NMR Analysis of Oligosaccharides Containing Fructopyranoside

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ABSTRACT

This review focuses on the NMR methods for the oligosaccharides containing fructopyranoside that were previously isolated from the fermented beverage of an extract from 50 kinds of fruits and vegetables. The ^{1}H and ^{13}C-NMR signals of each saccharide were assigned using 2D-NMR including COSY, HSQC, HSQC-TOCSY, CH$_2$-selected HSQC-TOCSY, and CT (constant time)-HMBC. The fructose in pyranosyl form showed different ^{13}C chemical shifts from those of furanosyl form. Further confirmation of the pyranosyl form could be obtained from the HMBC correlation peak between C-2 and H-6 of fructose residue (Fru), whereas the C-2 of Fru in furanosyl form chould give the HMBC correlation peak between H-5 of Fru. Problems encountered were signal overlapping of protons and low peak separation. The key correlation peak between C-2 and H-6 of Fru was overlapped by the correlation peak indicating a glycosidic linkage between the C-2 of Fru and the H-6 of the glucose residue (Glc, or Glc'). These were solved using HSQC and CT-HMBC spectra rather than HMQC and conventional HMBC spectra, which have an inherent broad-line-shape in the carbon dimension.

CONTENTS

INTRODUCTION

The NMR spectra of oligosaccharides are too complex to analyze using routine methods, COSY, HSQC, HSQC-TOCSY, and HMBC. The problems are signal overlapping of protons and low peak separation. The key HMBC correlation peak, which indicates a sugar-linkage, tends to be overlapped by intra-residual correlation peaks. Therefore, we created special methods to overcome this problem. Several oligosaccharides containing fructopyranoside were isolated from fermented beverage of an extract from 50 kinds of fruits and vegetables (Okada *et al.* 2006; Kawazoe *et al.* 2008). Their structure had been analyzed in a same procedure by using 1D and 2D NMR methods. Since the conventional NMR methods could not complete the analysis of their structure, the high-resolution 2D methods were applied. The established protocol performed the structural analysis of related oligosaccharide. In this paper, the procedure of these NMR analysis and efficiency of utilization of advanced NMR methods will be demonstrated using saccharide **1** (**Fig. 1**) as a model compound.

General procedure for NMR analysis

Fig. 2A shows a one-dimensional (1D) carbon (^{13}C)-NMR spectrum of saccharide **1**. NMR detects the nuclear magnetic resonance while 1D NMR spectrum provides a record about one kind of nucleus, in this case, the nucleus of car-
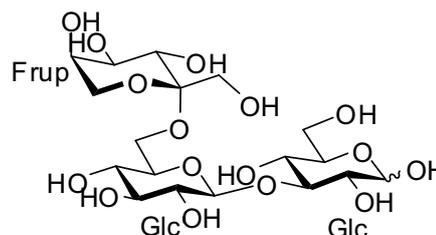

Fig. 1 Structure of saccharide 1.

bon. The horizontal axis, which indicates the chemical shift (in ppm) is an important value of NMR in which each carbon placed in a different environment, shows a different chemical shift. **Fig. 2B** is a 1D proton (^{1}H)-NMR spectrum. The fine structure of the signal because of the proton-proton coupling indicates information about neighboring protons. The anomer configuration of α- or β-aldose can be determined by the coupling constant (splitting width) of the anomeric proton.

