A new approach for the determination of oligosaccharide structures

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1. Introduction

The 1H NMR spectra of oligosaccharides are relatively complex. Therefore, the difficulties encountered in the structural determination of oligosaccharides mostly involve overlapped saccharide ring proton signals, which cannot be detected effectively using general methods. The sugar component sub-spectrum is determined by one-dimensional TOCSY technology, which is sometimes referred to as 1D-HOHAHA. Protons with a sound separation degree (usually anomic protons) are selected in the laboratory for simulation, and through a spin-lock pulse, related signals of spin coupling can be separated from highly overlapped spectra, forming the sub-spectrum. The high-quality sugar component sub-spectrum shares a high similarity with the conventional 1H NMR spectrum of the monosaccharide. Therefore, it can be used to identify different types of sugars. Aided by a one-dimensional NOE spectrum, the method can serve as a very effective approach to determine the connection location and connection sequence of glycosylation in oligosaccharide compounds.1–5 The approach determines the connection among sugar components as well as those between the aglycone and the sugar component using experimental evidence rather than speculation based on experiences. Therefore, its results are reliable and easy to use without the need for chemical degradation and derivatization of the oligosaccharides.

The quality of the sugar component sub-spectrum is crucial in oligosaccharide structural analysis because the identification of the connection location of the monosaccharide is determined by the correct attribution of related signals in the sub-spectrum. A major difficulty encountered in oligosaccharide sub-spectroscopy detection, however, is the existence of some sugar components with weak spin coupling, such as arabinose. In arabinose, the self-spin coupling constants among H-4 and H-5a and H5-b are so small that the magnetic signal transmission is blocked, resulting in the low sensitivity of remote magnetization transfer signals which are difficult to be detected. Therefore, experimental results often present the following problems. First, some signals cannot show themselves sufficiently, hindering a complete sub-spectrum. Second, the phase and intensity of multi-peaks are distorted, leading to difficult signal attribution. Although the z-filtering method can be used to improve the quality of the sub-spectrum,6 the operation procedures involved are relatively cumbersome and inconvenient to use.

The spin lock pulse is a key part in the production of the sugar component sub-spectrum. In the early stage, the laboratory adopted long pulses of 3 ms7 and later, the mixing sequence of MLEV-I78 and DIPSI-29,10 was developed. In 1992, Cavanagh and Rance confirmed that, compared with MLEV-17, the coherent magnetization transfer of the DIPSI-2 sequence is more effective.10 Experiments illustrated that the latest MMDY sequence5 can significantly improve the quality of the sugar component sub-spectrum. In recent years, new development methods that used gradient pulse for coherence pathway selection were employed.11 Only one single scan is needed for the samples with high concentrations, and it can decrease experimental acquisition times, but its chief drawback is the sensitivity losses,12 and it is not conducive to measure a sample with a low concentration. In this paper, a new approach to detect the oligosaccharide chain structure based on the D60 hybrid sequence is introduced, which is also known as the D60 one-dimensional TOCSY method.
by using this method, coherent remote magnetic transfer is more effective than the previous DIPSI-2,9 MMDY5 and XROESY13 methods, which facilitates sub-spectroscopy detection for the existence of weak spin coupling sugar components in the saccharide ring. Overall, it is a more effective and reliable method for the analysis of oligosaccharide structure.

2. Experiment

2.1. Devices and reagents

NMR experiments were carried out on a JEOL 400 MHz JNM-MCA instrument. The device was equipped with a Linear wave generator that can produce a selective pulse and a 5-mm Z-axis gradient pulse multi-core probe. The new pulse sequence in this approach was obtained by editing on the Delta application equipped in the device. Compound 1 was separated and obtained from Teasle by Zhang and Xue from the Sino-Japan Friendship Institute of Clinical Medicine; its structure was determined by stepwise hydrolysis.14 The sample concentration was 110 mg/mL, with a spectral width of 18.4 ppm, data position of 32,768, relaxation delay of 1.2 s and non-sampling pulse number of 2.90°. The pulse width was 11 μs. The carrier frequency was positioned at the center of the spectrum, and the signals were collected by the cross-detection method. The 180° Gaussian-shaped soft pulse had a width of 50 ms, whereas the 90° spin lock pulse had a width of 23.1 μs. The spin lock field strength was about 11.5 kHz. The signal was repeatedly accumulated according to a multiple of 4. The repetition time of the pulse was 1.2 s. Other parameters will be discussed in the next section.

3. Results and discussion

The pulse sequence emitted from the D60 one-dimensional TOCSY is shown in Figure 1(A). The method applied a 90° hard pulse (P1) for the stimulation. Through the gradient field pulse (G1 and G2) due to the poly-phase, 180° Gaussian soft pulse (P2 and P3) sugar component anomeric signals were retained, whereas other irrelevant signals were clear due to the bulk phase. During the mixing time, the stimulated signals were locked by the D60 sequence, leading to coherent magnetization relay transfer and sub-spectrum generation. The gradient pulse of the method was used for the selective excitation pulse rather than coherent path selection, thereby not presenting the defect of the loss of half of the sensitivity. According to the phase difference between D and 60, 4 combinations were available: Dx60, Dx60y, Dy60y and Dy60x, which were demonstrated by D60-1, D60-2, D60-3 and D60-4, respectively. According to the following experimental results, the performance and effects of coherent magnetization relay transfer were found to be different, in which D60-1 and D60-4 performed better. If not specified, the D60 sequence referred to D60-1 or D60-4.

