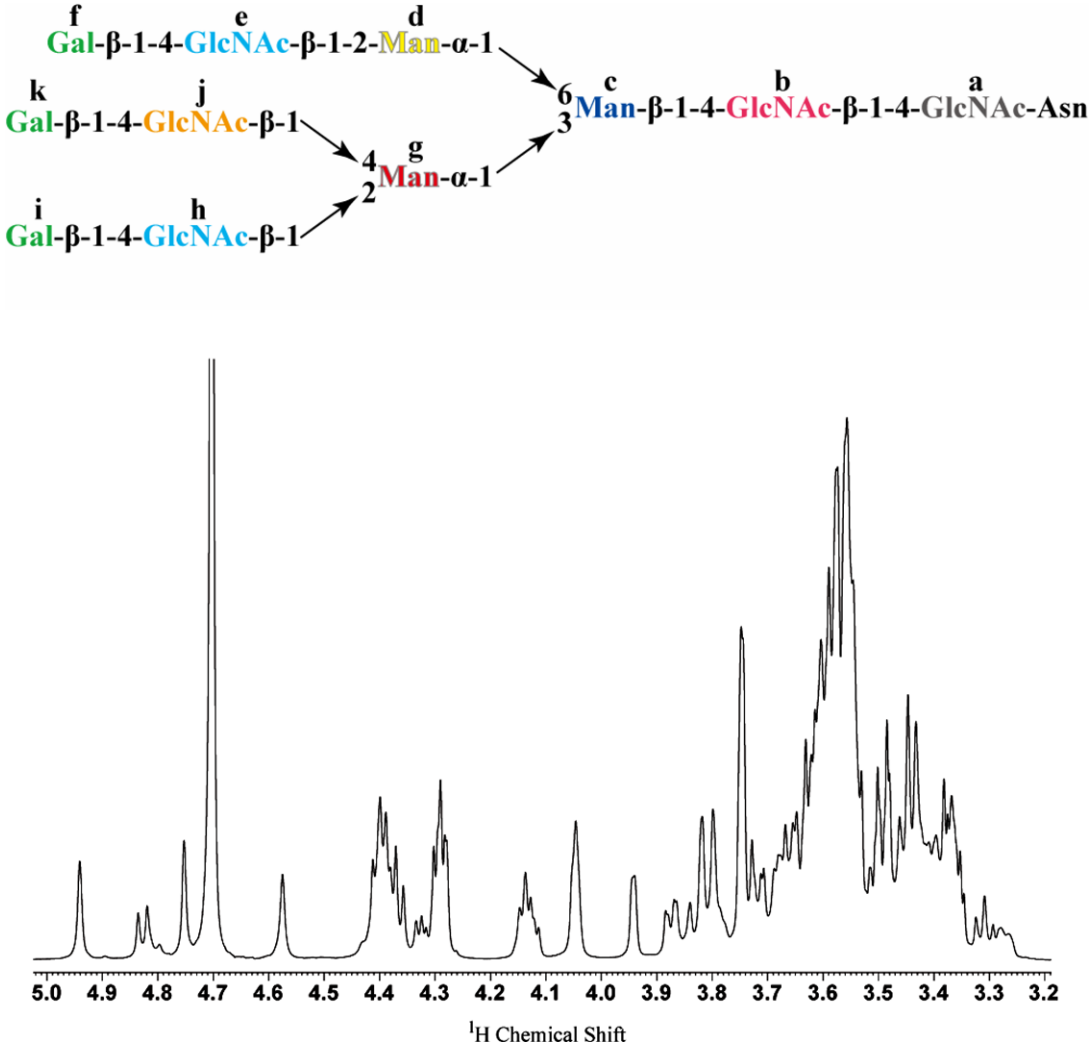

Figure 1. Primary structure and the 600-MHz ^{1}H NMR spectrum of the triantennary undecasaccharide.

sponding to the desired monosugar in the biantennary undecasaccharide. Non-empirical and systematic procedures in assigning the ^{1}H and ^{13}C signals can be carried out by use of these measurements. Therefore, the glycosyl shift phenomena[24–26] in the ^{13}C-dimension were easily found in the 2D selective-TOCSY–HSQC spectrum.

Different strategies and different NMR experiments are required for structural analysis toward more complex large oligosaccharides. In order to evaluate utility of the 2D selective-TOCSY based experiments, we examined structural analysis toward complex large oligosaccharide, human triantennary complex type undecasaccharide. In this paper, we will describe the assignment of ^{1}H and ^{13}C NMR signals of a triantennary undecasaccharide and discuss the utility of the 2D selective-TOCSY based experiments.

