Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 2060–2063
Carbohydrate RESEARCH

Note

Identification of bound water molecules in the cyclic tetrasaccharide, *cyclo*-{→6}-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→)

Kazuo Furihata,[a] Takashi Fujimoto,[b] Ayumi Tsutsui,[b] Tomoya Machinami[b,*] and Mitsuru Tashiro[b,*]

[a]*Division of Agriculture and Agricultural Life Sciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan*
[b]*Department of Chemistry, College of Science and Technology, Meisei University, Hino, Tokyo 191-8506, Japan*

Received 14 April 2005; accepted 15 June 2005

Abstract—A structural characterization of bound water molecules in the cyclic tetrasaccharide, *cyclo*-{→6}-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→), was carried out by NMR spectroscopy. H-1′, 2′-OH, H-3′, and 4′-OH of the 3-*O*-glycosylated residue and H-1 of the 6-*O*-glycosylated residue were found to cross-relax with protons of bound waters using the double-pulsed field-gradient spin–echo ROESY experiment. In the crystal structure, one water molecule is located in the center of the plate, and its temperature factor is very low, indicating that this water molecule is an intrinsic component.

Keywords: Cyclic tetrasaccharide; NMR spectroscopy; ROESY; Bound water

Internal and ordered surface waters of biological molecules often play significant roles in stabilizing structures through the formation of bridging hydrogen bonds and also in enzymatic mechanisms.[1,2] Much effort has been put into the kinetic and structural characterization of these water molecules. Large numbers of water molecules are often identified in high-resolution X-ray crystal structures of proteins and nucleic acids;[3,4] however, it is not possible to determine from X-ray crystallography whether these water molecules arise from crystal packing forces or are intrinsic components of the macromolecules. Recent NMR methods to study the location of macromolecular bound water molecules in solution have taken advantage of selective excitation of the water resonance, and 2D ROESY–HMQC and ROESY–HSQC methods have been presented for ^{15}N- or ^{13}C, ^{15}N-enriched protein.[5–9] Although hydration studies using NMR spectroscopy have often been carried out for proteins, this technique has been little used for saccharides. One of the reasons could be the difficulty in preparation of isotopically enriched oligosaccharides that are useful for unambiguous assignments, while overexpression systems to obtain enriched proteins is established as a routine method. In the present work, NMR techniques have been applied to identify bound water molecules of the cyclic tetrasaccharide, *cyclo*-{→6}-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→) (**1**) (Fig. 1), which is the major compound obtained by the action of *endo*-alternases on the alternan polysaccharide. Alternan is produced from sucrose by *Leuconostoc mesenteroides* NRRL B-1355.[10] It is composed predominantly of an alternating sequence of α-(1→3)-linked and α-(1→6)-linked D-glucose residues, with approximately 10% branching.[11]

In NMR studies, ROESY experiments can distinguish spin–spin cross-relaxation from chemical exchange, as these two processes give rise to cross-peaks of opposite sign.[12–15] Recently, we presented several ROESY pulse sequences[6,9] that incorporate the double-pulsed field-gradient spin–echo (DPFGSE) sequence[16,17] to identify

* Corresponding authors. Tel.: +81 42 591 5597; fax: +81 42 591 7485; e-mail addresses: machinam@chem.meisei-u.ac.jp; tashiro@chem.meisei-u.ac.jp

0008-6215/$ - see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.carres.2005.06.013

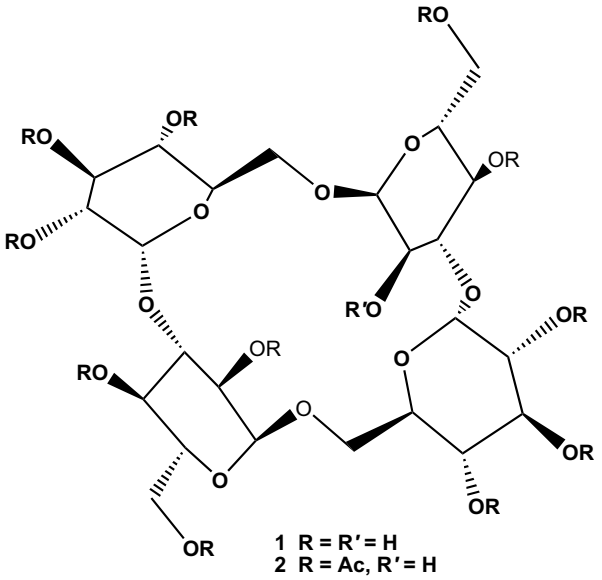

Figure 1. Structure of the cyclic tetrasaccharide.

