^{13}C and ^1H Nuclear Magnetic Resonance for Structural Investigation of Some Glucans

Taichi USUI, Naotaka YAMAOKA, Kazuo MATSUDA, Katura TUZIMURA,
Hiroshi SUGIYAMA and Shuichi SETO

*Faculty of Agriculture, Tohoku University, Tsutsumidori-Amamiya-machi, Sendai,
Chemical Research Institute of Non-Aqueous Solution, Tohoku University, Sendai*

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The ^{13}C and ^1H NMR technique was applied for the structural elucidation and characterization of D-glucan. Particularly peak assignments of the anomeric carbon and the positional carbon of linkage were indicated in comparison with the spectra of monosaccharides and oligosaccharides.

Spectrometric methods (IR, ORD and ^1H NMR) have been used for establishing the configuration of the glycosidic linkage of oligosaccharides and polysaccharides. Also, positions of linkage in oligosaccharides and polysaccharides have been determined by chemical methods and by biochemical degradation. Recent development of ^{13}C NMR spectroscopy encouraged us to apply the technique for the study of polysaccharides.^{1,2)} ^{13}C NMR spectroscopy of monosaccharides^{3~5)} and oligosaccharides^{1,6~8)} has been reported by several investigators including the authors. Although proton NMR provided crucial information for the structural elucidation of mono- and oligosaccharides, the size of the molecule gave rise to the problem of overlapping peaks. On the other hand, ^{13}C NMR, in addition to providing a different type of structural information, is expected to show a high degree of resolution, because carbon resonances occur in a wide range exceeding 200 ppm. We have reported ^{13}C NMR spectroscopy concerning the structural feature of glucobioses and glucotrioses.¹⁾ Moreover, ^{13}C Fourier transform (FT) spectroscopy revealed itself as an excellent technique for the investigation of carbohydrate macromolecules.^{2,9~11)} We now describe the application of the combined use of ^{13}C and proton NMR spectroscopy to elucidate the structural feature of some glucans, having different

kinds of linkage.

RESULTS AND DISCUSSION

The ^{13}C NMR spectra were shown to be applicable to the assignment of polyglucans by the generalization of the resonance of gluco-oligosaccharides. The resonance near 91 ~ 105 ppm downfield from tetramethylsilane could be attributable to anomeric carbon atoms. And the resonances of the positional carbon involved in glycosidic linkage (except for 1, 6-linkage) were found to occur in the range of 79 ~ 88 ppm, which was 4 ~ 12 ppm downfield of the usual range for the carbon 2 ~ 5 of pyranose carbon atoms.¹⁾ On the other hand, in proton NMR glycosidic protons were focused upon the region of δ 5.00 ~ 5.45 ppm downfield from DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate) by α -linkage and were focussed upon the region of δ 4.50 ~ 4.80 ppm by the corresponding β -linkage.¹²⁾ The coupling constants ($J_{1,2}$) were near 3.0 Hz for α -linkage having *cis* configuration and near 7.5 Hz for β -linkage having *trans* configuration. Thus, the assignments of glucan resonances have been made by the ^{13}C and proton spectra of a series of related gluco-oligosaccharides.

Water-soluble glucan of barley endosperm

As an example, a water-soluble glucan from the cell wall of the endosperm of naked barley was studied. Igarashi *et al.* had indicated

FIG. 1. ^{13}C FT NMR Spectrum of a Linear Water-soluble β -Glucan from the Endosperm of Naked Barely at 90°C in Deuterium Oxide.

by a chemical method that this glucan was a linear β -glucan consisting of (1-3) and (1-4)-linked β -glucopyranose residues with a proportion of approximately 1:2.5.¹³⁾ The proton NMR spectrum of this glucan was measured at 90°C in deuterium oxide containing 5% NaOD. The chemical shifts of the glycosidic protons were expected to correspond to those of laminaribiose (3-O- β -D-glucopyranosyl-D-glucopyranose) and cellobiose (4-O- β -D-glucopyranosyl-D-glucopyranose),¹²⁾ which were the smallest units of the structure. Hence, the resonances at δ 4.74 and 4.54 ppm were assigned to glycosidic proton due to the β -(1-3) and β -(1-4) linkage. The ratio of the intensity of the former to that of the latter peak was 1:2.5. The proportion of linkages indicated by proton NMR agreed well with the result of the chemical method. Furthermore, the coupling constants in signals of δ 4.74 and 4.54 ppm showed $J_{1,2}$ values near 8.0 Hz indicating *trans* configuration.