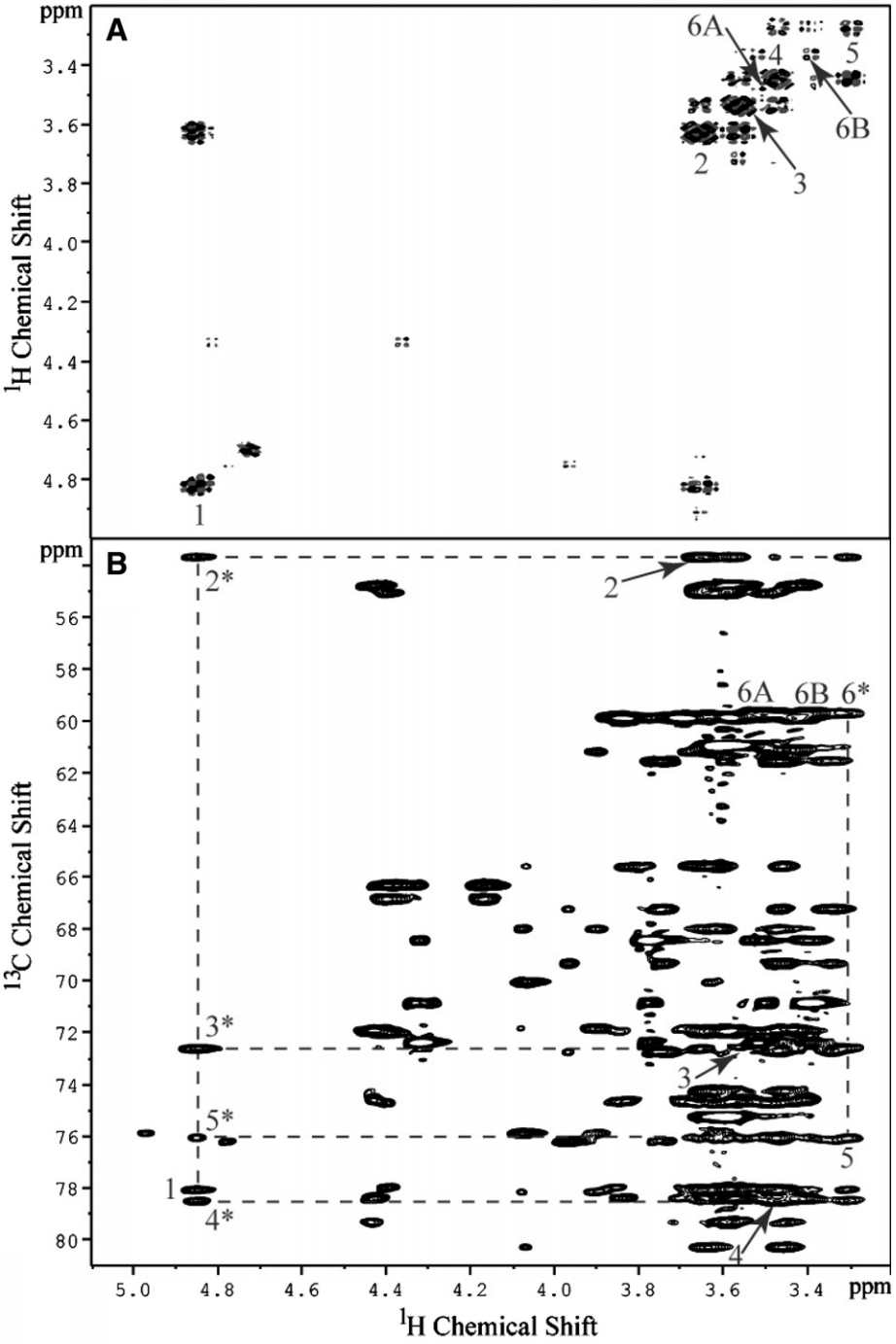

Figure 2. 2D Selective-TOCSY–DQFCOSY (A) and HSQC–TOCSY (B) spectra of GlcNAc-a. A and B were measured on 600 MHz and 400 MHz instruments, respectively. Assignment of the diagonal and the ^{1}H– ^{13}C 1-bond correlation signals of the GlcNAc-a residue were numbered from 1 to 6 on A and B, respectively. The long-range correlations of H-1 with C-2 to C-5 and of H-5 with C-6 were numbered from 2^{*} to 6^{*} on B.

2. Results

It is known that fetuin possesses complex triantennary oligosaccharides. We isolated one of the triantennary undecasaccharides and analyzed its structure by 2D selective-TOCSY–DQFCOSY and conventional HSQC–TOCSY experiments. The triantennary complex type undecasaccharide was dissolved in deuterium oxide and then was analyzed by ^{1}H NMR spectroscopy, as shown in Figure 1. Although several anomeric proton signals were observed in the 4–5 ppm area, almost all the sugar ring protons were observed from 3 to 4 ppm,

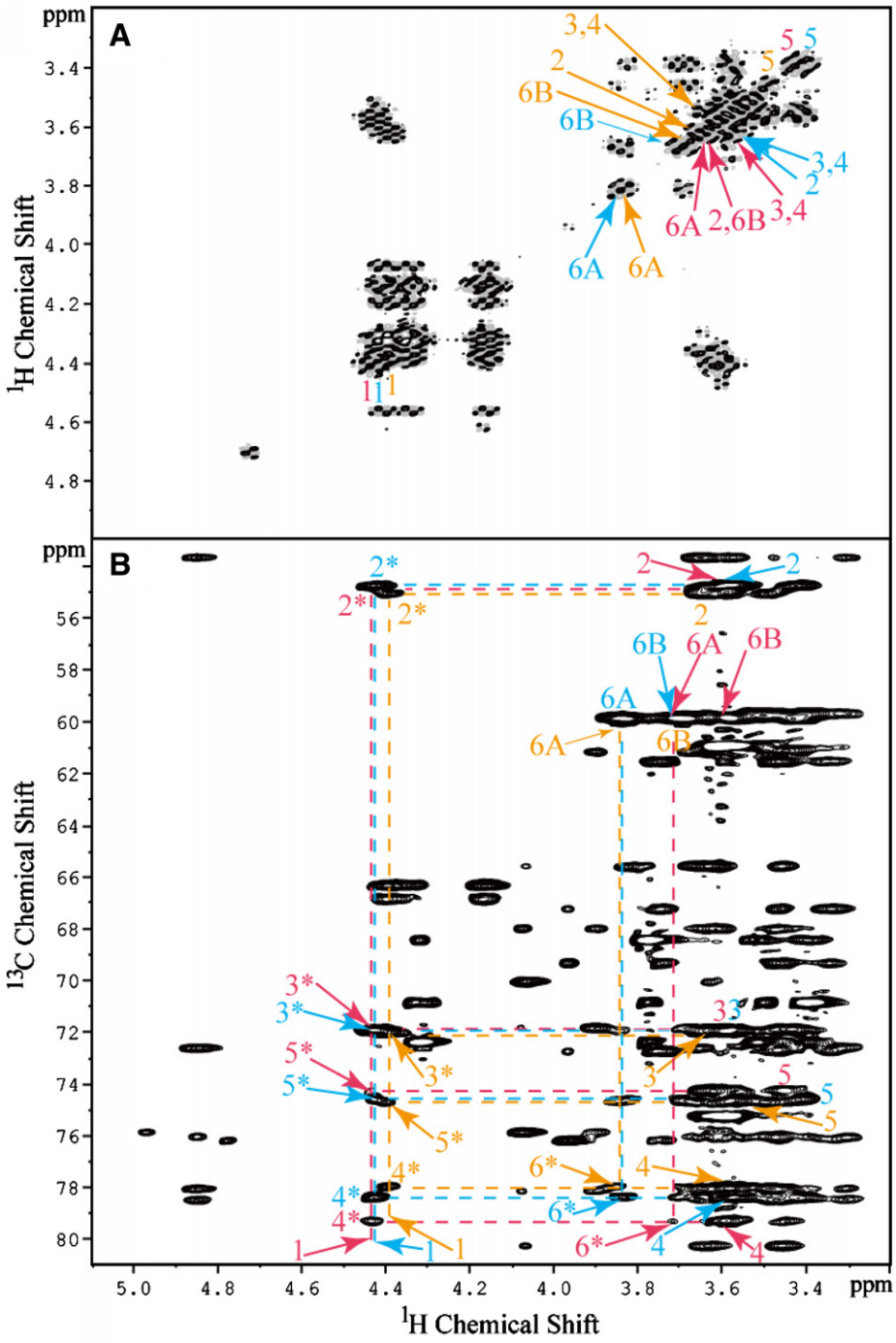

Figure 3. 2D Selective-TOCSY–DQFCOSY (A) and HSQC–TOCSY (B) spectra of GlcNAc-b,e,h,j. A and B were measured on 600 MHz and 400 MHz instruments, respectively. Assignment of the diagonal and the ^{1}H–^{13}C 1-bond correlation signals of the GlcNAc-b, GlcNAc-e,h, and GlcNAc-j residues were numbered from 1 to 6 and were colored magenta, light blue, and orange on A and B, respectively. The long-range correlations of H-1 with C-2 to C-5 and of H-4 with C-6 were numbered from 2* to 6* and colored as mentioned above on B. All signals of GlcNAc-e and GlcNAc-h were overlapped at each of the positions.

and these were heavily overlapping signals. Therefore, a selective shaped pulse excitation technique was applied in order to obtain the 1D TOCSY spectra corresponding to each monosaccharide in the triantennary undecasaccharide. Thus, the transverse magnetization obtained by 1D selective-TOCSY was developed to the second dimension by using DQFCOSY or HSQC pulse sequences. We repeated this 2D selective-TOCSY–DQFCOSY experiment corresponding to each monosaccharide in order to assign each monosaccharide in the triantennary undecasaccharide.

