ELSEVIER

Carbohydrate Research 270 (1995) 33–42

CARBOHYDRATE RESEARCH

Structure determination of galacto-oligosaccharides by pyridylamination and NMR spectroscopy

Kazumasa Kimura *, Keisuke Matsumoto, Chiyoko Ishihara, Katsuhisa Harada, Akihiko Miyagi

YAKULT Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186, Japan

Received 15 March 1994; accepted in revised form 18 November 1994

Abstract

Galacto-oligosaccharides formed from lactose by the action of some β-galactosidases were subjected to gel chromatography on Bio-Gel P-2, and the resulting oligosaccharide fractions were converted into pyridylamino (PA) derivatives. Each PA-oligosaccharide fraction, which consisted of several isomers in a given size-class, was then subjected to HPLC on an ODS column. Twenty-one individual galacto-oligosaccharide components were isolated in this way. The structures of most of these compounds, namely six disaccharides, five trisaccharides, two tetrasaccharides, and a pentasaccharide, were determined by ^{13}C-NMR spectroscopy. The results obtained will be useful for the study of the activity of various galacto-oligosaccharides on the growth of *Bifidobacterium* species.

Keywords: Structure determination; Galacto-oligosaccharides; Pyridylamination

1. Introduction

Galacto-oligosaccharides, that is, a series of galactosylated oligosaccharides formed from lactose by the transgalactosylating action of some β-galactosidases, show an ability to promote the growth of *Bifidobacterium* in the human intestine [1–3]. For this reason, galacto-oligosaccharides are now widely used as a foodstuff beneficial to human health [4]. The galacto-oligosaccharides are known to comprise a number of isomeric di-, tri-, tetra-, and penta-saccharides [3,5], but the structures of these compounds have not been investigated in detail, even though the information is required for the study of

* Corresponding author.

SSDI 0008-6215(94)00010-7

their bacterial metabolism in the intestine, and also for the control of their production. The lack of structural knowledge is mainly due to the absence of proper methods for separating the oligosaccharide isomers from each other. Analyses carried out thus far have involved the repeated use of charcoal or paper chromatography [6], and only a few di- and tri-saccharides have been identified, with only semiquantitative estimations. In the present research we applied the pyridylamination method reported by Hase et al. [7] to the separation of the isomers, and determined their structures by ^{13}C-NMR spectroscopy.

2. Experimental

Materials.—The galacto-oligosaccharide preparation used, Oligomate-50, is a product of Yakult Pharmaceutical Co. Ltd. It was made from lactose by the action of β-galactosidases from *Aspergillus oryzae* and *Streptococcus thermophillus* [4]. 2-Aminopyridine was purchased from Tokyo Kasei Co. Ltd. and borane–dimethylamine complex was obtained from the Aldrich Chemical Co. Other chemicals were of analytical grade.

Gel chromatography of galacto-oligosaccharides.—Gel-permeation HPLC on an analytical scale was performed on a Shodex KS-802 column (8.0 × 300 mm) at 80°C using water as an eluent. The flow rate was 0.5 mL/min. Gel chromatography on a preparative scale was carried out on a Bio-Gel P-2 open column (44 × 900 mm) at 60°C using water as an eluent. The flow rate was 0.83 mL/min. The eluate was monitored with a refractive index detector, Model Shodex RI SE-61.

Preparation of pyridylamino (PA) derivatives.—PA derivatives were prepared according to the method of Kondo et al. [8,9]. A sugar sample (20 mg, 10–60 mmol) was dried in a glass tube (13 × 100 mm) under reduced pressure at room temperature. To the residue 100 μL of coupling reagent (2.2 g of 2-aminopyridine in 1.3 mL of acetic acid) was added. The tube was sealed with a screw cap and heated at 90°C for 1 h, then 100 μL of freshly prepared reducing reagent (390 mg of borane–dimethylamine complex in 2 mL of acetic acid) was added. The tube was sealed again and heated at 80°C for 50 min. The resulting PA derivatives were isolated from the reaction mixture by electrodialysis using a Microacilyzer model S-1 (Asahi Kasei Co.); the detailed procedure will be published elsewhere.