bound water molecules of the ^{15}N-enriched proteins. DPFGSE can provide superior selective excitation but without the phase distortion problems that are often associated with the use of conventional selective excitation. Considering the convenience of ^{1}H assignments of **1**, DPFGSE 1D TOCSY and ROESY experiments (Fig. 2) were carried out for identification of bound water molecules. The excitation profile of the water signal in 99.9% dimethyl sulfoxide-d_6 in the DPFGSE 1D ROESY sequence is shown in Figure 3. The excitation width is 9 Hz corresponding to 0.018 ppm in a

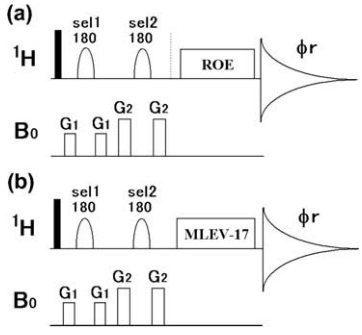

Figure 2. The DPFGSE 1D (a) ROESY and (b) TOCSY pulse sequences. The thin and thick bars represent 90° and 180° pulses, respectively. All pulses were along the *x*-axis unless otherwise shown. The experimental parameters used for the ROESY were ROESY mixing = 300 ms; G_1 = 5.8 g/cm; G_2 = 8.8 g/cm; data size = 64*k*; spectral width = 8000 Hz; number of scans = 1024. Gradient pulse widths were 1.5 ms for G_1 and G_2. The selective ^{1}H 180° pulses were 1.1 s Gaussian-shaped pulses. Phase cycling: sel1(180°) = x, y, $-x$, $-y$; sel2(180°) = 4x, 4y, 4($-x$), 4($-y$); ϕ_r = x, $-x$, x, $-x$, $-x$, x, $-x$, x. The exponential window function with line broadening of 5 Hz was used for processing spectra. In TOCSY, mixing = 60 ms. The other experimental parameters were identical in these measurements.

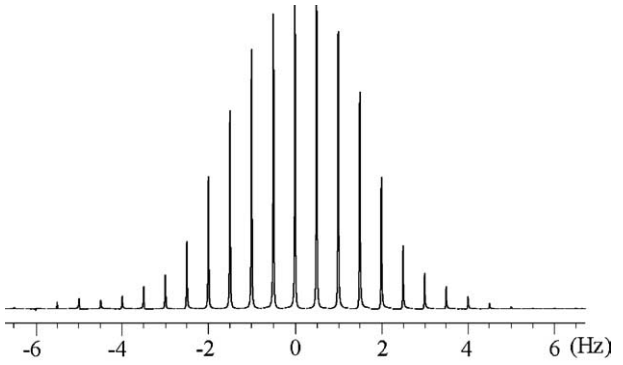

Figure 3. Excitation profiles of the water signal in the DPFGSE 1D ROESY sequence. Spectra were acquired on a 25.7 mM of the cyclic tetrasaccharide **1** at 15 °C dissolved in 99.9% dimethyl sulfoxide-d_6 without addition of water. Pulse width of the selective 180° pulses was 1.1 s. Resonance offsets were recorded at 0.5-Hz increments. The on-resonance signal was phased to absorption, and then the same correction was used for the rest of the spectra.

500 MHz spectrometer, which is narrow enough for the selective water excitation. As expected, the phase of the water signal is constant, and out-of-band suppression is excellent.