In the 2D selective-TOCSY–HSQC experiment, the 2 mg amount of the prepared triantennary undecasaccharide was not suitable to afford high quality spectra for each monosaccharide, because the sensitivity of the spectra was low. The 1D ^{1}H spectrum of the

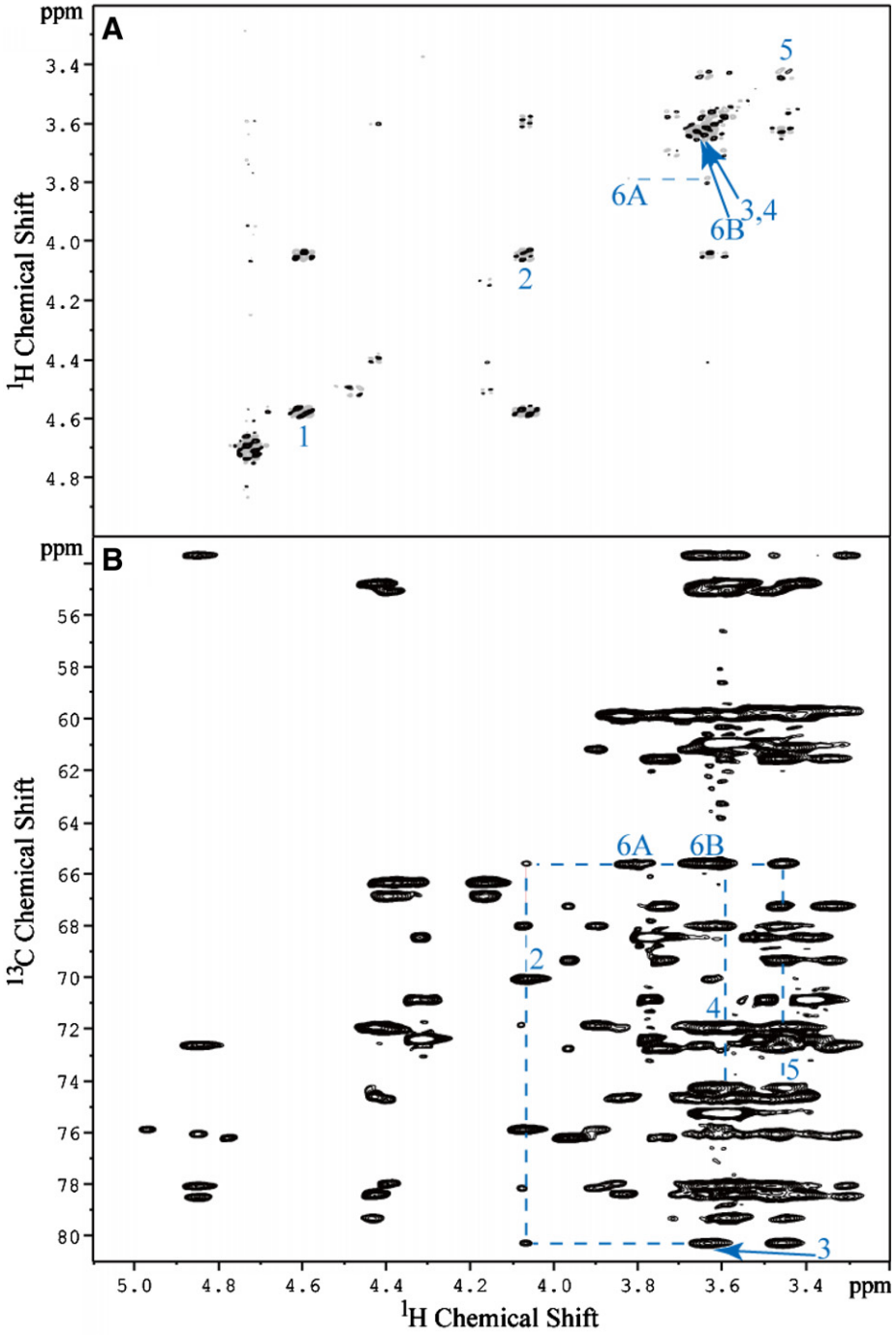

Figure 4. 2D Selective-TOCSY–DQFCOSY (A) and HSQC–TOCSY (B) spectra of Man-c. A and B were measured on 600 MHz and 400 MHz instruments, respectively. Assignment of the diagonal and the ^{1}H–^{13}C 1-bond correlation signals of the Man-c residue were numbered from 1 to 6 on A and B, respectively.

triantennary undecasaccharide was more complicated than that of the biantennary undecasaccharide; for example, H-2$^{Man\text{-}c}$ and H-2$^{Man\text{-}g}$ resonated at 4.06 and 4.07 ppm, respectively. A longer duration of the shaped pulses was needed to selectively excite an anomeric or H-2 (2-position) proton signal of a monosaccharide in the triantennary undecasaccharide. The net of the magnetization decayed due to the longer shaped pulses required for the selective excitation of a narrower region. We found that the method employing 2D selective-TOCSY–HSQC has a drawback in that it correlates with the naturally abundant ^{13}C nuclei in an experiment using large oligosaccharides. Therefore, a conventional 2D HSQC–TOCSY spectrum of the triantennary undecasac-