HPLC of PA oligosaccharides.—A Shimadzu Technoresearch Co. (STR) ODS-H column (4.6 × 250 mm, packed with 5 μm particles having 10 or 30 nm pore size), and a μ-Bondasphere ODS column (3.9 × 250 mm, packed with 5 μm particles having 12 nm pore size, Millipore Waters Japan Co.) were used for analysis. An STR ODS-H Prep column (20 × 250 mm, packed with 5 μm particles having 12 nm pore size) was used for preparative work.

A Waters Model 600E pump system with a Model 490 multi-wavelength detector was used with these columns. The eluate was monitored by measuring optical density at 310 nm, and the chromatogram was recorded with a Hitachi model D2500 integrator interfaced with a floppy-disk drive system.

As an eluent, sodium citrate buffer (0.2 M, pH 4.5–6.0), which was used by Takemoto [10] for the separation of PA alditols (PA monosaccharides), was adopted. The flow rate was 0.5 mL/min for analysis, or 9.0 mL/min for the preparative column. To desalt eluates containing PA oligosaccharides an electrodialysis instrument, Microacilyzer model G-1 (Asahi Kasei Co.) equipped with a 120-10 cartridge, was used.

Analysis of the sugar residue at the reducing end, and whole sugar composition of PA oligosaccharides.—A portion of the purified PA oligosaccharide was hydrolyzed in 2 N trifluoroacetic acid at 110°C for 1 h. The PA alditol released from the reducing end was identified by HPLC on an ODS column, using 1-deoxy-1-(2-pyridylamino)-D-gluctitol and -D-galactitol as reference substances. The free sugars contained in the hydrolyzate were converted into PA alditols, and analyzed in the same way to determine the whole sugar composition of the original PA oligosaccharide.

Determination of the molar absorption coefficients of PA oligosaccharides.—A portion of the purified PA oligosaccharide was dissolved in 1 M acetic acid and the absorbance at 310 nm was measured. The molar absorption coefficient was calculated from this absorbance and the molecular weight measured by mass spectrometry.

NMR spectroscopy.—^{1}H- and ^{13}C-NMR spectra were recorded at 30°C or 60°C with JEOL GX-400 and Alpha-400 spectrometers (^{1}H at 400 and ^{13}C at 100 MHz). The spectra were measured for 0.1 to 2% sugar solutions in D_2O. Chemical shifts, expressed on the δ scale, were measured from acetone (δ 3.216 at 30°C and 3.213 at 60°C for ^{1}H, and δ 30.5 for ^{13}C) as the internal standard.

The carbon signals, especially those for C-2 to C-5, of PA derivatives of mono-, di-, and tri-saccharides were generally assigned by the HH-COSY and CH-COSY (or HSQC [11]) methods. The signals for methylene carbons (C-6, C-1 at the reducing end) were assigned by the DEPT method. Also, the RELAY-COSY [12] and difference NOE methods were used in some cases. Additional signal assignments were made by reference to the spectral data for structurally related sugars. The signals for glycosylated carbons were confirmed by their markedly higher δ values compared with those for the corresponding nonglycosylated ones.

Measurement of molecular weight.—Molecular weights were measured with a JEOL DX-102 mass spectrometer. Fast atom bombardment (FAB) and ODS, liquid chromatography coupled to FAB methods were adopted for major and minor components, respectively.