DPFGSE 1D ROESY, TOCSY, and conventional ^{1}H spectra of **1** are shown in Figure 4. All NMR experiments were carried out at 15 °C because the spectral reproducibility and quality were not satisfactory for detailed analysis at higher temperatures. No evident signal was observed in the TOCSY spectrum (Fig. 4b) indicating that the water resonance was selectively excited and that all of ROE signals arose from bound water proton–proton of **1** spin–spin cross-relaxation. The TOCSY signals between **1** and water cannot be observed. If the TOCSY signals were observed, it means that proton resonances of **1** close to the water resonance were also excited, resulting in the misinterpretation of ROE signals. In the simple spin–echo experiment, it was demonstrated that the proton magnetization of **1** almost decayed in 1.5 s, while that of water still remained approximately 24% (data not shown). Therefore, proton magnetization of **1** completely decays during DPFGSE, which is approximately 2.2 s, and that of water only remains for observation of ROEs. Even if the resonances of **1** close to the water resonance were slightly excited, the magnetization of **1** cannot give rise to ROE signals. The low sensitivity of the ROESY spectrum could arise from the long-shaped pulses used in DPFGSE causing the loss of sensitivity due to the relaxation effects. Although high sensitivity in the ROESY experiment was not obtained, prominent ROE signals were observed for H-1′, 2′-OH, and 4′-OH of the 3-*O*-glycosylated residue, and weak ROE signals were observed for H-1 of the 6-*O*-glycosylated residue and H-3′ of the 3-*O*-glycosylated residue (Fig. 4a). These ROE signals, which are of interest in this study, arose from bound water

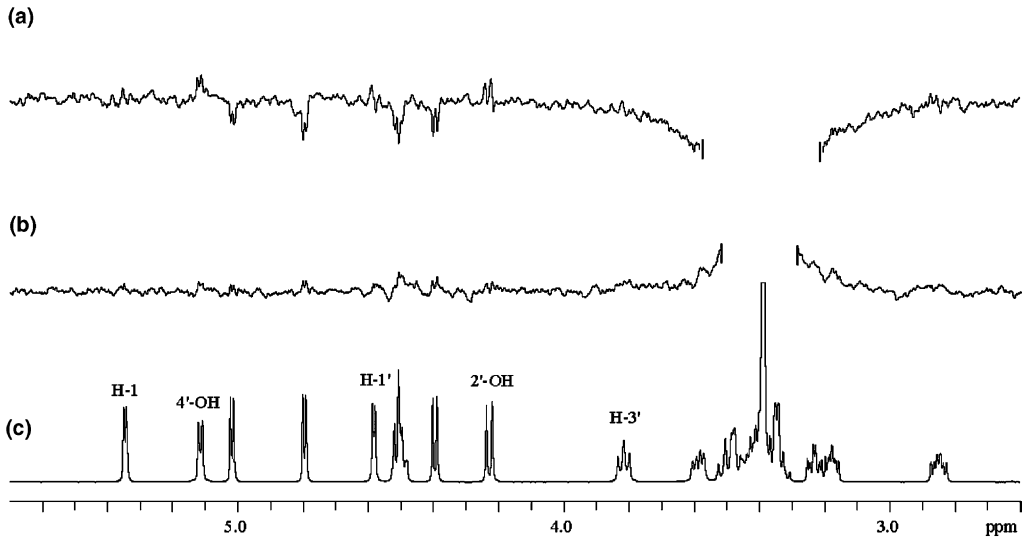

Figure 4. DPFGSE (a) ROESY, (b) TOCSY, and (c) conventional ^{1}H spectra of the cyclic tetrasaccharide **1**. Spectra were acquired on a 25.7 mM of the cyclic tetrasaccharide **1** at 15 °C in 99.9% dimethyl sulfoxide-d_6 without addition of water using a Varian Inova-500 spectrometer. In (a) and (b) the water resonance was selectively excited.

protons—the protons of **1** in a spin–spin cross-relaxation. The signals of opposite sign arose from exchange with the water protons. In the crystal structure,[18] one water molecule (OW1) is located in the center of the ring, and its temperature factor is very low. Its hydrogen atoms were clearly located by Fourier difference and were within hydrogen-bonding distances from two intra-ring oxygen atoms, O(5)2 and O(5)4. This water molecule neatly occupies the shallow depression at the center of the ring according to the calculation of the Connolly surface of **1**.[18] Two hydrogen atoms of OW1 point toward a region with negative electrostatic potential created by acetal oxygen atoms. The geometric information obtained in the crystal structure strongly indicates that OW1 is a bound water molecule of **1**. In the crystal structure (CCDC-146356), inter-proton distances between OW1 and H-1 of the 6-*O*-glycosylated residue are 2.59–4.18 (3.42 ± 0.67) Å, those between OW1 and H-3′ of 3-*O*-glycosylated residue are 2.23–2.83 (2.51 ± 0.27) Å, and those between OW1 and 2′-OH of the 3-*O*-glycosylated residue are 3.18–4.06 (3.59 ± 0.38) Å, which are reasonably short for observing ROE signals. On the other hand, those between OW1 and H-1′ of the 3-*O*-glycosylated residue are 4.04–5.22 (4.68 ± 0.49) Å, and those between OW1 and 4′-OH of the 3-*O*-glycosylated residue are 4.34 and 4.54 Å, in which observation of ROE signals is not expected. In comparison with the crystal structure, the results obtained by the ROESY experiment indicate that a bound water molecule exists in **1**, and its geometry in solution should be slightly different from that in the crystal form. In solution, it is very unlikely that the location of OW1 is completely fixed at the center of the ring as observed in the crystal structure. The distances between an oxygen of OW1 and C-1 of the 6-*O*-glycosylated residues are 3.56 and 3.72 Å, and those between an oxygen of OW1 and C-1 of the 3-*O*-glycosylated residues are 4.41 and 4.22 Å, which indicate that some mobility of the bound water molecule can satisfy inter-proton distances suggested by the ROESY experiment.