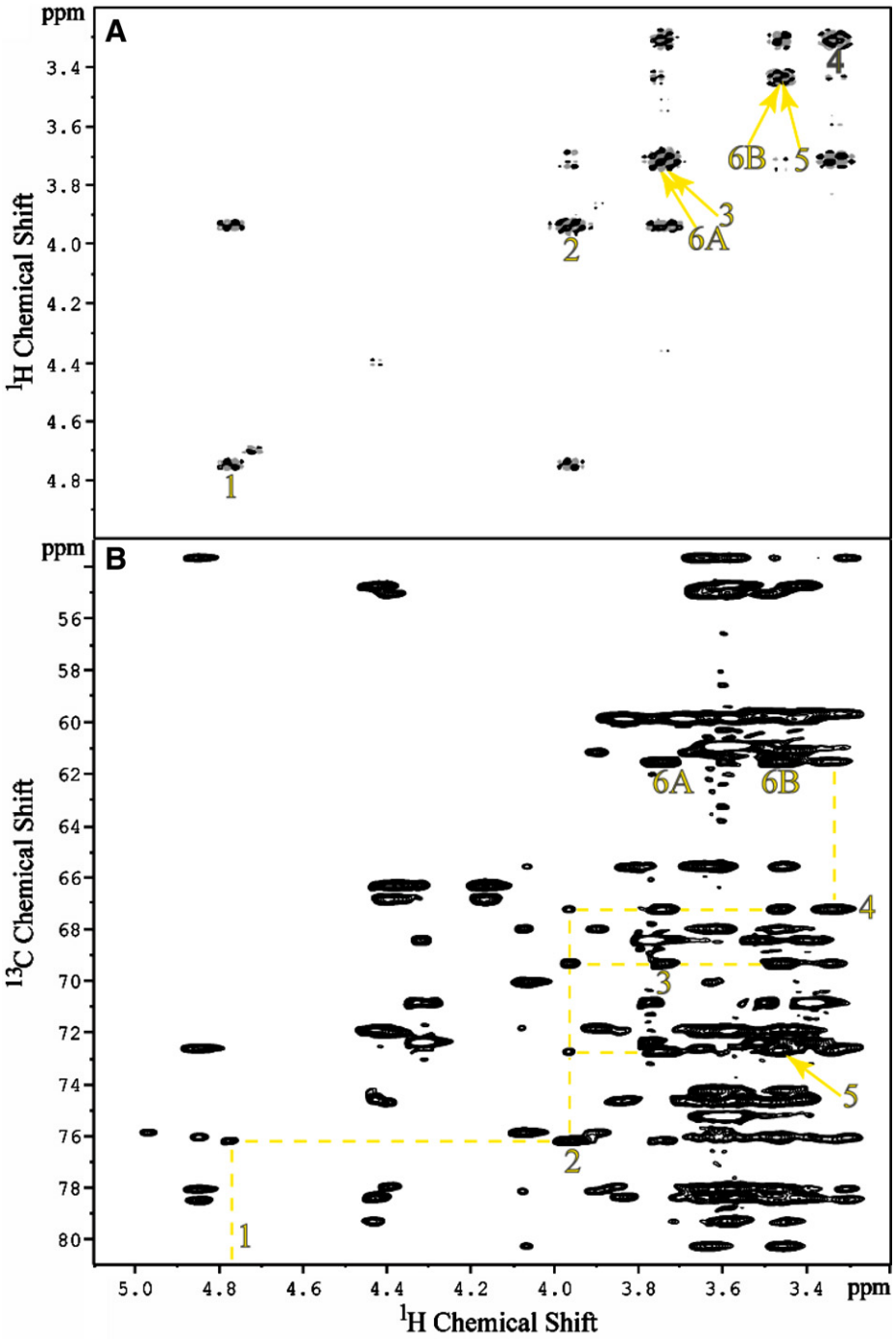

Figure 5. 2D Selective-TOCSY–DQFCOSY (A) and HSQC–TOCSY (B) spectra of Man-d. A and B were measured on 600 MHz and 400 MHz instruments, respectively. Assignment of the diagonal and the ^{1}H–^{13}C 1-bond correlation signals of the Man-d residue were numbered from 1 to 6 on A and B, respectively.

charide was used instead of the 2D elective-TOCSY–HSQC spectra. Since shaped pulses did not rely on this experiment and the net magnetization did not decay, the sensitivity of the spectrum was expected to be high.

In addition, we expected that the 2D selective-TOCSY–DQFCOSY of each monosaccharide would greatly help analyze the conventional 2D HSQC-TOCSY spectrum which exhibited the complicated signal pattern of the triantennary undecasaccharide. We also compared and evaluated differences in the signal pattern between the unambiguous assignment of the ^{1}H and ^{13}C signals of the biantennary and triantennary oligosaccharides. Furthermore, the glycosyl positions were revealed in the triantennary undecasaccharide.