3. Results and discussion

Separation of galacto-oligosaccharide components.—As noted above, the galacto-oligosaccharides prepared from lactose by the action of β-galactosidase consist of di-, tri-, tetra-, and penta-saccharides, each size-class being a mixture of several isomers [3–5]. In order to separate these complex mixtures, galacto-oligosaccharide preparations were first subjected to gel-permeation chromatography on a Bio-Gel P-2 column, and five fractions (F1–F5) were obtained (Fig. 1). F1–F5 were identified tentatively as monosaccharides to pentasaccharides, respectively, by referring to data reported for the malto-oligosaccharides [13]. Analytical gel-permeation HPLC showed F1 to be a

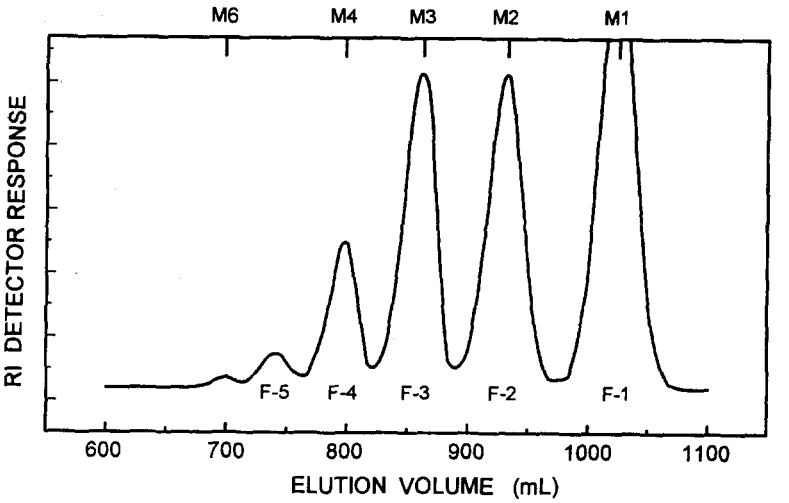

Fig. 1. Fractionation of the original galacto-oligosaccharide sample by gel-permeation chromatography on a Bio-Gel P-2 column. M1 and M2 to M6 indicate the elution positions of glucose and malto-di- to -hexa-saccharides, respectively.

mixture of D-glucose and D-galactose. F2–F5 were shown to contain oligosaccharides, each fraction representing a single size-class.

After conversion to PA derivatives F2–F5 were subjected to preparative HPLC on an ODS column to separate their isomeric components. Twenty-one PA-oligosaccharide fractions were obtained. These consisted of six fractions from F2 (Fig. 2A), five fractions from F3 (Fig. 2B), nine fractions from F4 (Fig. 2C and D), and a single fraction from F5. In the case of F4, rechromatography at pH 6.0 was needed to separate peaks F4-4 and F4-5. All the components obtained were homogeneous as seen from their ^{1}H- and ^{13}C-NMR spectra.

Analyses of the sugar composition showed that all of these oligosaccharide derivatives were PA-galacto-oligosaccharides, consisting solely of galactose or galactose plus glucose. The number of sugar residues in each PA-galacto-oligosaccharide molecule was determined from the number of anomeric carbon signals in its NMR spectrum, and further confirmed by molecular weight measurement.

The amounts of components F1–F5 in the original sample were calculated from their peak areas in an analytical gel-permeation HPLC. Further, the molar ratios of the isomers in each of the fractions F2–F5 were calculated from the peak areas in an analytical ODS-HPLC of their PA derivatives. Since the molar absorption coefficients of the different PA oligosaccharides had been shown to be almost the same (6724–6907) at 310 nm, the amounts of the various galacto-oligosaccharide components in the sample could be calculated and are shown in Table 1.

