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# An unambiguous assignment method by 2D selective-TOCSY-HSQC and selective-TOCSY-DQFCOSY and structural analysis by selective-TOCSY-NOESY experiments of a biantennary undecasaccharide

Hajime Sato[a,*] and Yasuhiro Kajihara[b]

[a]*Bruker Biospin K.K., 3-21-5 Ninomiya, Tsukuba, Ibaraki 305-0051, Japan*
[b]*Graduate School of Integrated Science, Yokohama City University, 22-2 Seto Kanazawa-ku, Yokohama 236-0027, Japan*

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**Abstract**—In this paper we present a newly developed 2D selective-TOCSY-HSQC experiment for unambiguous assignment of individual sugar components of a biantennary undecasaccharide. The correlation signals between the protons and carbons of a selectively excited sugar component in the undecasaccharide were observed in a 2D selective-TOCSY-HSQC experiment. The network of proton signals of this sugar was observed in a 2D selective-TOCSY-DQFCOSY experiment. These two experiments enabled us to perform unambiguous sequential assignment of both protons and carbons of the individual sugar components in the undecasaccharide. In addition, a three-dimensional structural analysis was performed by a 2D selective-TOCSY-NOESY experiment, which clearly distinguishes between inter-sugar and intra-sugar ring NOE signals.


*Keywords:* Biantennary undecasaccharide; NMR spectroscopy; Selective-TOCSY-HSQC; Selective-TOCSY-DQFCOSY; Selective-TOCSY-NOESY

## 1. Introduction

Both the sensitivity and resolution of NMR spectroscopy have been increasing year by year. Many new NMR experiments for isotopically labeled proteins[1,2] and nucleic acids[3–5] have been discovered and developed. However, structural analysis of oligosaccharides remains extremely difficult, since the sugar components, the hexoses of the oligosaccharides, have structures that are very similar to one another. In addition, the $^{1}$H signals are observed together in a very narrow region of the $^{1}$H NMR spectrum, except for the anomeric proton signals, which are observed downfield due to the electron-withdrawing acetal structure of the anomeric position.[6] Using this characteristic signal pattern and focusing on the anomeric proton as the starting point for assignment, 2D TOCSY or HOHAHA experiments[7,8] are useful methods for analysis of individual sugar components in oligosaccharides. These techniques can also be run as HSQC-TOCSY or TOCSY-HSQC experiments[6,9–12] in order to separate signals into the $^{13}$C-dimension. In these experiments, however, the signals are produced by employing both short and long isotropic-mixing times for observing protons neighboring to and remote from the anomeric proton, respectively. Since proton signals overlap, assignment is empirically based, and this often leads to mistaken assignments. In order to obtain a high-resolution signal, a 1D version of the TOCSY experiment using a selective excitation pulse has been developed.[12–15] However, in this case assignment can also be empirically based when the signals heavily overlap.

Assignment of the oligosaccharide carbon signals is a technique used to determine which hydroxyl group is

* Corresponding author. Tel.: +81 29 852 1235; fax: +81 29 858 0322; e-mail: hajime.sato@bruker-biospin.jp

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glycosylated by using the glycosyl shift in the $^{13}C$ NMR spectrum.[16–18] Although assignment of these glycosyl linkages has been performed on small oligosaccharides in which all carbon signals can be assigned, full carbon assignment of large oligosaccharides having over five sugar residues remains difficult due to the extreme overlap in both the $^{1}H$ (3.3–4.1 ppm) and $^{13}C$ (59–82 ppm) regions in the HSQC spectrum. Heteronuclear 3D experiments have been applied to a monosaccharide,[19] a tetrasaccharide,[20] and several disaccharides,[21,22] and appear to be useful, but spectral analysis of 3D spectra is not easy and spectral resolution is not sufficient for assignment of all protons and carbons in the oligosaccharide due to low digital points in both the time and frequency domains. We propose an efficient method combining the newly developed 2D selective-TOCSY-HSQC and the 2D selective-TOCSY-DQFCOSY[23] experiments (Fig. 1A and B, respectively) in order to perform unambiguous assignment of the oligosaccharides. Both experiments can measure 2D spectra of a desired sugar residue in the oligosaccharide. The mechanism of the 2D selective-TOCSY-HSQC experiment is similar to that of the 2D selective-TOCSY-DQFCOSY experiment. After extraction of magnetization of the desired protons of the sugar component in the oligosaccharide by the selective TOCSY pulse sequence, the magnetization is developed to the second dimension by an HSQC pulse sequence to afford a simple HSQC spectrum of the sugar residue. Using multiple experiments aimed at the different sugar components of the oligosaccharide, we were able to unambiguously assign all proton and carbon signals in large oligosaccharides. In addition, we also modified a selective TOCSY-NOESY[24] experiment (Fig. 1C) in order to perform concise conformational analysis.

In this paper, we describe an efficient measurement method combining 2D selective-TOCSY-HSQC, 2D selective-TOCSY-DQFCOSY, and 2D selective-TOCSY-NOESY to assign all proton and carbon signals of an asparagine-linked biantennary undecasaccharide[25] (Fig. 2A), which is a typical glycoprotein oligosaccharide, in order to gain insight into its conformational properties.