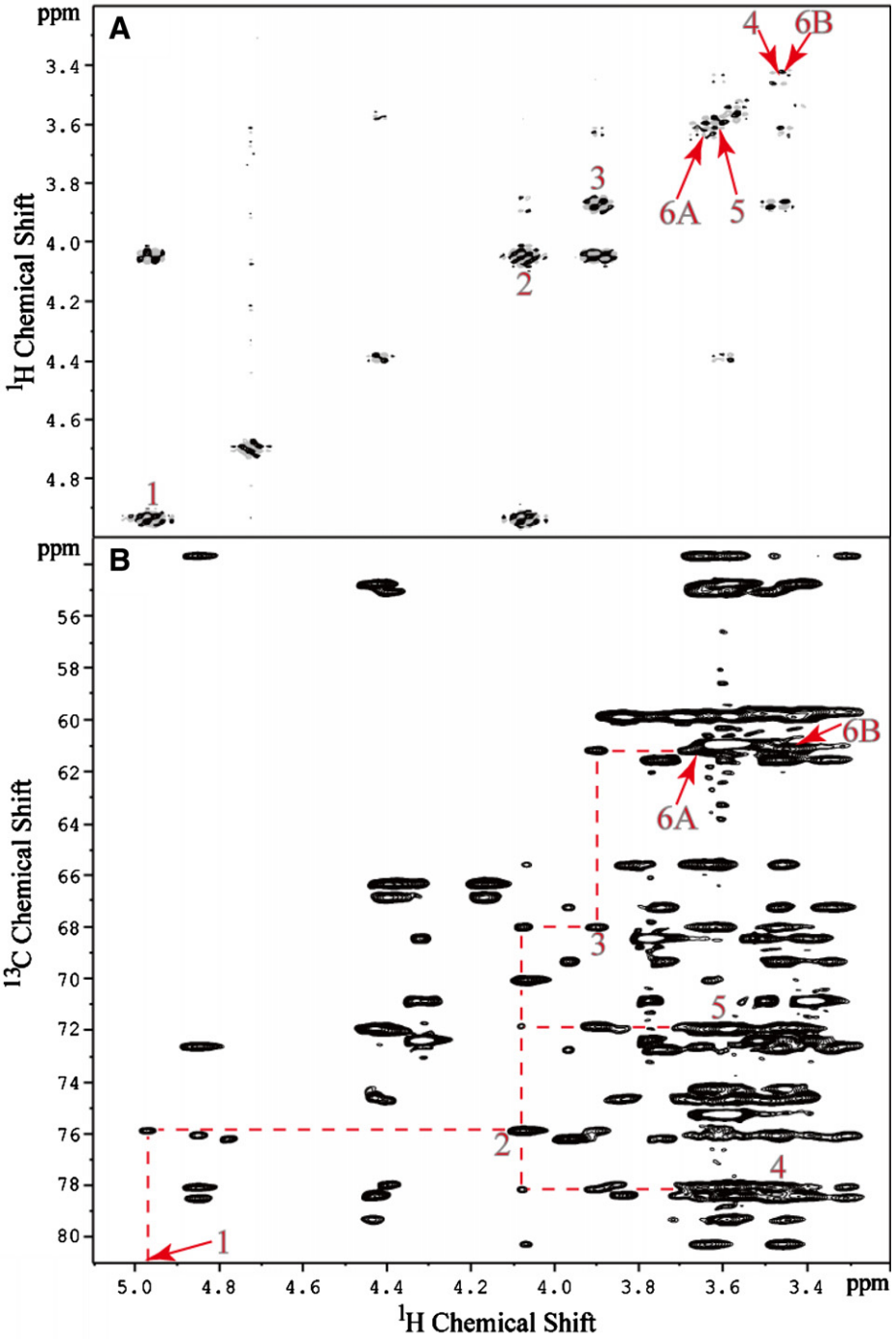

Figure 6. 2D Selective-TOCSY–DQFCOSY (A) and HSQC–TOCSY (B) spectra of Man-g. A and B were measured on 600 MHz and 400 MHz instruments, respectively. Assignment of the diagonal and the ^{1}H–^{13}C 1-bond correlation signals of the Man-g residue were numbered from 1 to 6 on A and B, respectively.

2.1. Assignment of GlcNAc-a

The selective excitation pulse (RE-BURP) was focused on the anomeric proton (H-1$^{GlcNAc-a}$: 4.84 ppm) in order to achieve selective-TOCSY magnetization and then its transverse magnetization was developed to the second dimension by a DQFCOSY pulse sequence. As shown in Figure 2A, a well-separated 2D DQFCOSY spectrum was obtained, and all proton signals were sequentially assigned by connecting diagonal peaks to cross peaks and vice versa on the spectrum. ^{1}H-^{13}C 1-bond correlations were numbered in the conventional 2D HSQC–TOCSY spectrum from 1 to 6A and 6B for convenience (Fig. 2B). The signals indicated by 2*, 3*, 4*, and 5* were

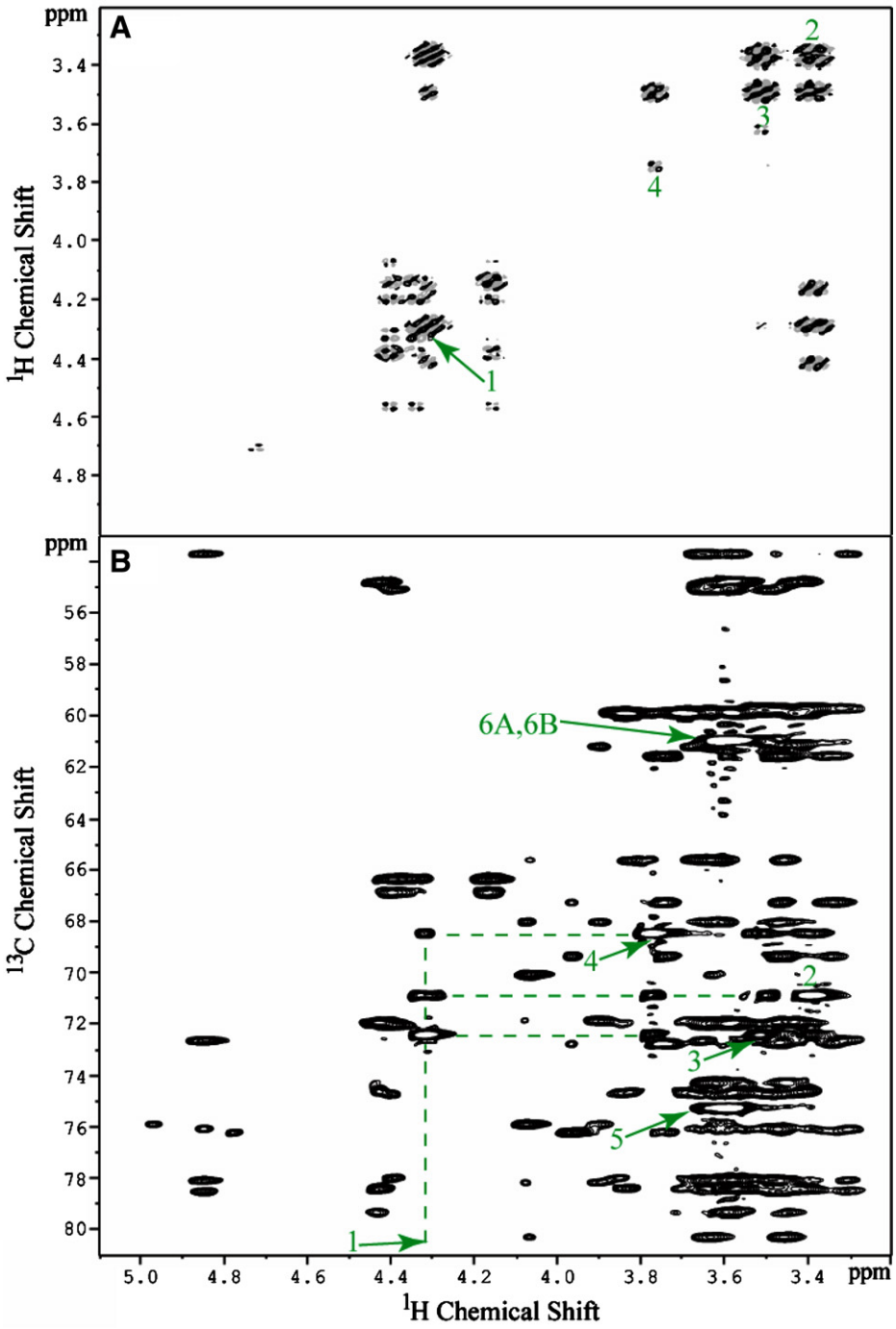

Figure 7. 2D Selective-TOCSY–DQFCOSY (A) and HSQC–TOCSY (B) spectra of Gal-f,i,k. A and B were measured on 600 MHz and 400 MHz instruments, respectively. Assignment of the diagonal and the ^{1}H–^{13}C 1-bond correlation signals of the Gal-f,i,k residue were numbered from 1 to 6 on A and B. All signals from Gal-f, Gal-i, and Gal-k were overlapped at each of the positions.

correlations between H-1 and C-2, H-1 and C-3, H-1 and C-4, and H-1 and C-5, which are ^{1}H TOCSY-correlated with 2, 3, 4, and 5, respectively. An assignment of the ^{13}C signals of the residue was easily obtained by these correlation signals, which were perpendicularly observed in the same chemical shift of ^{1}H (4.84 ppm). A TOCSY correlation with 6 was not observed on the perpendicular line at 4.84 ppm. 6* was useful for the assigning of C-6, which was correlated between H-5 and C-6. All signals in Figure 2B were reconfirmed in the assignment of ^{1}H in Figure 2A and were similar to an unambiguous assignment of the biantennary undecasaccharide.