The proton-noise-decoupled ^{13}C FT NMR spectrum of this glucan dissolved in deuterium oxide containing 5% NaOD has been recorded

at 90°C (Fig. 1). Since this glucan was not sufficiently soluble in neutral deuterium oxide, it was necessary to use the alkaline solvent under elevated temperature for the spectral determination. All signals have been assigned as illustrated in Fig. 1 by analogy with the ^{13}C spectra of laminaribiose and cellobiose since the linkage of these glucans is known.¹⁾ But if the linkage is unknown, the following consideration is possible. Comparing with the spectra of glucobioses, the peak at 104.0 ppm can be assigned to the resonance of glycosidic carbons of β -linkage because the C-1 signal of β -linkage appears at the range of 103~105 ppm, while that of α -linkage appear at 97~101 ppm. The signals of positional carbons substituted by β -glucosyl group appear at the range of 80~87 ppm, except for substituted C-6.¹⁾ The resonance at 87.4 ppm could be assigned to the C-3' involved in β -(1-3) linkage. (see the Fig. 1) The resonance at 80.0 ppm could be attributed to the C-4 involved in α -(1-4) linkage in combination with glycosidic carbon resonance. Although the recorded peak height is accurately proportional to the content of carbon by ^{13}C NMR, the relative peak height of these two peaks does not conflict with the ratio, 1:2.5 of β -(1-3) to β -(1-4) linkages calculated from the anomeric proton signals of proton NMR. Since the resonance of C-4' of the 3-O-substituted moiety was expected to shift about 1.0 ppm upfield from C-4 (71.4 ppm) of D-glucopyranose due to the β -effect of 3-O-substitution,^{1,3)} the resonance at 69.7 ppm was assigned to the non-linked C-4' of 1,3-di-O-substituted residues. This peak had a peak height similar to C-3' peak. From the above assignment, the presence of a larger amount of β -(1-4) linkage and a less amount of β -(1-3) linkage can be assumed. So that larger peaks should be compared with cellobiose and small peaks should be compared with laminaribiose without conflict. Moreover, upfield shift of the C-5 peak is explained by the β -effect of the C-4 substitution. The peak at 76.1 ppm should be an overlapped peaks of C-3 and C-5, and peak 77.2 ppm is assigned to C-

5'. That is, the resonance of C-5 of 1,4-di-O-substituted residues can be separated from C-5 of 1,3-di-O-substituted residues. A similar is seen with C-2 and C-2'. The resonance of C-2' should be upfield compared with that of C-2. Hence, peak 73.6 ppm is C-2', and peak 74.5 ppm is C-2. These interpretation might reflect a linear of this glucan.

*β -Glucan from *Lentinus edodes* ("shiitake" mushroom)*

This glucan was studied as an example having three different kinds of linkage. This glucan was reported to consist of β -linked glucose units by the chemical method. The amount of (1-6) linkage was twice as large as compared with the total amount of (1-3) and (1-4) linkages. The proton NMR in deuterium oxide showed the glycosidic proton signals clearly differentiated in the lowest field. The signal at δ 4.54 ppm ($J_{1,2}$ 8.0 Hz) should be assigned to the overlapped resonance of β -(1-4) and β -(1-6) linkages, since the glycosidic signal of the corresponding glucobioses is very close each other.¹²⁾ The signal at δ 4.74 ppm ($J_{1,2}$ 8.0 Hz) should also be assigned to the glycosidic proton of β -(1-3) linkage. The ratio of the intensities of the former to the latter was 5:1. Since this ratio was reliable, the ratio of (1-6), (1-3) and (1-4) linkages was calculated as 4:1:1, on the basis of the chemical study and proton NMR. The ^{13}C NMR spectrum in deuterium oxide showed one glycosidic carbon signal at 104.0 ppm due to β -linkage. The signal at 86.0 ppm was assigned to C-3 resonance involved in β -(1-3) linkage (in comparison with the data on glucobioses). The signal at 81.0 ppm should be attributed to C-4 resonance involved in β -(1-4) linkage in combination with glycosidic carbon resonance, but this resonance was also close to C-2 involved in β -(1-2) linkage. The peak height at 81.0 ppm was smaller than that of 86.0 ppm, and the former peak was too small to estimate the intensity ratio. The peak at 70.0 ppm was attributed to C-6 resonance involved in β -(1-6) linkage. No useful information with respect to the branch-

ing point could be obtained. This might due to the limited amount of β -(1-3) and β -(1-4) linkage relative to the main chain of β -(1-6) linkage.