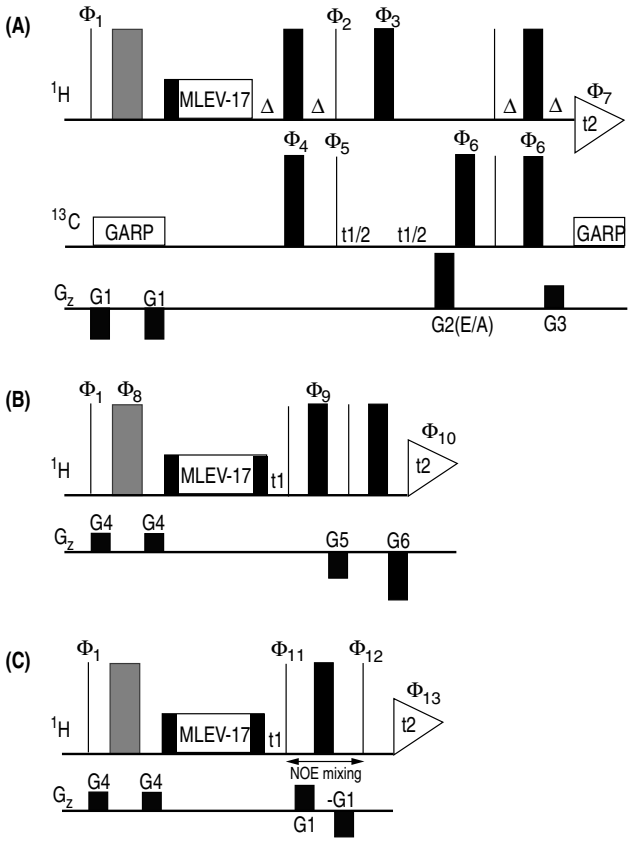


**Figure 1.** Pulse scheme for 2D selective-TOCSY-HSQC (A), 2D selective-TOCSY-DQFCOSY (B), and 2D selective-TOCSY-NOESY (C). Narrow and filled wide rectangular pulses denote 90° and 180° flip angles, respectively. The shaded wide rectangular pulse denotes a 180° flip angle with a shaped pulse. The shape of the 180° selective pulse is RE-BURP and the duration is 50–150 ms. The duration of the TOCSY isotropic-mixing time was set to 120–150 ms. Quadrature detection was accomplished by incrementing phases of $\Phi_4$ and $\Phi_5$ via the Echo-Antiecho method in (A) and of $\Phi_1$ and MLEV-17 via the States-TPPI method in (B) and (C). The following phase cycling was applied unless $x$ indicated otherwise: $\Phi_1 = x, -x$; $\Phi_2 = y$; $\Phi_3 = 4x, 4(-x)$; $\Phi_4 = x$; $\Phi_5 = 2x, 2(-x)$; $\Phi_6 = 8x, 8(-x)$; $\Phi_7 = 4x, 2(-x), 2x, 2(-x), x, -x, 2x, 2(-x), 2x, -x$. $\Phi_8 = 2x, 2y, 2(-x), 2(-y)$; $\Phi_9 = 8x, 8(-x), 8y, 8(-y)$; $\Phi_{10} = x, 2(-x), x$; $\Phi_{11} = 8x, 8(-x)$; $\Phi_{12} = 2x, 2(-x), 2y, 2(-y)$; $\Phi_{13} = x, 2(-x), x, y, 2(-y), y, -x, 2x, -x, -y, 2y, -y$. The sinusoidal gradient pulse length was 1 ms and the ratio 17.5:40:10.5:7.5:−11.5:−23 G/cm for G1:G2:G3:G4:G5:G6.

## 2. Results

### 2.1. Pulse sequences

**2.1.1. 2D selective-TOCSY-HSQC (Fig. 1A).** The 2D selective-TOCSY-HSQC experiment was produced by modification of the 2D selective-TOCSY-DQFCOSY experiment. After extraction of magnetization of the protons desired from a sugar component in the oligosaccharide by the selective TOCSY pulse sequence, the magnetization is developed in the second dimension by an HSQC pulse sequence to afford a simple HSQC spectrum of the sugar residue. In this case, it is essential to start a carbon decoupling after the first 90° pulse and to stop the decoupling before the MLEV-17 sequence in order to selectively excite both proton signals attached to $^{12}C$ and $^{13}C$ nuclei at the same time. Magnetization of the selectively excited H–$^{12}C$ is transferred to remote protons in the selective-TOCSY period, and is developed to the attached $^{13}C$ nuclei by the HSQC pulse sequence. In this case, this selectively excited proton (H–$^{12}C$) is not correlated to a $^{13}C$ nucleus because this proton is bound to a $^{12}C$ nucleus. When the decoupling

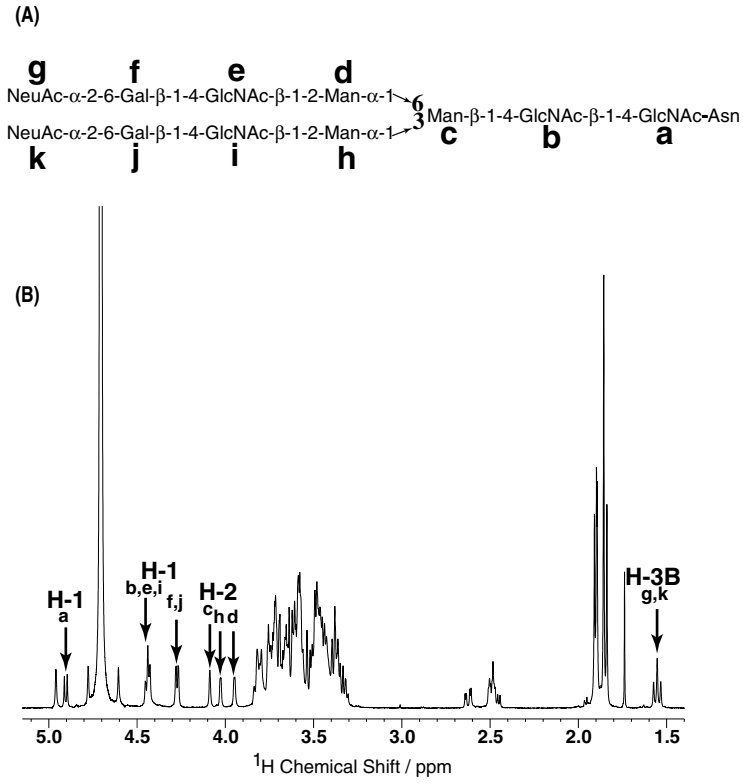


**Figure 2.** (A) Primary structure of the undecasaccharide. A 20 mg sample of the undecasaccharide was prepared in $D_2O$ (33 mM) for the 400-MHz instrument, and 2 mg of the same undecasaccharide was prepared (3.3 mM) for the 600-MHz instrument with a CryoProbe™. (B) 600-MHz $^1H$ NMR spectrum for the undecasaccharide. The arrows indicate the seven signals selected in the 2D selective-TOCSY-HSQC, the 2D selective-TOCSY-DQFCOSY, and the 2D selective-TOCSY-NOESY experiments.

is applied as described above, the satellite signal (H–$^{13}$C) of this selectively excited proton is also excited, and a correlation signal is observed. In contrast, when a satellite signal (H–$^{13}$C) from the other sugar residue is resonated at the same chemical shift of the selectively excited proton, a correlation signal from the other sugar residue infects the HSQC spectrum.

**2.1.2. 2D selective-TOCSY-DQFCOSY (Fig. 1B).** This experiment has been published and was applied to lactose (β-D-Gal-(1→4)-D-Glc).[23] We have applied this experiment to the biantennary undecasaccharide[25] shown in Figure 2A.

**2.1.3. 2D selective-TOCSY-NOESY (Fig. 1C).** This experiment is an improved version of a previously reported experiment.[24] The previous experiment did not employ a pulsed-field gradient at NOE-mixing time. By introducing a gradient pulse, the resulting spectrum does not have any artifacts, and it is possible to distinguish between inter- and intra-sugar ring NOE signals. The NOE signal for intra-sugar ring signals connect diagonal peaks via cross-peaks, and inter-sugar ring signals connect diagonal peaks to invisible diagonal peaks of the other residue.