2.2. Assignment of GlcNAc-b,e,h,j

Assignment of the GlcNAc-b,e,h,j residues was performed using the same process as with GlcNAc-a. However, the H-1 signals of these four GlcNAc-b,e,h,j residues unfortunately overlapped around 4.4 ppm. Since individual H-1 signals could not be selectively excited in the 2D selective-TOCSY–DQF-COSY experiment, the spectrum was obtained as a mixture of these four GlcNAc residues. As shown in Figure 3A, the H-1$^{GlcNAc\text{-}e}$ and the H-1$^{GlcNAc\text{-}h}$ completely overlapped. Connecting the diagonal peaks of three H-1 signals overlapped to the individual cross peak of H-2 was easy. However, connecting the three diagonal peaks of H-2 to the individual cross peak of H-3 and H-3 to the individual H-4 was very difficult. In this case, three perpendicular lines from the correlation signals of the 1-position were useful on the 2D HSQC–TOCSY spectrum (Fig. 3B). Both the diagonal in Figure 3A and correlation signals in Figure 3B were GlcNAc-b, GlcNAc-e,h, and GlcNAc-j, colored magenta, light blue, and orange, respectively. Three sets of signals which contain GlcNAc-b, GlcNAc-e,h and GlcNAc-j as indicated by 2*, 3*, 4*, and 5* are correlations between H-1 and C-2, H-1 and C-3, H-1 and C-4, and H-1 and C-5, which are ^{1}H TOCSY-correlated with 2, 3, 4, and 5, respectively. An assignment of the ^{13}C signals of the residues was easily obtained by these correlation signals, which were perpendicularly observed in the same chemical shift of ^{1}H (4.43, 4.42, and 4.38 ppm), respectively. Three sets of TOCSY correlations with the 6's were not observed on the perpendicular lines at 4.43, 4.42, and 4.38 ppm. 6*'s were useful for assigning the C-6's, which were correlated between H-6's and C-4's. All signals in Figure 3B were reconfirmed in the assignment of ^{1}H in Figure 3A and

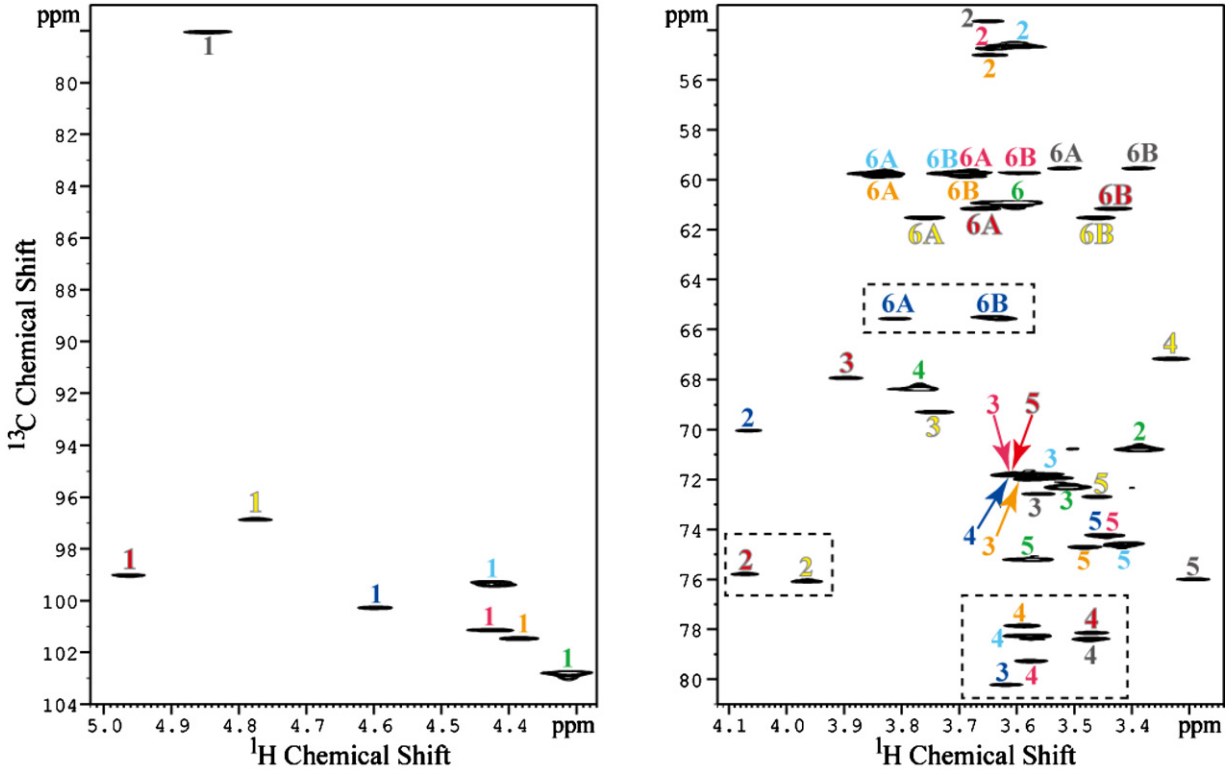

Figure 8. 600 MHz HSQC spectra of the triantennary undecasaccharide. Complete assignment was shown on the anomeric (left) and the other (right) region. The colored number indicating the position corresponding to the individual residue shown in Figure 1. The signals related to the glycosidic linkage are indicated by dotted quadrangles. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

resembled an unambiguous assignment of the biantennary undecasaccharide.

2.3. Assignment of mannoside-c

Application of a selective excitation pulse to H-2$^{Man\text{-}c}$ residue has an advantage over excitation to H-1$^{Man\text{-}c}$ because the coupling constant between H-1$^{Man\text{-}c}$ and H-2$^{Man\text{-}c}$ is very small and magnetization of H-1$^{Man\text{-}c}$ is not efficiently transferred to H-2$^{Man\text{-}c}$ in the TOCSY period. However, the H-2 signals of Man-c and Man-g residues overlapped at 4.06 and 4.07 ppm, respectively. Selective excitation for H-1$^{Man\text{-}c}$ was, therefore, performed in the 2D selective-TOCSY–DQFCOSY experiment. Although the resulting spectrum gave a low sensitivity, a sequential assignment was achieved, as shown in Figure 4A. All of the ^{13}C correlation signals were also assigned on the 2D HSQC–TOCSY spectrum (Fig. 4B) and were confirmed by comparison with the proton chemical shifts assigned (Fig. 4A). The assignment of this residue was found to be similar to an unambiguous assignment of the biantennary undecasaccharide.

2.4. Assignment of mannoside-d

Selective excitation of H-2$^{Man\text{-}d}$ was performed in the 2D selective-TOCSY–DQFCOSY experiment because H-2$^{Man\text{-}d}$ was separately observed at 3.96 ppm. A sequential assignment was easily achieved, as shown in Figure 5A. All of the ^{13}C correlation signals were also assigned on the 2D HSQC–TOCSY spectrum (Fig. 5B) and were confirmed by comparison with the proton chemical shifts assigned (Fig. 5A). The assignment of this residue was similar to an unambiguous assignment of the biantennary undecasaccharide.