Glycogen

We have previously reported that the ratio of α -(1-6) to α -(1-4) linkages of β -limit dextrans from glycogen and amylopectin agreed well with the data reported by the chemical method.¹⁵⁾ Previous study has shown that intact glycogen or amylopectin was difficult to give a spectrum by CW ^{13}C NMR, because of their low solubility and high viscosity.^{1,6)} However, the glycogen was found to be sufficiently soluble to give a spectrum by FT ^{13}C NMR, while the amylopectin from Waxy maize or potato was insufficient to give the spectrum.

Proton NMR spectra of rabbit liver glycogen and oyster glycogen were measured at 90°C. The peaks at δ 5.34 and 4.95 ppm could be attributed to the glycosidic proton involved in α -(1-4) and α -(1-6) linkages, respectively, because the H-1' and H-1'' resonances of panose (corresponding to the smallest unit of the branched structure) lay in the same positions.¹²⁾ The relative intensity of these two peaks should reflect the ratio of α -(1-4) to α -(1-6) linkages. The ratio of the linkages in rabbit liver glycogen was 14:1, and that of oyster glycogen was 9:1.

The ^{13}C FT NMR spectrum of rabbit liver glycogen in deuterium oxide showed five main peaks due to α -(1-4)-linked glucose units. The peak at 72.6 ppm was twice as intense as those at the other peaks. The peaks at 101.0, 72.6, 74.4, 78.8 and 61.9 ppm could be easily assigned to C-1, C-4, C-3, C-2 and -5, and C-6, respectively, in comparison with maltose and maltotriose.¹⁾ However, it was laborious to make the peaks attributed to α -(1-6) linkage, because the content of α -(1-6) linkage was less than 10% relative to α -(1-4) linkage. The peaks at 99.5 and 67.5 ppm, which were attributed to C-1 and C-6 carbons in α -(1-6)-linked glucose units, were observed only slightly by the signal ac-

FIG. 2. Proton and ^{13}C NMR Spectra of a Water-soluble Dextran from *Leuconostoc mesenteroides* at 90°C in Deuterium Oxide.

(a) Proton NMR; (b) ^{13}C FT NMR.

cumulation of 15,000 times.

Water-soluble dextran from *Leuconostoc mesenteroides* NRRL-1299

The structural feature of this dextran have been discussed mainly on the basis of the results of chemical studies.^{16,17)} Methylation analysis indicated that this dextran contained about 60% of α -D-(1-6), 33% of α -D-(1-2), and 7% of α -D-(1-3) linkages.

The proton NMR spectrum of this dextran was measured at 90°C . Four doublets with a coupling constant of $J_{1,2}$ 3.0 Hz appeared in the α -anomeric proton (δ 4.95~5.30 ppm). Peak A (δ 5.28 ppm) was close to the C-1' of nigerose (3-O- α -D-glucopyranosyl-D-glucopyranose) (5.38 ppm). Peak D (4.95 ppm) was close to the C-1' of isomaltose (6-O- α -D-glucopyranosyl-D-glucopyranose) (4.99 ppm). Both peaks B (5.14 ppm) and C (5.08 ppm) resembled the C-1' of α -kojibiose (2-O- α -D-glucopyranosyl- α -D-glucopyranose) (5.13 ppm). As shown in Fig. 2(a), the ratio of the intensities of peak A, B+C, and D is about 8: 52: 40. If peaks B and C denote α -(1-2) linkage, the indicated proportion of such a linkage is in conflict with the result of the methylation

study. The signal of the glycosidic proton of the 1,2,6-tri-O-substituted residues involved in the branching point should differ from that of the 1,6-di-O-substituted residues in the linear portion, because the glycosidic proton in α -(1-6) linkage intersected by α -(1-2) linkage would be expected to undergo steric interaction generated by the *cis* relationship of substituents at C-1 and C-2. Therefore, it is assumed that either one of signal B or signal C belongs to the anomeric proton involved in α -(1-6) linkage intersected by α -(1-2) linkage. And the other might be attributed to the anomeric proton involved in α -(1-2) linkage. Figure 2(a) indicates that signals B and C are present in equal proportion. From these facts and assumption, the ratio of (1-3), (1-2) and (1-6) linkages was calculated to be 8: 26: 66, which is close to the results of methylation studies.^{16,17)}