## 2.2. Assignment of individual sugar residues

**2.2.1. GlcNAc-a.** GlcNAc-a is a residue located at the reducing end and is linked to the amino group of asparagine. Therefore, the anomeric proton is observed at 5.0 ppm and is different from that of the other GlcNAc residues (b, e, and i). The selective excitation pulse was focused on H-1. Extraction of protons in GlcNAc-a skeleton was performed by a 1D selective-TOCSY experiment in which the shape of the selective pulse was RE-BURP,[26] and the isotropic mixing for the TOCSY period was employed by an MLEV-17 sequence. The 1D TOCSY spectrum was obtained as shown in Figure 3A and then developed to the second dimension according to the pulse sequence of the 2D selective-TOCSY-DQFCOSY (Fig. 1B). Selective excitation of this proton results in a well-separated selective-TOCSY-DQFCOSY spectrum. As shown in Figure 3B, although H-6A overlaps with H-2, and H-4, H-5, and H-6B appear in the narrow region, all proton signals of GlcNAc-a could be assigned. The magnetization of the 1D TOCSY was developed to the second dimension according to the 2D selective-TOCSY-HSQC pulse program (Fig. 1A). Since the anomeric carbon (C-1) is linked to the amino group, a correlation signal for

C-1 was observed at 78 ppm in the selective-TOCSY-HSQC spectrum (Fig. 3A), which is different from that of the other sugar residues in the undecasaccharide. The intensity of the correlation with C-6 is small, but clearly observed. C-4 was observed at low field in the $^{13}$C-dimension due to the typical glycosidic shift, which is evidence of glycosylation at the 4-position.

**2.2.2. GlcNAc-b,e,i.** Assignment of the GlcNAc-b,e,i residues was performed using the same conditions as with GlcNAc-a. The H-1 signal of the three GlcNAc-b,e,i residues overlapped at 4.5 ppm. Since individual H-1 signals could not be selectively excited using a RE-BURP pulse, a selective-TOCSY-DQFCOSY spectrum was obtained as a mixture of three GlcNAc-b,e,i residues. As shown in Figure 3E, TOCSY development was achieved from H-1 to H-6. However, H-2 and H-3 of the three GlcNAc-b,e,i residues overlapped at 3.7 ppm. In addition, H-4 and H-5 were observed in the narrow region. In these sugar residues, unambiguous assignment was difficult using only selective-TOCSY-DQFCOSY. This problem was solved using the selective-TOCSY-HSQC spectrum in Figure 3D. Since the H-1 signals of three GlcNAc-b,e,i residues overlapped in the $^{1}$H NMR spectrum, a selective-TOCSY-HSQC experiment was also performed on the three GlcNAc-b,e,i residues by the excitation of the three H-1's. Although C-1's and C-6's were observed with low sensitivity, the other ring carbons were clearly observed as shown in Figure 3D. In this case, the correlation signals of the 1-position and 6-position were assigned from a conventional HSQC spectrum and a conventional HSQC-TOCSY spectrum (data not shown). Although the correlation signals of the 3- and 5-positions in the three GlcNAc-b,e,i residues were observed at the same chemical shift in the $^{1}$H and $^{13}$C spectra, the correlation signals of the 1-, 2-, 4-, and 6-positions for GlcNAc-b were separated from those for GlcNAc-e,i in a conventional HSQC-TOCSY spectrum in $H_2O$ (data not shown) and assigned by the correlation from three separated protons of the AcNH-group. C-4 was observed at low field in the $^{13}$C-dimension due to the typical glycosidic shift, which is evidence of glycosylation at the 4-position.

**2.2.3. Mannoside-c.** Mannoside-c has a β-glycosyl linkage, but the coupling constant is small between H-1 and H-2. Selective excitation was focused on H-2 and then