The D60 sequence has not been reported, of which D represented 2 (RRRR), whereas 60 represented 60°. R was the 180° pulse combination of the alternating pulse of 13 different elements of the phase, and was a reverse pulse of R. For the sequence of D60-1, R was 110°-260°-(-x)50°(-y)300°(-x)160°-(-x)20°(-x)50°(-y)10°(-x)185°(-x)60°(-x)175°(-x)130°(-x) for D60-4, R was 110°(-y)260°(-y)50°(-y)300°(-y)160°-(-y)20°(-y)50°(-y)10°(-y)185°(-y)60°(-y)175°(-y)130°(-y) and was 110°(-y)260°(-y)50°(-y)300°(-y)160°-(-y)20°(-y)50°(-y)10°(-y)185°(-y)60°(-y)175°(-y)130°(-y). In Figure 1, the only difference between (A) and (B) was their trim pulse. When the trim width was zero, the two pulse sequences became identical. In Figure 1, the (B) pulse sequence was used to study the performance and features of different spin lock pulses. The experiments illustrate that if the trim pulse was not used, the sensitivity of the remote magnetization transfer signals of the MMDY and DIPSI-2 sequences or the sub-spectrum quality showed a significant decline. The new approach applied in this paper avoided the defect. The experimental results of the glycoside 1 were used for illustration purposes in Figure 2.

The saccharide chain structure of compound 1 was previously determined by a relatively complex hydrolysis method. It contained three types of sugar components: one rhamnopyranosyl, one arabinopyranosyl and one glucopyranosyl. Figure 3 illustrates the experimental result for compound 1. Figure 3a demonstrates the 1H NMR signals of the proton part of the saccharide ring. Except for the anomeric proton signals, the saccharide ring proton signals of three types of sugar components appeared between δ 3.5 and 5.0, which contained a majority of overlapped signals, leading to

![Figure 1. Pulse sequences for obtaining the subspectra of the sugar components of the oligosaccharide chain; P2 and P3 are selective 180° Gaussian pulses; G1 and G2 are sinusoidal-shape pulsed field gradients; G1/G2 = 2:3. All gradients are of 1 ms duration. Pulse and receiver phase: P1 = x, -x, y, -y, -x, -y, y; P2 = P3 = x, y, -y, -x, -y, y (see text for details).](image)

![Figure 2. Chemical structure of compound 1.](image)
the difficulty in its analysis using general methods. Figure 3(b–d) were obtained with the application of the D60 one-dimensional TOCSY method (Fig. 1A pulse sequence). Under the role of the D60 spin lock pulse sequence, the overlapped signals can present isolation according to the coupling of each sugar component, generating three high-quality sugar component sub-spectra. Their basic characteristics were similar to the general $^1$H NMR spectra of the corresponding monosaccharide without any missing signals (methyl rhamnose showed $\delta$ 1.56, but it is not indicated in the figure). However, the intensity and phases of the spin multiple peak were distorted, enabling clear attribution, as shown in the figure. The types of the sugar components were rhamnopyranosyl (b), glucopyranose (c), and arabinopyranosyl (d), respectively, which were consistent with the saccharide chain structure of compound 1. The connection sequence and location among glycosylations and between the aglycone and the glycosyl unit can be identified with the integration of the experimental results for one-dimensional NOE measurements.

In arabinose, the spin coupling constants among H-4, H-5a, and H-5b were very small, leading to hindered magnetic signal transmission. Therefore, the H-5a and H-5b signal strengths became weak as shown in Figure 3(d–f). Although these results were detected under the same mixing time of the same compound, comparison, the results from different methods were still different. The differences in signal strength and shapes of the multiple peaks of H-5a and H-5b were particularly prominent. Among them, Figure 3(d) shows the result determined by the new method proposed in this paper, which presents two characteristics. First, the phase and intensity of multiple peaks essentially presented no distortion, which indicated that a high-quality sub-spectrum can be gained without trim pulse $z$-filtering. Second, the sensitivity of the remote magnetization transfer signal was relatively high, of which H-5a and H-5b presented the strongest signals, almost double that shown in Figure 3(f) with the DIPSI-2 method. This indicates that this method was most effective in its coherent magnetization relay transfer. Figure 3 illustrates the H-5b signals of the $\delta$ 4.38 double-peak glucopyranose sub-spectrum. These signals can generate the anomeric proton of glucose through selective excitation after coherent magnetization relay transfer up to five chemical bonds (H1→H2→H3→H4→H5→H6). These signals belonged to remote magnetization transfer signals whose shapes and intensity were affected by the performance and effects of the coherence magnetization relay transfer of the spin lock pulse. Therefore, these results were applied to examine the performance of different sequences or methods.