One-dimensional ^{1}H and ^{13}C NMR signals are assigned using two-dimensional (2D) NMR spectroscopy. **Fig. 3** shows a 2D HSQC (Bodenhausen *et al.* 1980; Willker *et al.* 1993) spectrum. The first dimension (F_1), the vertical axis, is a carbon chemical shift (δ_C). The second dimension (F_2), the horizontal axis, is a proton chemical shift (δ_H). Proton and carbon 1D spectra are attached. The HSQC detects the
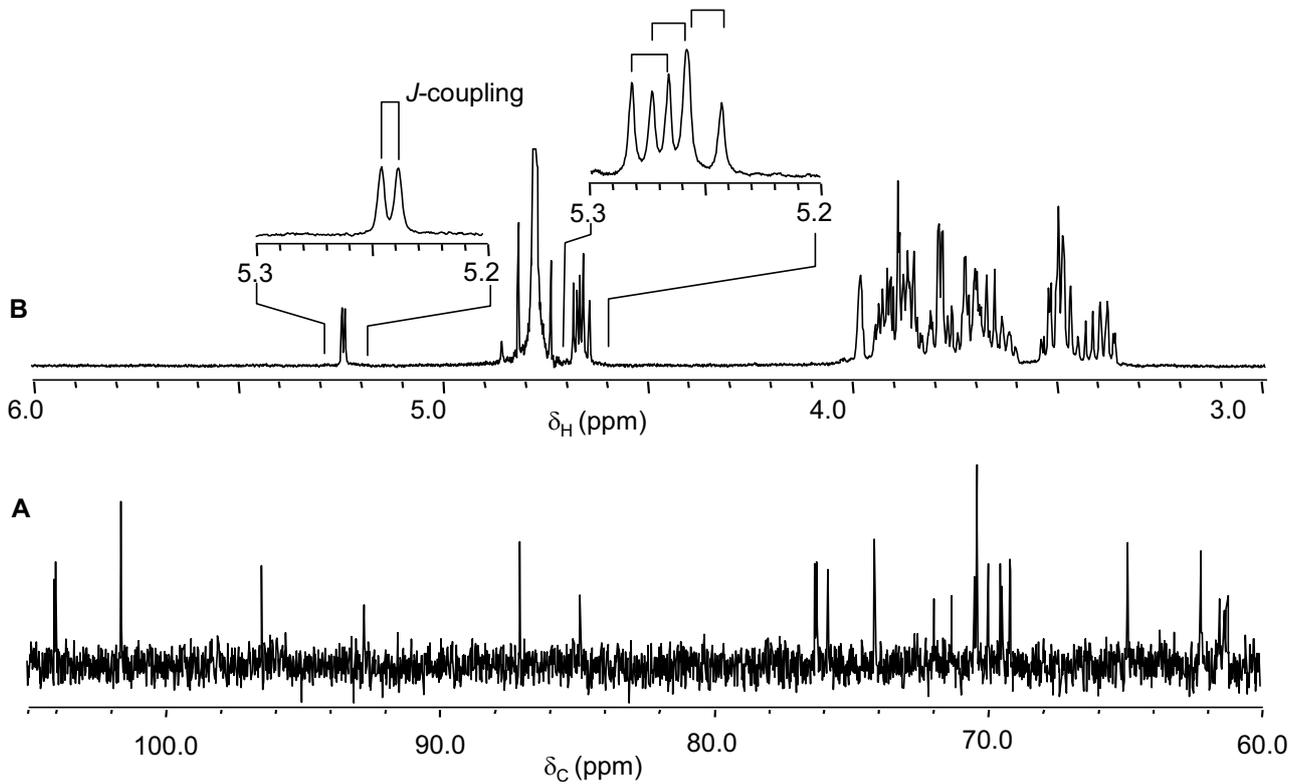