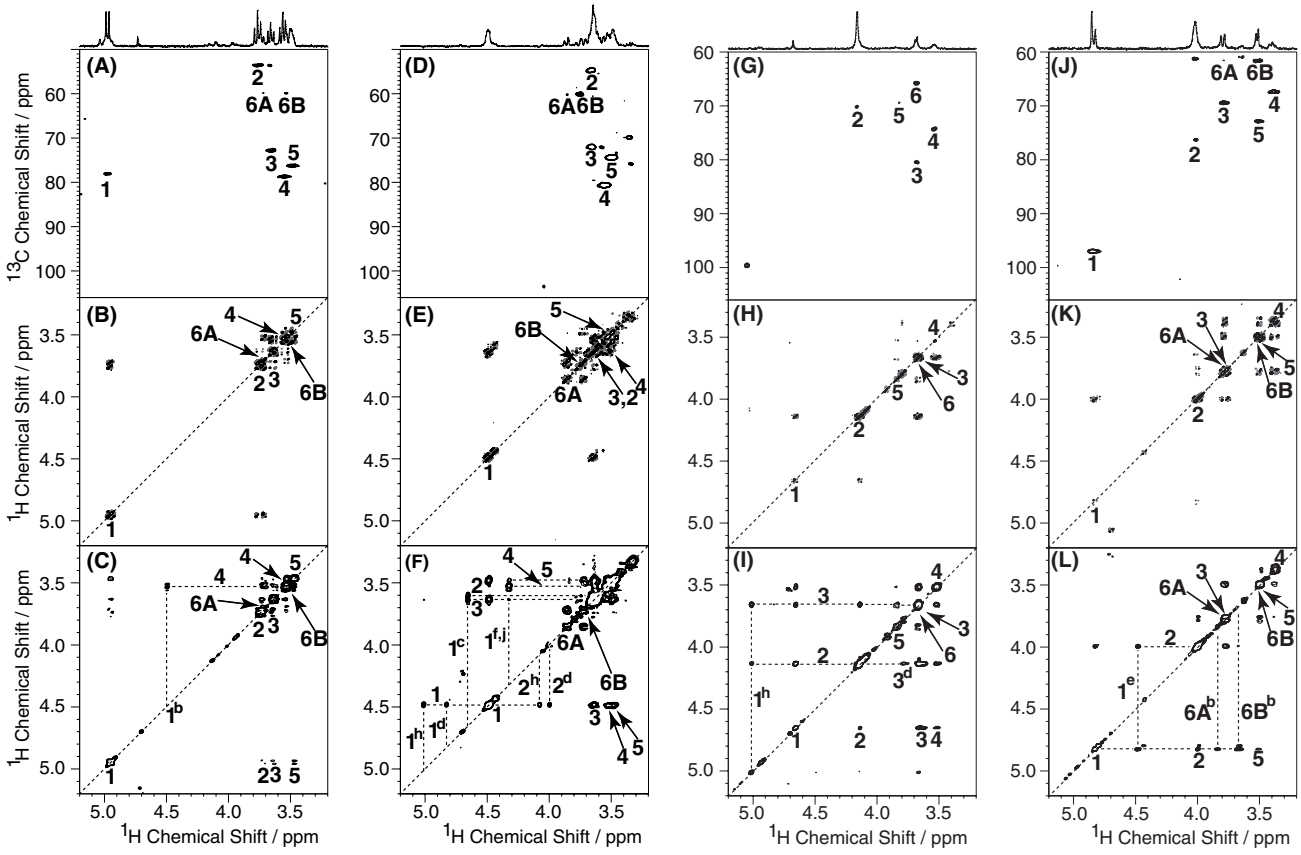


**Figure 3.** Contour plots of the 2D selective-TOCSY-HSQC (A, D, G, J, M, P, and S), the 2D selective-TOCSY-DQFCOSY (B, E, H, K, N, Q, and T), and the 2D selective-TOCSY-NOESY (C, F, I, L, O, R, and U) spectra of the undecasaccharide on a 400-MHz instrument. A, B, and C are GlcNAc-a. D, E, and F are GlcNAc-b,e,i. G, H, and I are Man-c. J, K, and L are Man-d. M, N, and O are Man-h. P, Q, and R are Gal-f,j. S, T, and U are NeuAc-g,k. Each 1D selective-TOCSY spectrum is shown on the 2D spectrum.

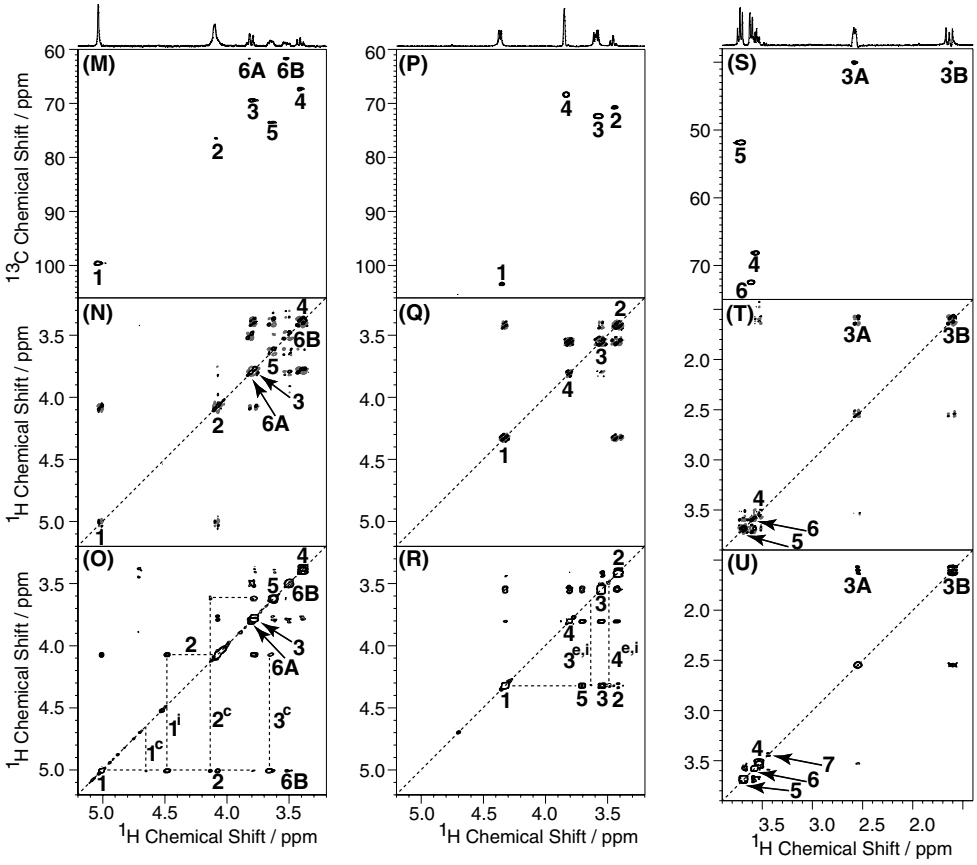


**Figure 3.** (*continued*)

developed to H-6 in the selective-TOCSY experiment. Although assignment from H-1 to H-4 can be performed as shown in Figure 3H, the correlation signals of H-3 and H-6 were not separated. This problem was solved by acquisition of the selective-TOCSY-HSQC spectrum in Figure 3G. The selective-TOCSY-HSQC spectrum shows clear correlation signals for mannoside-c. Proton chemical sifts of all correlations except the 1-position in Figure 3G were identified from the 1D TOCSY and selective-TOCSY-DQFCOSY spectra. Assignment of the 1-position was reconfirmed by a conventional HSQC spectrum and a conventional 2D TOCSY-HSQC spectrum (data not shown). C-3 and C-6 were observed at low field in the $^{13}$C-dimension due to the typical glycosidic shift, which is evidence of glycosylation at the 3- and 6-positions.

**2.2.4. Mannoside-d.** In order to take a 1D selective-TOCSY, the H-2 proton was excited by a shaped pulse. Both H-1 and H-2 are well separated in the NMR spectrum, but the coupling constant between them is small (<1 Hz). Therefore, TOCSY development from H-2 to H-6 gives better results than development from H-1. As shown in Figure 3K, a well-separated 2D DQF-COSY spectrum was obtained, and all protons were easily assigned. In addition, the H-3 and H-6A, as well as H-5 and H-6B, overlap in the 1D spectrum, but both signals are easily separated in the DQFCOSY spectrum. In the selective-TOCSY-HSQC spectrum, H-2 was also excited. As shown in Figure 3J, all carbon signals were clearly observed and can be assigned. Although the coupling constant between H-6A and H-5 was small, correlation signals between C-6 and H-6A/H-6B can be clearly observed. C-2 was observed at low field in the $^{13}$C-dimension due to the typical glycosidic shift, which is evidence of glycosylation at the 2-position.

**2.2.5. Mannoside-h.** Since all proton signals are well separated in the 1D TOCSY spectrum, exciting H-2 gives a clear selective-TOCSY-DQFCOSY spectrum as shown in Figure 3N. In this selective-TOCSY-HSQC spectrum (Fig. 3M), all correlation signals were observed as in the case of mannoside-d. C-2 was also observed at low field in the $^{13}$C-dimension due to the typical glycosidic shift, which is evidence of glycosylation at the 2-position.