First, the performance of D60-1, D60-2, D60-3 and D60-4 was examined. Figure 4 presents the pulse sequence as Figure 1B, wherein the trim was taken as a variable (0–51 µs) for the detection of H-6b signals. The results show that in the D60-2 (b) and D60-3 (c) sequences, the phases and intensity of the H-6b signals at both ends of the spectra underwent significant but anomalous changes. However, in D60-1 (a) and D60-4 (d), the situation was different: all H-6b signals shared basically the same phases and intensity without any anomaly. This indicated that they had good compensation function and low sensitivities of errors caused by the fluctuations in RF. The performance was stable and repeatable.

Second, the performance of the three different methods (D60, DIPSI-2 and MMDY) was examined. Figure 5 demonstrates the pulse sequence in accordance with Figure 1B wherein the trim was taken as a variable for the selective excitation of glucose anomeric protons. The shape and intensity changes of the H-6b signal ($\delta$ 4.38 bimodal) sub-spectra of glucopyranose were recorded. Noticeably, in Figure 5, the results of the trim with 0 data position showed great differences in H-6b signal intensity among the three methods. Figure 5(a) was determined using the D60 method wherein the H-6b signal strength was the most significant, whereas Figure 5(b and c) were detected, respectively, in accordance with the DIPSI-2 and MMDY methods wherein the H-6b signals were very weak.

Figure 3. The NMR spectrum of compound 1: (a) $^1$H spectrum; (b–d) the subspectra of individual sugar components obtained by applying pulse sequences as shown in Figure 1(A); (e) and (f) the subspectra, obtained using the MMDY sequence and DIPSI-2 sequence, respectively. Mixing time 0.17 s. Each spectrum was obtained with 32 scans.
or difficult to observe. In addition, in Figure 5(a), the H-6b signal strengths of 18 data points were different from one another. The distortion in phases and peak shapes of some H-6b signals showed some abnormality. These results suggested that the MMDY and DIPSI-2 methods have some defects. If the experimental conditions are inappropriately chosen, obtaining satisfactory results would be
difficult. However, the new approach proposed in this paper does not have these drawbacks. It can prove that the D60 spin lock pulse itself has a good compensation role and is not sensitive to the errors caused by the fluctuations in RF. It is effectively stable and repeatable, covering a relatively broad range of applications. Good results can be obtained without compensation from the trim pulse.

In the TOCSY experiment, the mixing time is set to determine the repetition frequency of the spin lock pulse, which directly affects the steps and signal strength of coherence magnetization relay transfer. Therefore, this is an important parameter. To achieve the expected results, in the detection, a certain length of mixing time is required. In general, increasing the mixing time can increase the steps of coherent magnetization relay transfer and improve the strength of the remote magnetization transfer signal. However, the longer the mixing time is, the greater the signal loss. For compounds with less relaxation time of mapping, the signal loss is particularly serious. For example, in Figure 3(a) for the conventional 1H spectrum, in the experiment involving the selective excitation of δ 5.33 glucopyranosyl anomeric proton signals, the signals will definitely be excited. However, due to the short relaxation time, the water singlet under a great spin lock of the sub-spectrum of glucose in Figure 3(c) will completely disappear. Despite this, the double bond aglycone signals at δ 5.38, which are generated by partial resonance, will still appear for a long relaxation time. In addition, the experiments have proven that the anomeric proton signals of the glucose sub-spectra of compound 1 (δ 5.33 bimodal) can receive selective excitation, and the length of the mixing times are set at 10, 50 and 100 ms when the dry signal ratios (S/N) are 743, 584 and 361, respectively. The required mixing time is increased from 10 to 100 ms when the sensitivity is decreased to about 50%. Therefore, the ideal approach is under the premise of obtaining similar results. The required mixing time should be as short as possible so that it will not only reduce the signal loss but also save time. Further, the scope of application of sample detection with a relatively low concentration and relatively large molecular weight can be expanded.

Figure 6 demonstrates the experimental results of H-6b signal strength variation using three different methods in which the mixing time was used as a variable for the selective excitation of the glucose anomeric proton. A significant difference was observed. Figure 6(a) presents the detection results using this method wherein the mixing time produced the maximum signal strength of H-6b. Meanwhile, the method proposed in this paper produced the shortest, which is 185 ms, whereas the results of MMDY (b) and DIPSI-2 (c) were longer at 205 and 245 ms, respectively. This indicates under the premise of gaining similar results of the same compound that the mixing time of the method is reduced by approximately 20 and 60 ms compared with that by MMDY and DIPSI-2. The above results suggest that coherent magnetization relay transfer in this new method is more effective, enabling a higher sensitivity of remote magnetization transfer signals, which can also significantly improve the quality of the sugar component sub-spectra with weak spin coupling in the saccharous ring. Further, a more complete D60 sequence performance can be achieved with good compensation characteristics and no trim pulse. Therefore, a sugar component sub-spectrum of better quality can be obtained compared with MMDY and DIPSI-2. As the mixing time is relatively short, for compounds with a relatively short relaxation time, the sensitivity loss can be reduced, enabling a wider application scope. The method is easy to operate and can quickly receive pure absorption-shaped signals without Z-filtering.

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