Fig. 2 One dimensional (1D) ^{13}C(A, 126 MHz)- and ^{1}H(B, 500MHz)-NMR spectra of 1 (5 mg / 0.5 ml of D$_2$O). Chemical shifts of ^{1}H (δ_H) and ^{13}C (δ_C) in ppm were determined relative to the external standard of sodium [2,2,3,3-^{2}H$_4$]-3-(trimethylsilyl) propanoate in D$_2$O (δ_H 0.00 ppm) and 1,4-dioxane (δ_C 67.40 ppm) in D$_2$O.
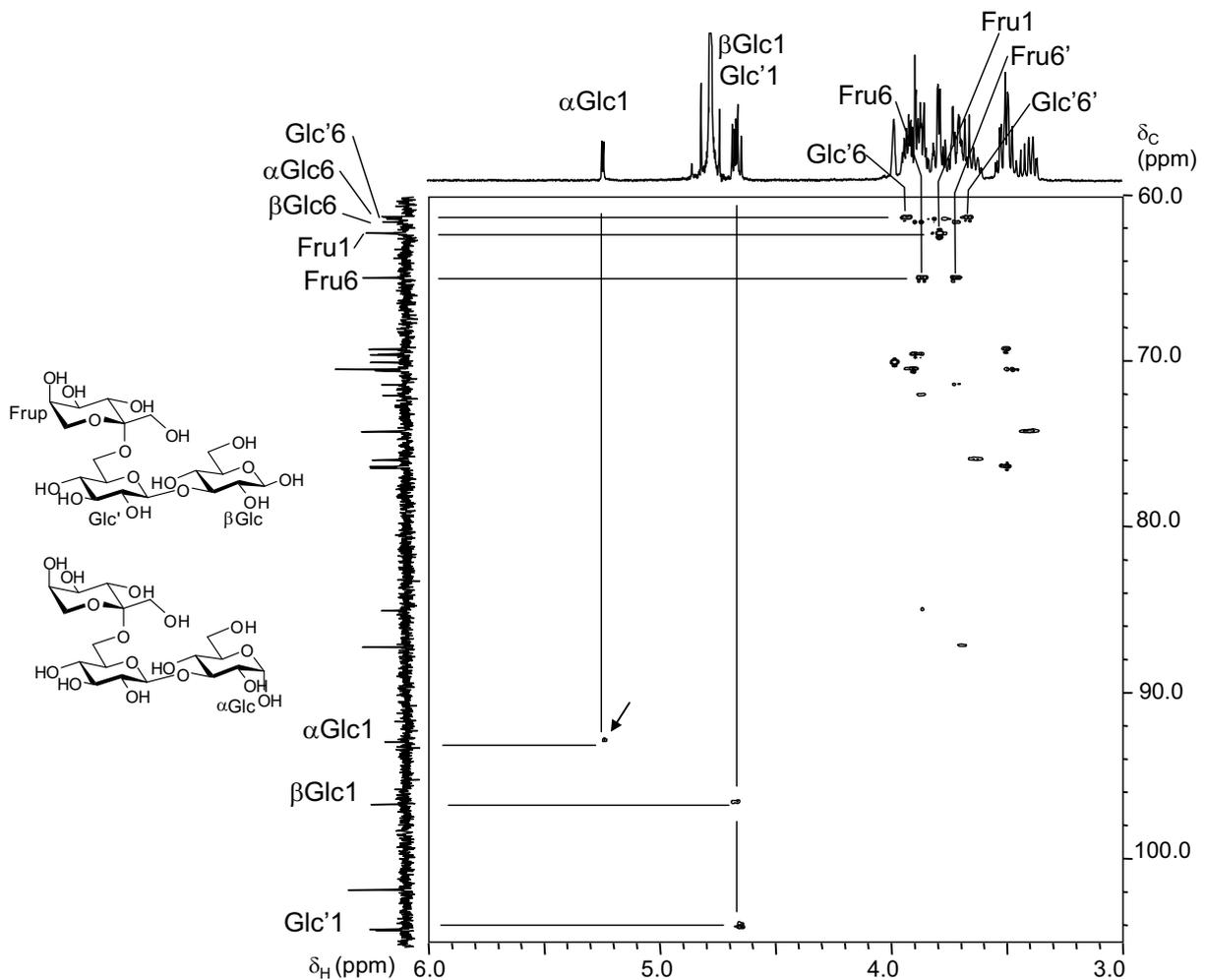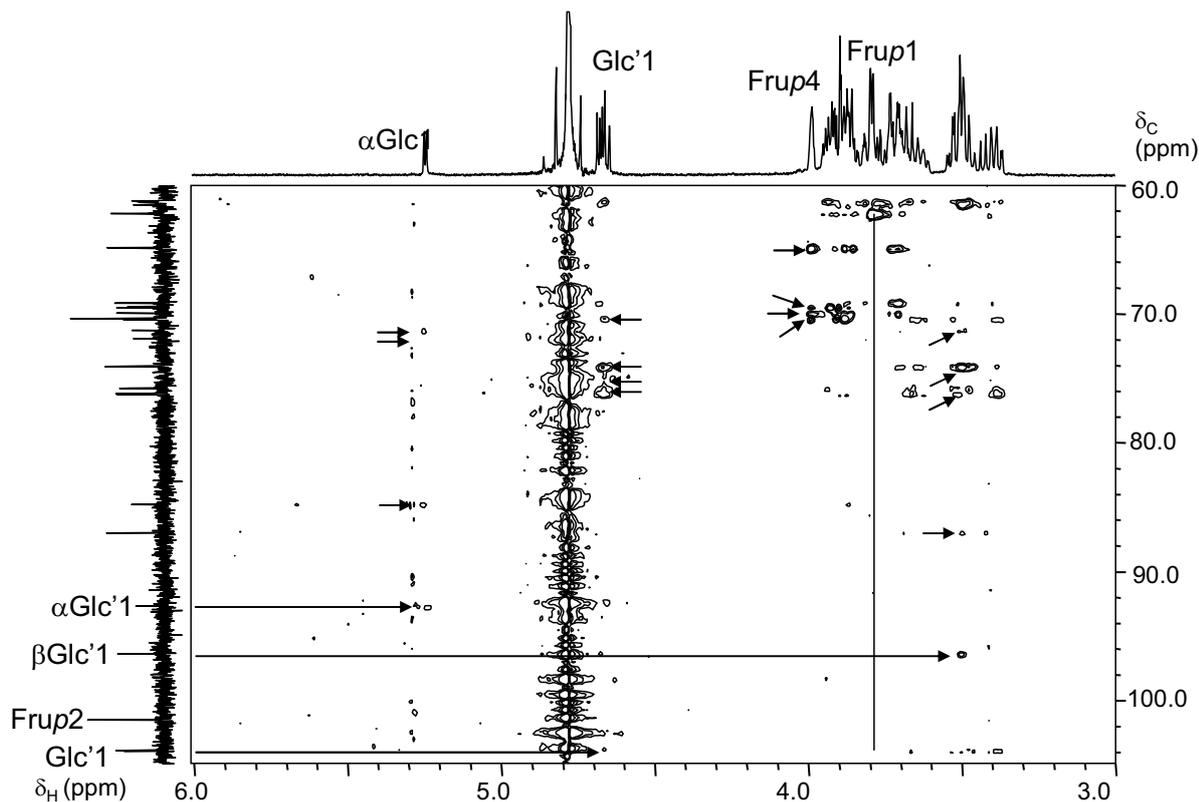