The ^{13}C FT NMR spectrum of this dextran in deuterium oxide have three more clearly differentiated peaks (1, 2, 3) in the region of α -linked anomeric carbon and three peaks (13, 14, 15) in the region of C-6 carbon as shown in Fig. 2(b). Peak 1 at 99.4 ppm can be assigned to the glycosidic carbon due to α -(1-6) linkage by analogy with isomaltose.¹⁾ Therefore, either one of peak 2 (98.0 ppm) or peak 3 (97.2 ppm) should be attributed to the glycosidic carbon involved in α -(1-2) linkage and the other is probably involved in α -(1-6) linkage intersected by α -(1-2) linkage when proton NMR is taken into consideration. The chemical shift of peak 15 at 62.3 ppm is identical with the non-substituted C-6 carbon of glucose and glucobioses.^{1,3)} Peak 13 at 67.6 ppm is identical with the C-6 carbon involved in the glycosidic linkage of isomaltose and of clinical dextran (a dextran containing only α -(1-6) linkage).¹⁾ The remaining peak 14 at 64.9 ppm should be C-6 involved in α -(1-6) linkage intersected by α -(1-2) linkage, which would be expected to undergo steric interaction generated by the *cis* relationship of substituents at C-1 and C-2. The intensity ratio of peaks 1, 3, and 2 was 41: 26: 33, and that of peaks 13, 14, and 15 was 39: 24: 37.

This relative intensity almost coincides with the result of proton NMR. The legitimacy of this determination is based on the knowledge that the same-numbered carbons in glucan are in the same chemical environment and should experience identical Overhauser enhancement.¹⁸⁾ So, peak 3 should be attributed to the glycosidic carbon involved in α -(1-6) linkage intersected by α -(1-2) linkage, suffering from steric interaction. This interpretation might be supported by the result from α -kajibiose which has the same pattern of chemical shift due to 1, 2 steric interaction.¹⁾ Therefore, peak 2 might be assigned to the glycosidic carbon involved in α -(1-2) linkage. The glycosidic carbon signal of α -(1-3) linkage might be hidden under noise signals or overlapped with peak 1, under the present experimental condition. Peak 4 at 83.0 ppm and peak 5 at 77.7 ppm should be assigned to C-3 involved in α -(1-3) linkage and C-2 involved in α -(1-2) linkage, respectively, by the analogy with nigerose and kojibiose. The relative peak heights due to α -(1-2) linked units indicated by peaks 2 and 5 correspond roughly to those due to 1,2,6-tri-O-substituted units indicated by peaks 3 and 14, suggesting that most of the α -(1-2) linkage of this dextran are at branching points, and that no long chain of α -(1-2) linkage exists. It should be noted that the identity of the chemical shifts with the corresponding glucobioses by ^{13}C and proton NMR indicates the *CI* (D) chair conformation of each D-glucose residue of this glucan. From these interpretation a hypothesis might be proposed about the construction of this dextran. Our data provide information on a highly branched structure. The frame is the chain of α -(1-6) linked glucoses. Among this consecutive units mostly α -(1-2) linked units and in some cases α -(1-3) linked units branch out. The branches are short in length, mostly consisting of one glucose residue or two. The total structure is like a comb.

Thus, the combination of ^{13}C and proton NMR spectroscopy provides useful informations about the composition and sequence of homopolymer with mixed linkage. This

technique should become one of the indispensable means for structural analysis of polysaccharides.

EXPERIMENTAL

Measurement of proton NMR spectra

The proton NMR spectra were measured at room temperature or at 90°C in deuterium oxide at 100 MHz, by using a JNM PS-100 spectrometer. It was generally found necessary to heat the solution and to shift the interfering HOD signal upfield, in order to observe the signal of anomeric protons. All chemical shifts were expressed in ppm from DSS as an internal standard. The first-order coupling constants (*J*) are expressed in Herz (Hz). The volume and concentration for the spectral measurement of polysaccharides were as follow, (a) β -glucan from the *Endosperm* of naked barley, in deuterium oxide containing 5% NaOD, 25 mg/0.3 ml; (b) β -glucan from *Lentinus edodes*, 40 mg/0.3 ml in D_2O ; (c) glycogen of rabbit liver and oyster, 40 mg/0.3 ml in D_2O ; (d) dextran from *Leuconostoc mesenteroides*, 20 mg/0.3 ml in D_2O . To exchange labile hydroxyl protons with deuterons, samples were dissolved in deuterium oxide, and then freeze-dried. This process was repeated two times.