Acetylation (Ac_2O–pyridine, 100 °C) of **1** afforded undecaacetate **2**, whose structure was determined to be only 2′-OH in one of the 3-*O*-glycosylated residues not acetylated. In the crystal structure of **1**, both 2′-OHs are in close proximity (d_{O-O} = 2.73 Å) and presumably involved in hydrogen bonding, which could be the reason for protection from acetylation. These hydroxyl groups are located at the bottom of the shallow depression on the plate shape, the other side of OW1 sitting on the plate. The global structure of **1** is considered to be stabilized by the water molecule OW1, which bridges intramolecular hydrogen bonds.

The detection of water molecules by NMR spectroscopy depends on observation of the correlation between protons of macromolecules and those of water molecules providing geometrical information on the proton atoms. The present results indicate that this method is highly amenable to the structure-based interpretations of bound water in oligosaccharides, and leads to a better understanding of the functional role of water in macromolecules.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (No. 15550076) from the Ministry of Education, Culture, Sports, Science, and Technology.

References

1. Westhof, E. *Int. J. Biol. Macromol.* **1987**, *9*, 186–192.
2. Westhof, E. *Annu. Rev. Biophys. Biophys. Chem.* **1988**, *17*, 125–144.
3. Steitz, T. A. *Q. Rev. Biophys.* **1990**, *23*, 205–280.
4. Baker, E. N.; Huddard, R. E. *Prog. Biophys. Mol. Biol.* **1984**, *44*, 97–179.
5. Kriwacki, R. W.; Hill, R. B.; Flanagan, J. M.; Caradonna, J. P.; Prestegard, J. H. *J. Am. Chem. Soc.* **1993**, *115*, 8907–8911.
6. Tashiro, M.; Furihata, K.; Shimotakahara, S.; Shindo, H. *Magn. Reson. Chem.* **2002**, *40*, 559–562.
7. Grzesiek, S.; Bax, A. *J. Am. Chem. Soc.* **1993**, *115*, 12593–12594.
8. Grzesiek, S.; Bax, A. *J. Biomol. NMR* **1993**, *3*, 627–638.
9. Furihata, K.; Shimotakahara, S.; Tashiro, M. *Magn. Reson. Chem.* **2003**, *41*, 955–958.
10. Côte, G. L.; Robyt, J. F. *Carbohydr. Res.* **1982**, *101*, 57–74.
11. Misaki, A.; Torn, M.; Sawai, T.; Goldstein, I. J. *Carbohydr. Res.* **1980**, *84*, 273–285.
12. Bothner-By, A. A.; Stephens, R. L.; Lee, J. T.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811–813.
13. Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207–213.
14. Davis, D. G.; Bax, A. *J. Magn. Reson.* **1985**, *64*, 533–535.
15. Otting, G.; Wüthrich, K. *J. Am. Chem. Soc.* **1989**, *111*, 1871–1875.
16. Stott, K.; Stonehouse, J.; Keeler, J.; Hwang, T. L.; Shaka, A. J. *J. Am. Chem. Soc.* **1995**, *117*, 4199–4200.
17. Stott, K.; Keeler, J.; Van, Q. N.; Shaka, A. J. *J. Magn. Reson.* **1997**, *125*, 302–324.
18. Bradbrook, G. M.; Gessler, K.; Côte, G. L.; Momany, F.; Biely, P.; Bordet, P.; Pérez, S.; Imberty, A. *Carbohydr. Res.* **2000**, *329*, 655–665.