Fig. 3 Two dimensional (2D) HSQC spectrum of 1.

Fig. 4 2D HSQC-TOCSY spectrum of 1.

directly bonded proton and carbon. For example, the cross peak marked by an arrow is on the crossing point of the horizontal line from the carbon at δ_C 92.71 ppm (C-1 of αGlc) and the vertical line from the proton at δ_H at 5.25 ppm (H-1 of αGlc). This means that this proton and this carbon are connected directly. The NMR spectra of saccharide **1** show that it was an anomeric mixture at the glucose residue. The β anomer is predominant and some signals of the α anomer were distinct and could be assigned.

Fig. 4 shows a 2D ^{1}H–^{13}C HSQC-TOCSY (Willker *et al.* 1993; Domke *et al.* 1991). It is the combined method of HSQC and TOCSY (Braunschweiler *et al.* 1983; Hurd 1990; Parella *et al.* 1997). Each protonated carbon gives a HSQC peak for the directly coupled proton, and in the same row at this carbon, additional peaks for protons belonging the same spin–spin network with that proton. The HSQC-TOCSY spectrum can assign protons and carbons in the same spin–system. In an oligosaccharide spectrum, almost every signal in the same sugar unit can be identified. This spectrum revealed the ^{1}H-spin systems of each sugar residue. The isolated methylene was assigned as H-1 and C-1 of Fru. The quaternary carbon was assigned as C-2 of Fru.