Measurement of ^{13}C NMR spectra. ^{13}C NMR spectra (25.15 Hz) were measured by the pulse FT method with JEOL PFT-100 and EC-6 computer system at 90°C. All protons were decoupled. The concentration of the samples and the acquisition of the spectra were: (a) 150-mg/ml in deuterium oxide containing 5% NaOD, 30,000 acquisitions; (b) 250 mg/ml in D_2O , 20,000 acquisitions; (c) 500 mg/ml in D_2O , 15,000 acquisitions; (d) 150mg/ml in D_2O , 30,000 acquisitions. Deuteron was used as the source of the lock signal. All chemical shifts were expressed in ppm downfield from TMS as an external standard.

Materials. A linear water-soluble β -glucan from the *Endosperm* of naked barley was generously given by Prof. O. Igarashi of the Ochanomizu University. β -Glucan from *Lentinus edodes* was prepared by the same procedure as reported previously.¹⁴⁾ Animal glycogens (rabbit liver and oyster) were purchased from Sigma Chemical Company, U.S.A. The water soluble dextran elaborated by *Leuconostoc mesenteroides* NRRL B-1299 was prepared by the same procedure as reported previously.¹⁹⁾

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REFERENCES

- 1) T. Usui, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto *J. Chem. Soc., Perkin I*, **1973**, 2425.
 - 2) T. Usui, M. Kobayashi, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto, *Tetrahedron Lett.*, **1973**, 3397.
 - 3) D. E. Dorman and J. D. Roberts, *J. Amer. Chem. Soc.*, **92**, 1355 (1970).
 - 4) A. S. Perlin, B. Casu and H. J. Koch, *Can. J. Chem.*, **48**, 2596 (1970).
 - 5) A. S. Perlin and B. Casu, *Tetrahedron Lett.*, **1969**, 2921.
 - 6) D. E. Dorman and J. D. Roberts, *J. Amer. Chem. Soc.*, **93**, 4463 (1971).
 - 7) D. Doddrell and A. Allerhand, *ibid.*, **93**, 2779 (1971).
 - 8) P. A. J. Gorin, *Can. J. Chem.*, **51**, 2375 (1973).
 - 9) A. S. Perlin, N. M. K. Ng Ying Kin, S. S. Bhat-tacharjee and L. F. Johnson, *ibid.*, **50**, 2437 (1972).
 - 10) H. J. Jennings and I. C. P. Smith, *J. Amer. Chem. Soc.*, **95**, 606 (1973).
 - 11) D. R. Bundle, I. C. P. Smith and H. J. Jennings, *J. Biol. Chem.*, **249**, 2275 (1974).
 - 12) T. Usui, M. Yokoyama, N. Yamaoka, K. Matsuda, K. Tuzimura, H. Sugiyama and S. Seto, *Carbohydr. Res.*, **33**, 105 (1974).
 - 13) O. Igarashi, M. Igoshi and Y. Sakurai, *Agr. Biol. Chem.*, **29**, 678 (1965).
 - 14) M. Shida, T. Mase, Y. Sasagawa and K. Matsuda, *Nippon Nôgeikagaku Kaishi*, **45**, 454 (1971).
 - 15) P. J. Manners, *Advan. Carbohydr. Chem.*, **12**, 287 (1957).
 - 16) E. J. Bourne, R. L. Sidebotham and H. Weigel, *Carbohydr. Res.*, **22**, 13 (1972).
 - 17) M. Kobayashi, K. Shishido, T. Kikuchi and K. Matsuda, *Agr. Biol. Chem.*, **37**, 2763 (1973).
 - 18) J. B. Stothers, (1972) in *Carbon-13 NMR Spectroscopy*; pp. 19~45, Academic Press, New York.
 - 19) M. Kobayashi, K. Shishido, T. Kikuchi and K. Matsuda, *Agr. Biol. Chem.*, **37**, 357 (1973).
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