2.5. Assignment of mannoside-g

Since H-2$^{Man\text{-}c}$ and H-2$^{Man\text{-}g}$ overlapped, selective excitation pulse was focused on H-1$^{Man\text{-}g}$ in the 2D selective-TOCSY–DQFCOSY experiment. A sequential assignment for proton signals was easily achieved, as shown in Figure 6A. All ^{13}C correlation signals were also assigned on the 2D HSQC–TOCSY spectrum (Fig. 6B) and were confirmed by comparing them with the proton chemical shifts assigned (Fig. 6A). The assignment of this residue was found to be similar to an unambiguous assignment of the biantennary undecasaccharide, except for the correlation at the 4-position.

2.6. Assignment of galactoside-f,i,k

Since H-1$^{Gal\text{-}f}$, H-1$^{Gal\text{-}i}$, and H-1$^{Gal\text{-}k}$ overlapped at 4.31 ppm, the 2D selective-TOCSY–DQFCOSY spectrum was obtained as a mixture of these three Gal residues. However, a sequential assignment based on 2D selective-TOCSY–DQFCOSY was easily achieved from H-1 to H-4, as shown in Figure 7A. In this case, H-5, H-6A, and H-6B are not seen in Figure 7A because the coupling constant between H-4 and H-5 is nearly zero. For ^{13}C signals of these galactosides, C-1 to C-4 were assigned as shown in Figure 7B, and confirmed by comparison with the proton chemical shifts (Fig. 7A). The signals that TOCSY-correlated with 5 and 6 were not observed on the perpendicular line at 4.29 ppm. These two signals were assigned by comparing them with the assignment of the biantennary undecasaccharide. The 2D selective-TOCSY–DQFCOSY and the HSQC–TOCSY experiments revealed that the proton and carbon signals in Gal-f, Gal-i, and Gal-k were overlapped at each of the positions.

Table 1. Assignment of ^{1}H and ^{13}C signals of the triantennary undecasaccharide

		1	2	3	4	5	6A	6B
GlcNAc-a	^{1}H (ppm)	4.84	3.65	3.56	3.47	3.29	3.51	3.39
	^{13}C (ppm)	78.08	53.69	72.62	78.42	76.04	59.59	59.59
GlcNAc-b	^{1}H (ppm)	4.43	3.64	3.60	3.58	3.44	3.69	3.59
	^{13}C (ppm)	101.19	54.79	71.86	79.33	74.29	59.78	59.78
GlcNAc-e,h	^{1}H (ppm)	4.42	3.60	3.54	3.58	3.41	3.84	3.69
	^{13}C (ppm)	99.39	54.72	71.99	78.32	74.62	59.81	59.81
GlcNAc-j	^{1}H (ppm)	4.38	3.64	3.57	3.59	3.48	3.83	3.68
	^{13}C (ppm)	101.52	55.05	72.03	77.91	74.75	59.92	59.92
Man-c	^{1}H (ppm)	4.60	4.06	3.62	3.60	3.44	3.81	3.63
	^{13}C (ppm)	100.33	70.08	80.27	71.88	74.29	65.62	65.62
Man-d	^{1}H (ppm)	4.77	3.96	3.74	3.33	3.46	3.76	3.46
	^{13}C (ppm)	96.93	76.13	69.35	67.23	72.75	61.57	61.57
Man-g	^{1}H (ppm)	4.96	4.07	3.90	3.47	3.57	3.66	3.43
	^{13}C (ppm)	99.07	75.85	68.00	78.20	71.85	61.20	61.20
Gal-f,i,k	^{1}H (ppm)	4.29	3.37	3.49	3.75	3.55	3.58	3.58
	^{13}C (ppm)	102.78	70.79	72.31	68.37	75.19	60.90	60.90

Carbon signals that are glycosylated (C-4: GlcNAc-a, GlcNAc-b, GlcNAc-e, GlcNAc-h, and GlcNAc-j; C-3 and C-6: Man-c; C-2: Man-d; C-2 and C-4: Man-g) were observed at low field in the ^{13}C-dimension due to the typical glycosidic shift. Unambiguous assignment of those by our experiments afforded evidence to confirm glycosylation positions. The typical glycosidic shift was not observed in Gal-f, Gal-i, and Gal-k because the residues are at the non-reducing end.

3. Discussion

A complete assignment of the triantennary undecasaccharide is shown in Figure 8. The assignment was obtained by the 2D selective-TOCSY–DQFCOSY and HSQC–TOCSY experiments, and was similar to the previously reported biantennary undecasaccharide obtained by 2D selective-TOCSY–DQFCOSY and selective-TOCSY–HSQC experiments. In this experiment, the 2D selective-TOCSY–DQFCOSY was found to be very useful for an unambiguous assignment of the ^{1}H signals of the triantennary oligosaccharide. Although the net magnetization of the monosugar component was strictly extracted by the shaped pulse (80 ms to 100 ms), the sensitivity of the magnetization decayed due to the T2 relaxation which occurred during the 1D selective-TOCSY experiment. This caused difficulties in obtaining high-quality heteronuclear 2D selective-TOCSY–HSQC spectra by the use of 2 mg of the triantennary undecasaccharide. Because the sensitivity of this experiment was low, the extracted magnetization was not well correlated with the naturally abundant ^{13}C nuclei. Therefore, we relied on a conventional 2D HSQC–TOCSY experiment, instead of the 2D selective-TOCSY–HSQC. The complicated 2D HSQC–TOCSY spectrum of the triantennary undecasaccharide contains signals of eleven monosugar components. In order to assign the ^{1}H and ^{13}C signals, a series of the 2D selective-TOCSY–DQFCOSY spectra were compared with the complicated spectrum, and ^{1}H TOCSY correlations were connected from the ^{1}H–^{13}C 1-bond correlations. As shown in Figure 2B, correlations (2* to 5*) were observed on the perpendicular line at 4.84 ppm of H-1$^{GlcNAc\text{-}a}$, and the ^{1}H-^{13}C 1-bond correlations were assigned by comparison with the assignment in Figure 2A. As expected, the 2D selective-TOCSY–DQFCOSY spectrum supported the assignment of the ^{13}C signals. Finally, the systematic procedure in assigning the ^{1}H and ^{13}C signals can be carried out by the use of this method.

In assigning ^{1}H and ^{13}C signals, H2BC is a very useful experimental procedure that exhibits three kinds of correlations, that is, $^{1}H_a$–$^{13}C_a$, $^{1}H_a$–$^{1}H_b$, and $^{1}H_b$–$^{13}C_b$ correlations through a $^{1}H_a$–$^{13}C_a$–$^{13}C_b$–$^{1}H_b$ bond network. In applying this procedure to the oligosaccharide analysis, connecting the correlations has sometimes been difficult, because the assignment of the $^{1}H_a$–$^{1}H_b$ correlation by the use of the $^{1}H_a$–$^{13}C_a$ correlation has a few alternative candidates due to the heavily overlapped H2BC signals in the complex triantennary oligosaccharide, and the H2BC signals sometime are of weak intensity.