Fig. 5 is the 2D COSY (Aue *et al.* 1975; von Kienlin *et al.* 1991) spectrum. It detects protons via two or three bonds. There is a scalar coupling (*J* coupling) between protons via two or three bonds. The NMR parameters, *J* values (in Hz) of such scalar couplings depend on the stereochemistry, can be used to determine the sugar type and its anomeric configuration. The starting signals to analyze this COSY spectrum are the anomeric protons (H-1) of glucoses. They are characteristic doublet signals at rather large chemical shifts in the ^{1}H-NMR spectrum. This spectrum is assigned from H-1 to H-3 in each glucose residue. Other signals could not be assigned because of signal overlapping.

2D ^{1}H–^{13}C HMBC (Bax *et al.* 1986; Summers *et al.* 1986; Hurd *et al.* 1991; Furihata *et al.* 1998) detects long-range J_{CH} couplings. This can also detect the connectivity between proton and carbon via oxygen and quaternary carbon. It is a useful tool in determining the glycosidic linkages and assigning the fructose unit. In the Fru unit, the HMBC correlations between C-3 and H-1, and C-2 and H-1
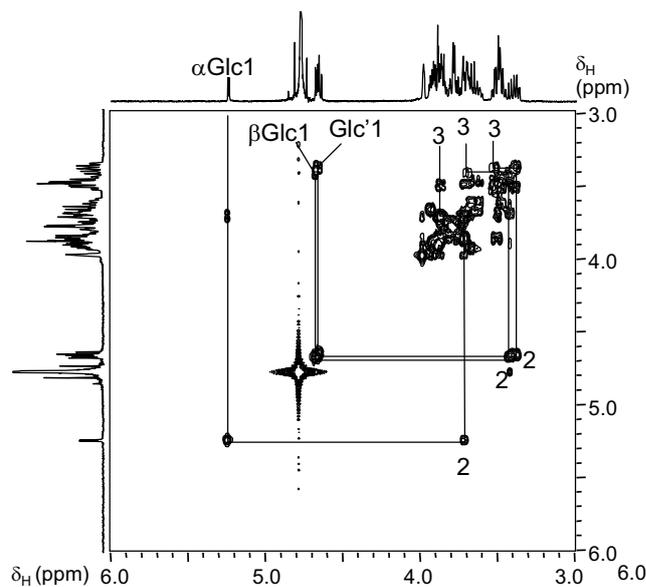

Fig. 5 2D COSY spectrum of 1.

confirm the assignment of these signals (**Fig. 6**). The other four methylene carbons were assigned as C-6 in these residues. As for glucose residues, the HMBC correlations between C-5 and H-1 revealed the assignment of C-5 in each residue. The remaining ^{1}H and ^{13}C signals in each glucose residues could be assigned as H-4 and C-4. These results clarified the assignment of ^{1}H and ^{13}C-NMR signals of each residue.