Determination of the structures of the PA oligosaccharides.—HPLC analysis of the hydrolysates of the PA oligosaccharides showed that the reducing-end units of F2-1 and F2-3 were galactose residues while those of the other fractions were glucose residues. Furthermore, sugar residues other than those at the reducing end were all galactose,

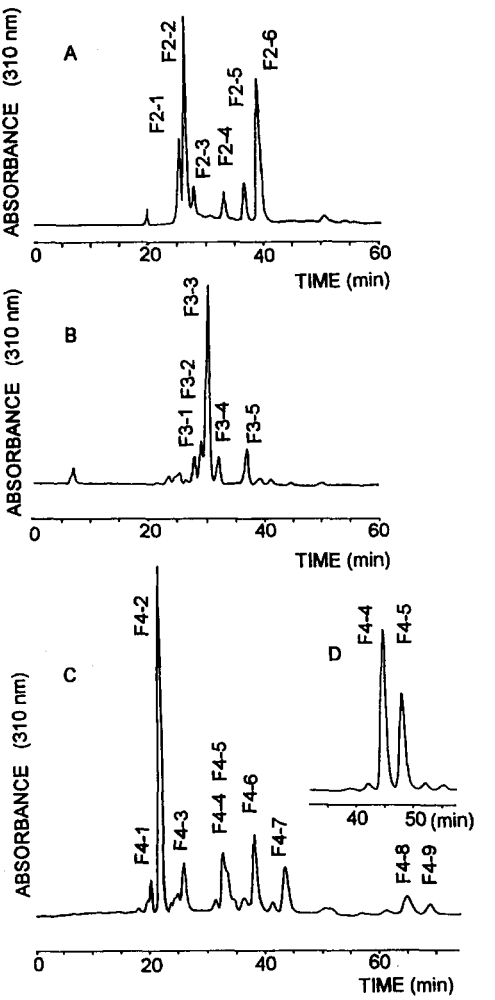

Fig. 2. Separation of PA-galacto-oligosaccharide components by HPLC on an ODS column. A, PA-disaccharides; B, PA-trisaccharides; C, PA-tetrasaccharides; D, rechromatography of the overlapped peak from C containing F4-4 and F4-5. The pH of the eluent was 5.7 in A, 4.5 in B and C, and 6.0 in D.

which indicated that almost all the oligosaccharides in the sample were formed by transgalactosylation; the transglucosylating action of the enzymes was negligible under the experimental conditions.

The ^{13}C-NMR signals of the PA derivatives of authentic D-glucose, D-galactose, and lactose were assigned first with a view to facilitating the assignment of the signals of the isolated PA oligosaccharides. The results are shown in Table 2.

Since the ring structure of the reducing residue is opened into that of an alditol upon reductive amination at C-1, the signals of the PA monosaccharide derivatives could be assigned most easily. In the ^{13}C-NMR spectrum of PA-D-glucitol, the C-1 signal was

Table 1
Amounts of galacto-oligosaccharide components in the original sample

Size class	Component	Content (%)
Disaccharide	F2-1	2.4
	F2-2	8.3
	F2-3	1.1
	F2-4	1.9
	F2-5	2.2
	F2-6	10.5
Trisaccharide	F3-1	1.8
	F3-2	2.4
	F3-3	12.7
	F3-4	1.9
	F3-5	2.6
Tetrasaccharide	F4-1	0.2
	F4-2	3.7
	F4-3	0.9
	F4-4	0.8
	F4-5	0.5
	F4-6	1.3
	F4-7	0.8
	F4-8	0.4
	F4-9	0.3
Pentasaccharide	F5-1	0.4