In order to overcome these problems, unambiguous proton assignment by 2D selective-TOCSY–DQF-COSY affords great help in finding the $^{1}H_a$–$^{1}H_b$ correlation in both the H2BC and conventional HSQC–TOCSY experiments. In addition, the problem related to sensitivity in the 2D selective-TOCSY–HSQC experiment, the shorter duration of the shaped pulse on higher field NMR instruments (>700 MHz), is solved. In this case, sensitivity does not decay due to T2 relaxation during the short period shaped pulse, because the higher field instrument affords well-separated anomeric signals of the oligosaccharide. In addition, a higher sensitivity probehead technology would solve the ^{13}C sensitivity problem, even though the introduction of ^{13}C labeling is still difficult on large oligosaccharides.

Structural analysis of oligosaccharides by NMR is different than that of proteins and nucleic acids, because oligosaccharides have antennary structures. Therefore, the key step is to find the glycosylation positions in the oligosaccharide. As shown in Figure 8, the signals shifted to the low field (dotted quadrangle) display the glycosylated ^{13}C-carbon in the oligosaccharide. A combined 2D selective-TOCSY–DQFCOSY and HSQC–TOCSY method assigns these as glycosylated ^{13}C-carbons. This assignment was found to be consistent with a typical structure for asparagine-linked triantennary complex type oligosaccharides.

4. Conclusions

All ^{1}H and ^{13}C signals of the triantennary undecasaccharide were completely assigned by the 2D selective-TOCSY–DQFCOSY and HSQC–TOCSY experiments on 600 MHz and 400 MHz NMR instruments as shown in Table 1. It was demonstrated that the 2D selective-TOCSY–DQFCOSY method is of great help in assigning the ^{13}C signals obtained in 2D HSQC–TOCSY. This powerful procedure easily finds the glycosyl positions in triantennary complex type oligosaccharides.

5. Experimental procedures

5.1. Preparation of tribranched oligosaccharide

The experimental details for the preparation of the complex type triantennary undecasaccharide will be presented elsewhere. To a solution of fetuin (1.0 g, SIGMA) and NaN_3 in a phosphate buffer (50 mM, pH

7.0, 20 mL) was added protease (100 mg, Orientase of aspergillus oryzae, Hankyu Bioindustry Co. Ltd, Osaka, Japan),[27] and this mixture was incubated at 50 °C for 24 h. After the reaction, the mixture was filtered through Celite and then lyophilized and purified by gel-permeation chromatography (Sephadex-G-25: ∅2.5 × 100 cm, H_2O) to afford crude glycopeptide. To a solution of this residue in Tris–HCl buffer (50 mM, $CaCl_2$ 10 mM, pH 7.5, 13 mL) was added Actinase-E (13 mg),[1] and this mixture was incubated for 60 h. After the reaction, the mixture was filtered through Celite and then lyophilized and purified again by gel-permeation chromatography (Sephadex-G-25: ∅2.5 × 100 cm, H_2O) to afford Asn-tribranched oligosaccharide. To a solution of this mixture (277 mg) in H_2O–DMF (1.4–1.9 mL) were added $NaHCO_3$ (77.8 mg, 0.93 mmol) and 9-fluorenylmethyl-*N*-succimidylcarbonate (188 mg, 0.56 mmol). This mixture was stirred at room temperature, and after 2 h the mixture was poured into acetone (50 mL) and filtered using a 1-μm glass filter. The filtered solids were dissolved in water, and desalted by an ODS-column (∅20 × 250 mm), eluting with H_2O (100 mL) and then MeCN (25%, 200 mL) to afford a mixture of Fmoc-oligosaccharides. This mixture was further purified by HPLC:ODS column (YMC-Pack ODS-AM SH-343-5AM, ∅20 × 250 mm, 82:18 50 mM NH_4OAc–MeCN, 8.0 mL/min, monitoring at 274 nm) to obtain the Fmoc-Asn-oligosaccharide. To the Fmoc-Asn-oligosaccharide was added HCl solution (40 mM, 4.1 mL), and this mixture was stirred at 50 °C for 6 h, followed by cooling to 4 °C and neutralization with aq $NaHCO_3$. This reaction mixture was purified by an HPLC:ODS column (YMC-Pack ODS-AM SH-343-5AM, ∅20 × 250 mm, 82:18 50 mM NH_4OAc–MeCN, 8.0 mL/min, monitoring at 274 nm) to obtain the Fmoc-Asn-asialooligosaccharide (15.8 mg).

5.2. NMR experiments

2D Selective-TOCSY–DQFCOSY measurements for the triantennary undecasaccharide (2 mg) in D_2O were acquired on a Bruker Avance-600 spectrometer (^1H frequency: 600.13 MHz) equipped with an inverse 5-mm TXI CryoProbe™ ($^1H/^{13}C/^{15}N$) fitted with a Z-gradient coil. A 2D HSQC–TOCSY for the same sample was acquired on a Bruker Avance-400 spectrometer (^1H frequency: 400.13 MHz) equipped with an inverse 5-mm TXI conventional probe fitted with a Z-gradient coil. The temperature was set to 293 K on both instruments. The duration of the TOCSY mixing time was 65 ms in the 2D HSQC–TOCSY. The duration of the mixing time depended on residues in the 2D selective-TOCSY–DQFCOSY. These were as follows: 50 ms for Gal-f, -i, and -k residues; 90 ms for GlcNAc-a, -b, -e, -h, and -j residues; 100 ms for Man-d and -g residues; 140 ms for Man-c.

The shape of the selective excitation pulse and the duration for the 2D selective-TOCSY–DQFCOSY experiment were RE-BURP and 100 ms, except for 80 ms on Man-c. The selectively excited signals were as follows: H-1 of GlcNAc-a, H-1 of GlcNAc-b,e,h,j, H-1 of Man-c, H-2 of Man-d, H-1 of Man-g, and H-1 of Gal-f,i,k. The number of scans and the experimental time were as follows: GlcNAc-a and Man-g; 64 scans and 11 h 28 min, GlcNAc-b,e,h,j and Gal-f,i,k; 32 scans and 5 h 43 m, Man-d; 128 scans and 23 h 4 m, Man-c; 208 scans and 38 h 2 m, respectively. The other parameters were referenced in the previous report.[24] The number of scans and the experimental time for the 2D HSQC–TOCSY experiment on the 400 MHz instrument were 480 scans and 3 d 6 h 52 m, respectively.

The ^1H chemical shifts were referenced to the residual HDO signal (4.7 ppm), while the ^{13}C chemical shifts were referenced indirectly to the absolute frequency ratio $^{13}C/^1H = 0.251450201$.

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