The position of the glycosidic linkage, and pyranoside-form of Fru were analyzed as follows. The C-3 in βGlc and αGlc showed HMBC correlations between H-1 in Glc', and C-1 in Glc' showed the HMBC correlations to H-3 in β-Glc and α-Glc (**Fig. 6**). The *J*-value taken from the 1D ^{1}H-NMR spectrum between H-1 and H-2 in Glc' was 7.7 Hz. These results indicate **1** has the Glc' α1->3 Glc linkage, namely, the laminaribiose moiety. The C-2 of Fru showed the
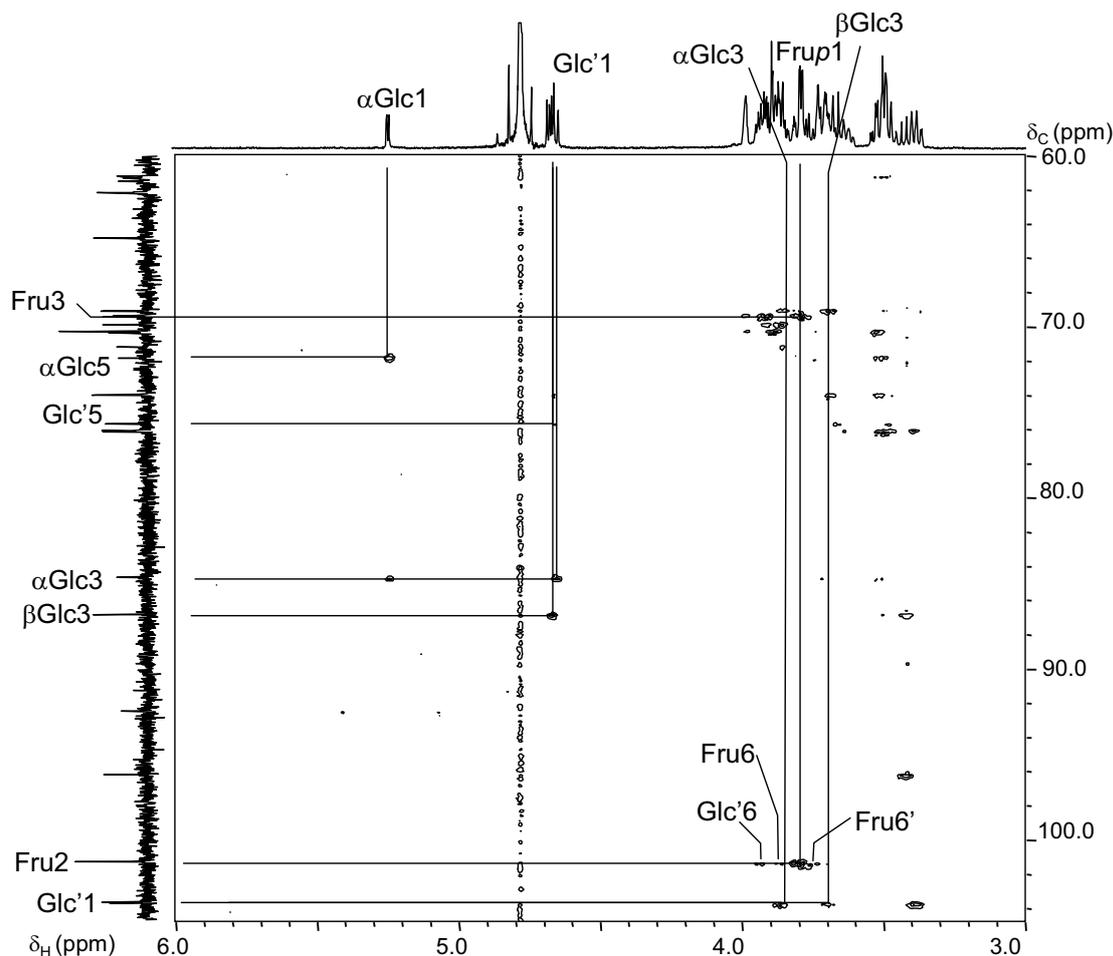
Fig. 6 2D HMBC spectrum of 1.

HMBC correlations to H-6 of Glc' and H-6 of Fru (**Fig. 6**). These results revealed the fructopyranoside residue and its 2->6 Glc' linkage. The anomeric configuration and pyranosyl/furanosyl forms can be confirmed by the ^{13}C chemical shifts (Shiomi *et al.* 2007).

NMR analysis with special methods for high resolution in the ^{13}C axis

The major problem of NMR of oligosaccharides is signal overlapping. In general, ^{1}H and ^{13}C-NMR signals spread at 0–10 and 0–220 ppm, respectively. However, in typical saccharides, they are concentrated at 3–5 and 60–110 ppm, respectively. Especially methylene carbons are concentrated in a few ppm. For determination of sugar linkages and fructopyranoside form, the assignment of each methylene was necessary. So, we created special methods for higher resolution in the ^{13}C dimension. That is, CH$_2$-selected (Fukushi *et al.* 2000) Editing(E)-HSQC (Willker *et al.* 1993; Davis *et al.* 1991) and CH$_2$-selected E-HSQC-TOCSY (Okada *et al.* 2003).

Peak separation is dependent on the spectral resolution. This is one of the key factors of spectral quality. For example, the expansion plot of the indicated region of the **Fig. 7A** is shown in **Fig. 7B**. In **B**, the correlation peaks also became larger so these peaks continue to overlap. The resolution is defined as spectral width per data point: the smaller the value, the higher the resolution. An increase of data points in the ^{13}C axis is effective for improving the resolution of the ^{13}C dimension. Narrowing the spectral width of the ^{13}C dimension is also effective for improving the resolution. However, the spectral width of the ^{13}C dimension for a conventional HSQC spectrum is usually set to cover the range of all protonated carbons. If there are signals outside the measuring range, they appear in the

spectrum as unwanted folded signals.