**2.2.6. Galactosides-f,j.** H-1's of the galactosides were easily assigned by 1D selective-TOCSY, since TOCSY development stopped at H-4 due to a small coupling constant between H-4 and H-5, and the coupling constants observed for H-1 to H-4 were identical to with

those of typical galactoside protons. Therefore, 2D selective-TOCSY-DQFCOSY (Fig. 3Q) between H-1 and H-4 was performed, and the correlation signals were clearly observed. In this case, however, resonance signals of two galactoside residues overlapped. Assignment of carbon signals was performed from the selective-TOCSY-HSQC spectrum (Fig. 3P). In this case, only C-1, C-2, C-3, and C-4 were observed even using several different isotropic-mixing times. Therefore, assignment of C-5 and C-6 was performed using conventional 2D HSQC, HSQC-TOCSY, and HSQC-NOESY spectra (data not shown). C-6 was observed at low field in the $^{13}$C-dimension due to the typical glycosidic shift, which is evidence of glycosylation at the 6-position.

**2.2.7. NeuAc-g,k.** NeuAc (sialic acid) is a characteristic sugar residue having a carboxyl group at the 2-position and a deoxygenated carbon atom at the 3-position. Since sialic acid does not have an anomeric proton, the selective excitation pulse was focused on the axial proton at H-3. In addition, since the coupling constant between H-6 and H-7 is very small (<1 Hz), correlation signals from the H-3 stopped at the H-6. In order to observe H-7, H-8, and H-9, the isotropic-mixing time was varied; however, the H-7 signal could not be observed. As shown in Figure 3T, protons from H-3 to H-6 were clearly observed on the DQFCOSY spectrum. The correlation signals from C-3 to C-6 can be clearly observed as expected in the HSQC spectrum (Fig. 3S). Correlation signals of the 7-, 8-, and 9-positions were assigned by conventional 2D HSQC, HSQC-TOCSY, and HSQC-NOESY spectra (data not shown). The chemical shifts of these carbons were almost identical to those reported for sialic acid in small oligosaccharides.

### 2.3. Table of chemical shift data

Assignment of $^{1}$H and $^{13}$C in the undecasaccharide is shown in Table 1. The $^{1}$H chemical shifts are referenced to the residual HDO signal (4.7 ppm), while the $^{13}$C

**Table 1.** Assignment of the $^{1}$H and $^{13}$C signals by 2D selective-TOCSY-HSQC and 2D selective-TOCSY-DQFCOSY experiments

| Excitation sugar and its positions | $^{1}$H (ppm) | $^{13}$C (ppm) | Excitation sugar and its positions | $^{1}$H (ppm) | $^{13}$C (ppm) |
|---|---|---|---|---|---|
| GlcNAc-a 1 | 4.97 | 78.15 | Man-d 1 | 4.84 | 96.90 |
| 2 | 3.74 | 53.47 | 2 | 4.01 | 76.21 |
| 3 | 3.65 | 72.74 | 3 | 3.78 | 69.35 |
| 4 | 3.54 | 78.61 | 4 | 3.38 | 67.84 |
| 5 | 3.48 | 76.14 | 5 | 3.50 | 72.76 |
| 6A | 3.72 | 59.75 | 6A | 3.78 | 61.56 |
| 6B | 3.53 | 59.75 | 6B | 3.51 | 61.56 |
| GlcNAc-b 1 | 4.52 | 101.22 | Man-h 1 | 5.03 | 99.50 |
| 2 | 3.68 | 54.81 | 2 | 4.09 | 76.38 |
| 3 | 3.66 | 71.98 | 3 | 3.79 | 69.33 |
| 4 | 3.65 | 79.51 | 4 | 3.41 | 67.25 |
| 5 | 3.51 | 74.34 | 5 | 3.63 | 73.42 |
| 6A | 3.78 | 59.85 | 6A | 3.82 | 61.62 |
| 6B | 3.66 | 59.85 | 6B | 3.52 | 61.62 |
| GlcNAc-e,i 1 | 4.50 | 99.23 | Gal-f,j 1 | 4.35 | 103.37 |
| 2 | 3.66 | 54.50 | 2 | 3.44 | 70.61 |
| 3 | 3.66 | 71.98 | 3 | 3.57 | 72.23 |
| 4 | 3.54 | 80.59 | 4 | 3.83 | 68.24 |
| 5 | 3.51 | 74.34 | 5 | 3.73 | 73.63 |
| 6A | 3.88 | 60.12 | 6A | 3.90 | 63.24 |
| 6B | 3.75 | 60.12 | 6B | 3.45 | 63.24 |
| Man-c 1 | 4.65 | 100.38 | NeuAc-g,k 3A | 2.58 | 39.93 |
| 2 | 4.16 | 70.09 | 3B | 1.62 | 39.93 |
| 3 | 3.68 | 80.34 | 4 | 3.56 | 68.12 |
| 4 | 3.53 | 74.21 | 5 | 3.71 | 51.77 |
| 5 | 3.82 | 69.34 | 6 | 3.61 | 72.45 |
| 6A | 3.68 | 65.68 | 7 | 3.46 | 68.29 |
| 6B | 3.68 | 65.68 | 8 | 3.79 | 71.65 |
| | | | 9A | 3.78 | 62.52 |
| | | | 9B | 3.54 | 62.52 |

Correlation signals of the 1- and 6-positions of GlcNAc-b,e,i and the 1- and 6A-position of Man-c were reconfirmed by conventional HSQC and HSQC-TOCSY spectra (data not shown). Although the correlation signals of the 3- and 5-positions in the three GlcNAc-b,e,i residues were observed at the same chemical shift on $^{1}$H and $^{13}$C, correlation signals of 1-, 2-, 4-, and 6-positions were separated GlcNAc-b from GlcNAc-e,i on a conventional HSQC-TOCSY spectrum in $H_2O$ (data not shown) and assigned by the correlation of three separated protons of the AcNH-group. Correlation of the 5- and 6-positions of Gal-f,j and the 7-, 8-, and 9-positions of NeuAc-g,k were assigned by conventional HSQC, HSQC-TOCSY, and HSQC-NOESY spectra (data not shown).