The E-HSQC gives each CH-, CH$_2$-, and CH$_3$-subspectrum. The spectral width of the ^{13}C dimension and data points can be optimized for each subspectrum. The chemical shifts of methylene carbons for compound **1** are limited to a narrow region, enabling the spectral range of the ^{13}C axis to be set narrower, so that a high resolution results. The C-6 in each residue can be assigned by this CH$_2$-selected E-HSQC spectrum (**Fig. 8**). In each glucose residue, the correlation peak appeared separately between C-6 and H-1.

PERSPECTIVES AND CONCLUSIONS

The NMR methods for the oligosaccharides containing fructopyranoside are demonstrated above. The NMR spectra of oligosaccharides are too complex to analyze using routine methods, COSY, HSQC, HSQC-TOCSY, and HMBC. Problems of signal overlapping and low peak separation can be solved using high resolution CH$_2$-selected HSQC-TOCSY, and CT (constant time)-HMBC spectra. The procedure of these NMR analyses is also efficient for other kinds of compounds.

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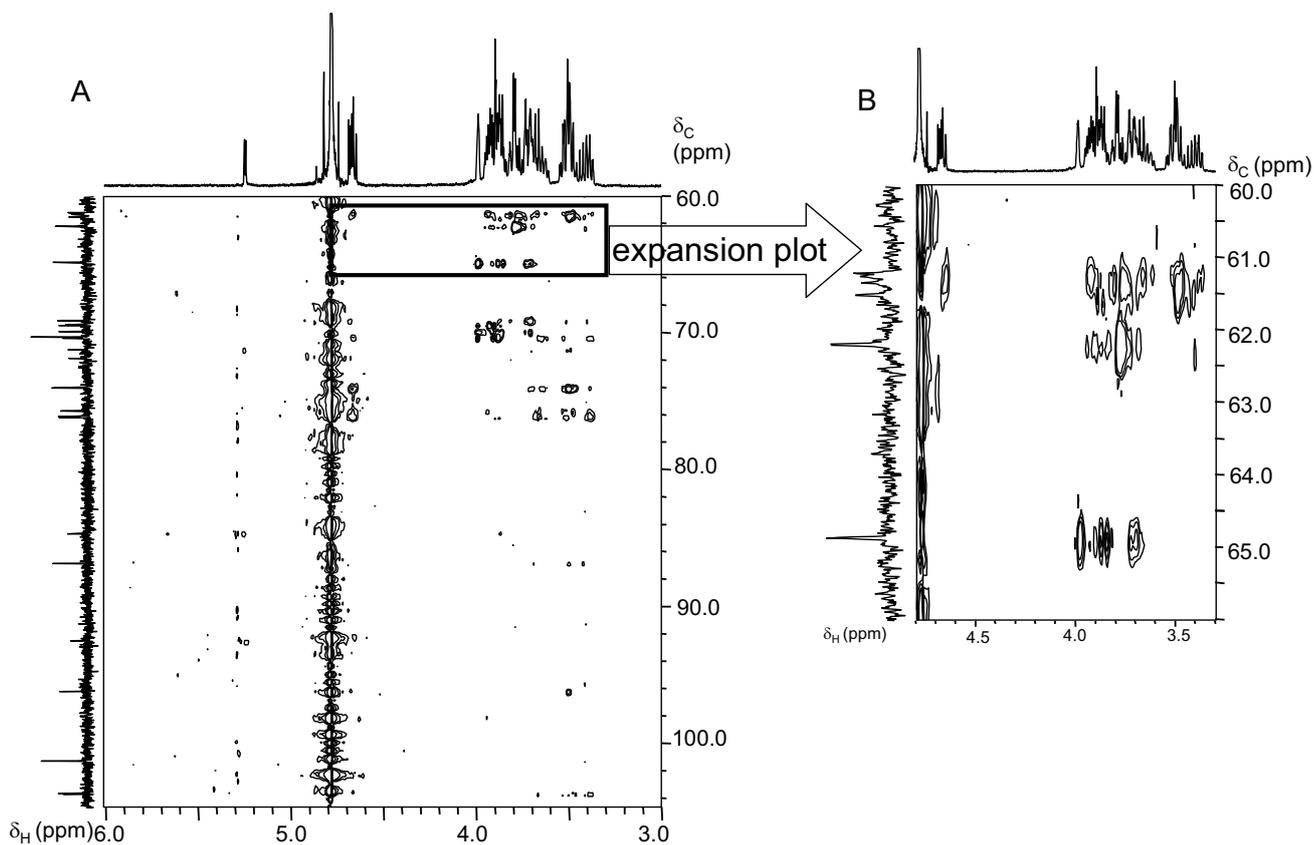
Fig. 7 2D HSQC-TOCSY (A) of <u>1</u> and expansion plot (B) of indicated area of (A).
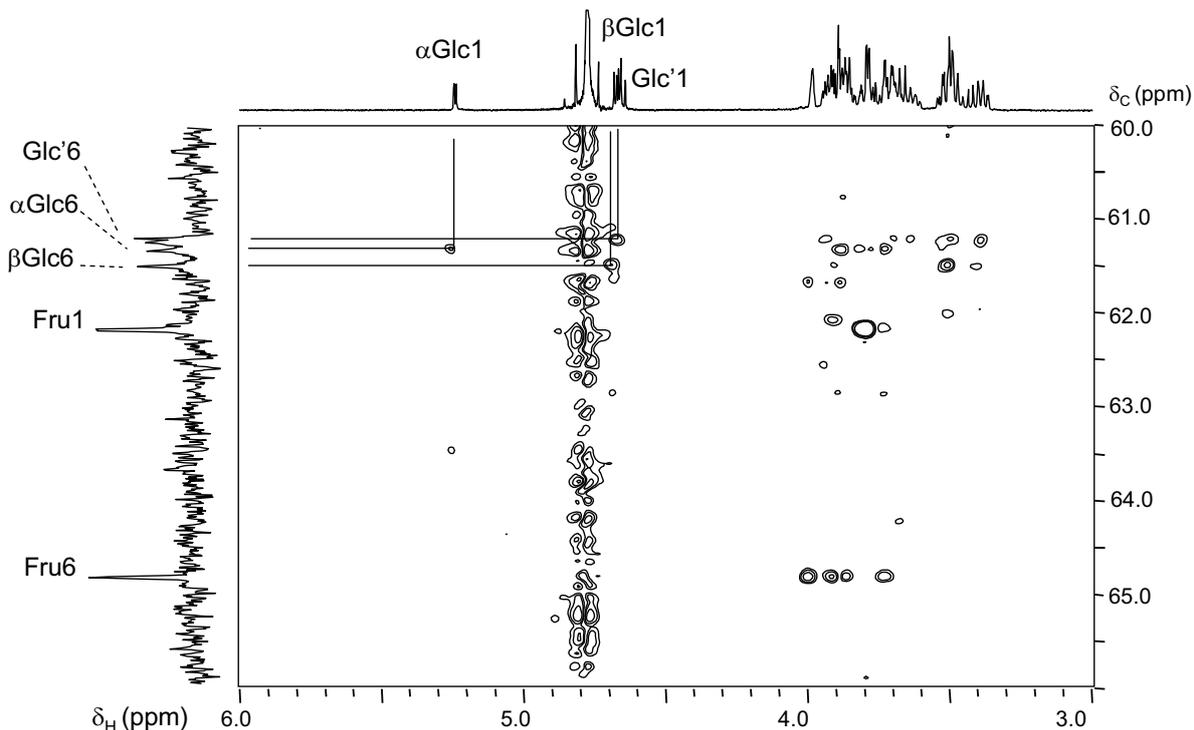

Fig. 8 CH$_2$-selected E-HSQC-TOCSY of <u>1</u>.

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