**Table 2.** A total of 25 inter- and 79 intra-NOE signals were observed by 2D selective-TOCSY-NOESY

| Excitation sugar and its positions | Inter-NOE | Intra-NOE | Excitation sugar and its positions | Inter-NOE | Intra-NOE |
|---|---|---|---|---|---|
| GlcNAc-a 1 | | 2a, 3a, 5a | Man-h 1 | 1c, 2c, 3c, 1i | 2h, 6′h |
| 2 | | 3a | 2 | | 1h, 3h, 6′h |
| 3 | | | 3 | | 2h, 4h, 5h, 6′ |
| 4 | 1b | | 4 | | 3h, 5h |
| 5 | | 6′a | 5 | 2c | 3h |
| 6 | | 3a, 6′a | 6 | | 2h, 3h, 4h, 5h, 6′ |
| 6′ | | 3a, 5a, 6a | 6′ | | 6 |
| GlcNAc-b,e,i 1 | 1d, 2d, 1h, 2h | 3, 4, 5 | Gal-f, j 1 | 3e, 3i, 4e, 4i | 2, 3, 5 |
| 2 | 1c | | 2 | | 3 |
| 3 | 1c, 1f, 1j | 5 | 3 | | 1, 2, 4, 5 |
| 4 | | 5 | 4 | | 1, 2, 3, 5 |
| 5 | | 1, 3, 4, 6, 6′ | 5 | | |
| 6 | | | 6 | | |
| 6′ | | 6 | 6′ | | |
| Man-c 1 | | 2c, 3c, 4c | NeuAc-g,k 3ax | | 3eq |
| 2 | | | 3eq | | 3ax |
| 3 | 1n | 3c, 4c | 4 | | 3ax |
| 4 | 1n | 1c, 2c, 4c, 5c | 5 | | 6 |
| 5 | | 1c, 3c | 6 | | 5, 7 |
| 6 | | | | | |
| 6′ | | | | | |
| Man-d 1 | 1e | 2d, 5d | | | |
| 2 | 1e | 1d, 3d | | | |
| 3 | | 2d, 4d, 5d | | | |
| 4 | | 5d | | | |
| 5 | | 6 | | | |
| 6 | | 6′ | | | |
| 6′ | | 6 | | | |

chemical shifts are referenced indirectly to the absolute frequency ratio $^{13}C/^{1}H = 0.251450201$.

### 2.4. Assignment of inter-sugar and intra-sugar NOE signals by a 2D selective-TOCSY-NOESY experiment

In order to investigate conformational properties of the oligosaccharide, a nuclear Overhauser effect (NOE) experiment is the most suitable for estimation of both the three-dimensional (3D) structure and the molecular dynamics. The experiment involves the NOESY method to find the through-space network in the molecule. However, a full assignment of the NOE signal is difficult when the number of NOE signals is many and the resonances overlap. The overlapping signals make it impossible to unambiguously assign the NOE signals. To overcome this problem, we modified the selective-TOCSY-NOESY experiment to enable unambiguous assignment of the NOE signals. This experiment involves a pulsed field gradient to eliminate unwanted signals. The mechanism of this experiment is similar to that of 2D selective-TOCSY-DQFCOSY and selective-TOCSY-HSQC experiments. The NOE signal for an intra-sugar ring connects from one diagonal peak to another diagonal peak via a cross-peak, and for inter-ring connects one diagonal peak correlates with an invisible diagonal peak of the other residue on the spectrum. This method enabled us to assign all the NOE signals (Table 2) and observe 2D NOESY spectra corresponding to each desired sugar as shown in Figure 3C, F, I, L, O, R, and U. NOE signals are well separated, and even the signals close to the diagonal peaks are clearly observed. For estimation of the distance between protons, NOE buildup rate curves are usually prepared,[27–29] but this selective TOCSY-NOESY system does not involve a linear relationship between the intensity of the NOE signal and the proton–proton distance, since the magnetization of the diagonal peaks is not homogenously transferred from selectively excited protons to remote protons. To solve this problem, the NOE buildup rate curve was prepared from a conventional NOESY experiment[30] focusing on the desired NOE signals.

## 3. Discussion

As shown in Figure 2B, H-1 of GlcNAc-a, GlcNAc-b,e,i, and Gal-f,j, H-2 of Man-c, Man-d, and Man-h, and H-3 of NeuAc-g,k are largely resolved in the $^{1}H$ spectrum of the undecasaccharide. By measuring the 2D selective-TOCSY-HSQC and the 2D selective-TOCSY-DQFCOSY spectra in which the H-1 of GlcNAc-a were selectively excited, the correlation signals

between protons and carbons became visible in the HSQC spectrum (Fig. 3A), and the proton signals from H-1 to H-6 of GlcNAc were sequentially assigned by connecting the diagonal peaks to the cross-peaks on the DQFCOSY spectrum (Fig. 3B). The assignment for each of the other sugar components was performed in the same way (Fig. 3). Assignment of the signals on the selective-TOCSY-HSQC and the selective-TOCSY-DQFCOSY spectra was not difficult because there is a one-to-one correspondence of these two spectra and the sugar components in the oligosaccharide. The spectral width of the indirect $^{13}$C-dimension and the offset of the $^{13}$C-frequency were individually optimized for each sugar component in the undecasaccharide. It is advantageous to employ the selective 2D excitation rather than the nonselective 3D excitation. The selective excitation pulse and number of scans can be optimized individually. In a 3D NMR experiment, optimization of NMR parameters such as TOCSY isotropic-mixing time, spectral width, and number of scans is not possible for each sugar residue. If NMR usage time is limited to half a day, several 2D selective-TOCSY-HSQC spectra, in contrast to one 3D spectrum, can be obtained and used for analysis. The resolution of the 2D selective-TOCSY-HSQC spectrum (Fig. 3Q) of Gal-f,j in the undecasaccharide is much better than that in the $^{1}$H–$^{1}$H plane (Fig. 4A) for Gal-f,j of a conventional 3D TOCSY-HSQC spectrum. Although the $^{1}$H signals of Gal-f,j are well-separated in the 1D selective-TOCSY spectrum (left side of Fig. 4A) of the undecasaccharide, assignment of H-2 is difficult (Fig. 4A). The acquired data points of the 3D experiment were 1024, 112, and 140 in the $t_3$ ($^{1}$H), $t_2$ ($^{13}$C), and $t_1$ ($^{1}$H) time domains, respectively. The experimental time was 84 h 52 min. The 3D spectrum was zero-filled and Fourier transformed to 1024, 512, and 1024, and the digital spectral resolution was 2.63, 11.49, and 2.63 Hz, in the $f_3$, $f_2$, and $f_1$ frequency domains, respectively. The three frequency domains showed relatively high spectral resolution, but a few correlation signals from Man-h were nevertheless mixed into the $^{1}$H–$^{13}$C plane (Fig. 4B) for GlcNAc-a of the 3D spectrum. In contrast to the $^{1}$H–$^{13}$C plane of the 3D spectrum, the 2D selective-TOCSY-HSQC spectrum showed the correlation signals for GlcNAc-a as shown in Figure 3A. Although this 2D experiment does not show high sensitivity, unambiguous assignment of the individual sugar components was possible. This measurement supports nonempirical procedure in assigning the signals and these data for the undecasaccharide results in different signal assignments from the previous report.[31,10]

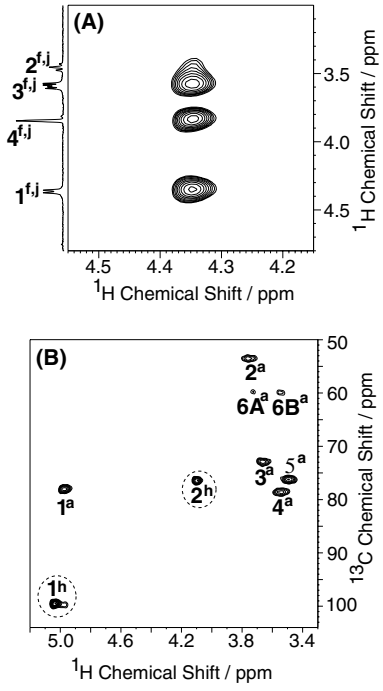


**Figure 4.** (A) Expanded plot of $^{1}$H–$^{1}$H plain for Gal-f,j in the 3D TOCSY-HSQC spectrum of the undecasaccharide. The 1D selective-TOCSY spectrum is shown on the left. (B) Expanded plot of $^{1}$H–$^{13}$C plain for GlcNAc-a of the 3D spectrum. Some signals from Man-h are indicated by the dotted circle.

In order to improve the sensitivity in the three experiments, the isotropic-mixing time in the selective-TOCSY period was adjusted to optimize the intensity of the signals from H-1 to H-6. As a result, the duration of 150 ms for the isotropic-mixing time was found to be the most suitable for the selective-TOCSY experiment. The same duration was therefore used for all three experiments. Because Gal and NeuAc normally show a very small scalar coupling between H-4 and H-5, and between H-6 and H-7, respectively, and also the magnetization transfer from H-4 to H-5 and from H-6 to H-7 in the above sugars is not efficient in these 2D selective-TOCSY-type experiments, H-5 and H-6 in Gal and H-7, H-8, and H-9 in NeuAc are not observed in the 2D selective-TOCSY-HSQC and the 2D selective-TOCSY-DQFCOSY experiments. These signals were assigned by comparison of the conventional 2D HSQC, 2D HSQC-TOCSY, and 2D HSQC-NOESY spectra. All proton and carbon signals were successfully assigned by these experiments.

In order to measure a low concentration sample of the same undecasaccharide (2 mg, 3.3 mM), the 2D selective-TOCSY-HSQC spectra were measured on a 600-MHz NMR instrument with a CryoProbe™ with a sensitivity up to four times that of a conventional inverse probe. The four spectra for GlcNAc-a, GlcNAc-b,e,i, Gal-f,j, and NeuAc-g,k were obtained in 2 days (Fig. 5). Optimization of the spectral width of the indirect $^{13}$C-dimension and the offset of the $^{13}$C-frequency for each of sugar component in the undecasaccharide was more efficient on the 600-MHz instrument than on the 400-MHz instrument because the acquisition time was

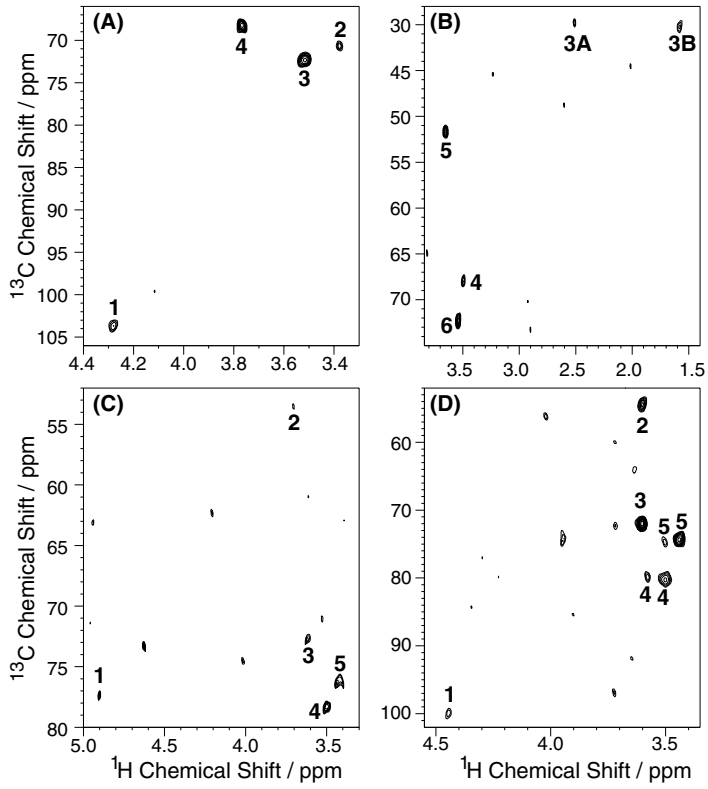


**Figure 5.** Contour plots of the 2D selective-TOCSY-HSQC spectra for Gal-f,j (A), NeuAc-g,k (B), GlcNAc-a (C), and GlcNAc-b,e,i (D) of the undecasaccharide (2 mg, 3.3 mM) with a CryoProbe™ on a 600-MHz instrument.

reduced and the digital resolution of the spectrum was sufficient for analysis. A tiny amount (<1 mg) of oligosaccharides isolated from a natural source may not be enough for analysis, but large, synthetic oligosaccharides are enough for this new and concise 2D selective-TOCSY-HSQC method.

Conformational analysis is essential to have an insight into the 3D structure of oligosaccharide that is a bioactive ligand toward proteins on a cellular or viral surface. In order to analyze the detailed conformation, an NOE experiment is suitable, but one should be able to assign all of the NOE network observed over the range of 3–4 ppm in the 2D NOESY spectrum. Assignment of enormous NOE signals is very laborious work due to heavily overlapped resonances. After the assignment of the signals by 2D selective-TOCSY-HSQC and selective-TOCSY-DQFCOSY, the enormous NOE signals were assigned using the selective-TOCSY-NOESY spectra. The 25 inter-ring NOE and the 79 intra-ring NOE signals (Fig. 3C, F, I, L, O, R, and U) were collected by experiment as shown in Table 2. Almost all inter-ring NOE networks are identical compared to that of di- or trisaccharides. Key NOE signals for the 3D structure of the biantennary oligosaccharide are around three branched mannose residues. NOEs of H-1$^{h}$ to H-2$^{c}$ and H-3$^{c}$ are sufficient to analyze the 3D structure around the α-D-Man-(1→3)-β-D-Man linkage. On the other hand, analysis of the α-D-Man-(1→6)-β-D-Man linkage is frequently confused due to mixture of two kinds of glycosyl conformers (*gt* and *gg* rotamers). However, since inter-ring NOE signals between H-1$^{d}$ and H-6B$^{b}$ and between H-3$^{d}$ and H-2$^{c}$ were observed, conformation of the α-D-Man-(1→6)-β-D-Man linkage appears to be biased in favor of the *gt* rotamer. Complete NOE assignment would serve to support computational analysis that would be useful in the design of a potent inhibitor to block virus invasion.

## 4. Conclusion

An unambiguous sequential assignment of proton and carbon signals of an undecasaccharide was achieved by 2D selective-TOCSY-HSQC and 2D selective-TOCSY-DQFCOSY experiments on a 400-MHz NMR instrument. This method can be used with the naturally occurring concentration of oligosaccharides in the CryoProbe™. We believe that this method is a powerful new tool for analyzing oligosaccharide samples. As shown in Figure 6 and Table 2, the 25 inter-ring NOE and the 79 intra-ring NOE signals were collected by 2D selective-TOCSY-NOESY experiments. The 3D structure of the biantennary undecasaccharide was deduced from these studies.

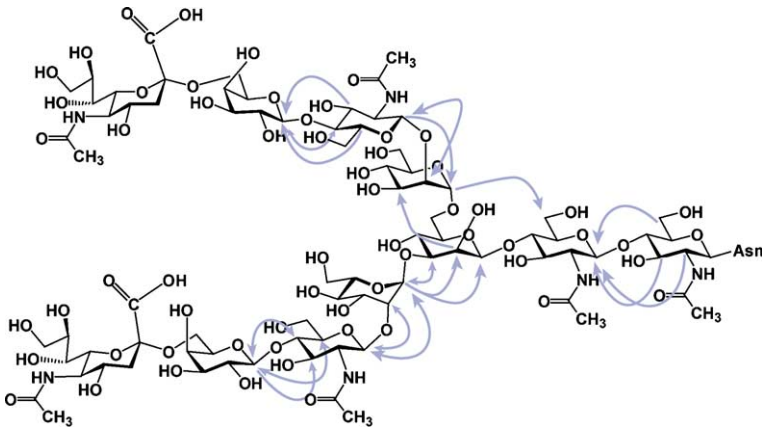

**Figure 6.** Inter-ring NOE connectivity is indicated by arrows.

## 5. Experimental

### 5.1. General methods

All measurements for the undecasaccharide (20 mg, 33 mM) in $D_2O$ were acquired on a Bruker AVANCE-400 spectrometer ($^1H$ frequency: 400.13 MHz) equipped with an inverse 5-mm BBI ($^1H$/Broadband) probe-head fitted with a Z-gradient coil. The 2D selective-TOCSY-HSQC experiment for the same sample at low concentration (2 mg, 3.3 mM) was 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. The temperature was set to 293 K on both instruments.

The shape of the selective excitation pulse for the 2D selective-TOCSY-HSQC experiment on the 400-MHz instrument was RE-BURP,[25] and the duration of the pulse was set to 70 ms for the Gal-f,j and NeuAc-g,k or 150 ms for the Man-c, Man-d, Man-h, GlcNAc-a, and GlcNAc-b,e,i residues. The number of scans and the experimental time were as follows: Gal-f,j; 128 scans and 6 h 13 min, NeuAc-g,k; 256 scans and 12 h 28 min; Man-c, Man-d, and Man-h; 800 scans and 41 h 30 min, GlcNAc-a and GlcNAc-b,e,i; 1024 scans and 52 h 37 min. The duration of the RE-BURP pulse was set to 150 ms for Gal-f,j and NeuAc-g,k or 200 ms for Man-c, Man-d, Man-h, GlcNAc-a, and GlcNAc-b,e,i residues in the 2D selective-TOCSY-DQFCOSY and the 2D selective-TOCSY-NOESY experiments on the 400-MHz instrument. The number of scans and the experimental time for the 2D selective-TOCSY-DQF-COSY were as follows: Gal-f,j and GlcNAc-b,e,i; 8 scans and 1 h 43 min, NeuAc-g,k; 32 scans and 6 h 50 min, GlcNAc-a and Man-h; 32 scans and 7 h 4 min, Man-c and Man-d; 64 scans and 14 h 7 min. The NOE-mixing time was set to 300 ms in the 2D selective-TOCSY-NOESY experiments. The number of scans and the experimental times were as follows: Gal-f,j; 8 scans and 2 h 6 min, NeuAc-g,k and Man-h; 16 scans and 4 h 6 min, GlcNAc-a, GlcNAc-b,e,i, Man-c, and Man-d; 32 scans and 8 h 25 min. In the 2D selective-TOCSY-HSQC experiment, the data were recorded with a spectral width of 2003 Hz in proton dimensions, with an FID of 512 data points and a $t_1$-increment of 128 points. The $^{13}C$ spectral width was individually set for each sugar component as follows: 3571 Hz for GlcNAc-a, 3731 Hz for NeuAc-g,k, 5000 Hz for Man-c, Man-d, and Man-h, 5556 Hz for Gal-f,j and GlcNAc-b,e,i. The data were recorded with a spectral width of 2003 Hz in both proton dimensions, with an FID and a $t_1$-increment of 512 data points in both the 2D selective-TOCSY-DQFCOSY and 2D selective-TOCSY-NOESY experiments. The data were zero-filled to 2k and 2k in both dimensions prior to Fourier transform. A $\pi/3$-shifted sine-bell window function was applied in both dimensions.

The experimental parameters for the 2D selective-TOCSY-HSQC experiment on the 600-MHz instrument were the same as those on the 400-MHz instrument, except the pulse length of the RE-BURP, the number of scans, the experimental time, the data points of the FID and $t_1$-increments, and the spectral width in $^1H$ and $^{13}C$. The duration of the RE-BURP pulse was set to 80 ms for the GlcNAc-a, GlcNAc-b,e,i, Gal-f,j, and NeuAc-g,k residues. The number of scans were 3200, 1024, 512, and 512 for the GlcNAc-a, GlcNAc-b,e,i, Gal-f,j, and NeuAc-g,k residues, respectively. The experimental times were 21 h 1 min, 13 h 29 min, 6 h 49 min, and 5 h 3 min for the GlcNAc-a, GlcNAc-b,e,i, Gal-f,j, and NeuAc-g,k residues, respectively. The data were recorded with a spectral width of 3592 Hz in proton dimensions, with an FID of 512 data points and a $t_1$-increment of 18, 36, 36, and 28 points for the GlcNAc-a, GlcNAc-b,e,i, Gal-f,j, and NeuAc-g,k residues, respectively. The $^{13}C$ spectral widths were individually set for the sugar components as follows: 4762, 8772,

6944, and 5747 Hz for the GlcNAc-a, GlcNAc-b,e,i, Gal-f,j, and NeuAc-g,k residues, respectively.

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