Table 2
^{13}C Chemical shifts for PA derivatives of mono- and di-saccharides

Derivative	Residue	Chemical shifts [a] (δ)					
		C-1	C-2	C-3	C-4	C-5	C-6
Glc-PA		44.7	71.6	70.8	72.0	71.7	63.1
Gal-PA		45.5	69.3	71.0	71.1	70.6	64.0
Lac-PA	Gal*p*	103.3	71.4	72.9	68.9	75.4	61.2
	Glc-PA	44.7	70.7	70.5	**79.7**	71.4	62.4
F2-1	Gal*p*	103.7	71.2	73.1	69.0	75.4	61.3
	Gal-PA	45.0	69.0	70.5	70.3	69.3	**72.0**
F2-2	Gal*p*	103.4	71.5	73.0	68.9	75.3	61.1
	Glc-PA	44.6	70.7	70.9	**80.2**	71.9	62.5
F2-3	Gal*p*	104.3	71.6	73.0	68.9	75.4	61.3
	Gal-PA	44.7	69.6	**78.9**	70.0	70.1	63.3
F2-4	Gal*p*	104.2	71.7	73.1	69.0	75.5	61.3
	Glc-PA	44.3	71.6	**79.7**	71.1	71.2	63.2
F2-5	Gal*p*	104.0	71.5	73.0	68.9	75.4	61.4
	Glc-PA	44.7	**81.0**	70.3	71.5	70.8	63.2
F2-6	Gal*p*	103.8	71.3	73.1	69.0	75.4	61.3
	Glc-PA	44.7	71.5	70.7	71.8	70.5	**71.6**

[a] Bold indicates signals shifted downfield markedly as compared with the corresponding signals in PA-D-glucitol or PA-D-galactitol.

shifted upfield markedly (δ 44.7) upon amination, compared with the C-1 signals of free α- and β-D-glucose, whereas the C-6 signal was almost unchanged (δ 63.1). Similarly, the C-1 signal of PA-D-galactitol shifted to δ 45.5, but the C-6 signal did not change (δ 64.0). The δ values for C-2, C-3, C-4, and C-5 in these alditols became closer to each other as a result of amination.

The C-4 signal of the PA-glucitol residue in PA-lactitol was observed at δ 79.7, which is a significant downfield shift, arising from galactosylation, as compared with that of PA-D-glucitol. The δ values for the carbon signals of the galactose residue were similar to those of methyl β-D-galactoside. The carbon signals of PA-lactitol also resembled those of lactitol [14] in their δ values.

Disaccharides.—Signal assignments in the ^{13}C-NMR spectra of F2-1 to F2-6 are shown also in Table 2.

F2-2 was identified as PA-lactitol on the basis of coinciding signal patterns. In the spectrum of F2-6, the C-6 signal of the PA-glucitol residue was observed to be markedly shifted downfield (δ 71.6), as compared with that of PA-D-glucitol, while the rest of the signal pattern resembled that of PA-lactitol very closely. Thus, the structure of F2-6 was determined as β-D-Gal*p*-(1 → 6)-D-Glc-PA (PA-allolactitol).

In a similar way, F2-4 was identified as β-D-Gal*p*-(1 → 3)-D-Glc-PA by recognizing the C-3 signal (δ 79.7) as shifted downfield, and F2-5 as β-D-Gal*p*-(1 → 2)-D-Glc-PA by an analogous shift of the C-2 signal (δ 81.0).

The individual signals of F2-1 and F2-3, which have PA-galactitol residues, were assigned particularly by the combined use of the RELAY-COSY [12] and CH-COSY methods. The proton signals of the PA-galactitol residues were well separated; accordingly the carbon signals of these residues were easily identified by the HH- and

Table 3
^{13}C Chemical shifts for PA-trisaccharides

Derivative	Residue	Chemical shifts [a] (δ)					
		C-1	C-2	C-3	C-4	C-5	C-6
F3-1	Gal*p*	103.3	70.9	72.8	68.6	75.2	61.1
	Gal*p*	103.0	71.2	72.7	68.8	75.0	60.8
	Glc-PA	44.3	70.6	70.3	**79.1**	70.3	**69.9**
F3-2	Gal*p*	103.6	71.7	72.9	68.9	75.3	61.2
	Gal*p*	103.4	70.6	72.8	68.9	74.0	**69.1**
	Glc-PA	44.4	71.4	71.0	70.4	71.0	**71.8**
F3-3	Gal*p*	103.7	71.0	73.0	68.7	75.2	61.1
	Gal*p*	103.1	71.4	72.8	68.8	73.8	**68.8**
	Glc-PA	44.6	70.8	71.8	**79.9**	71.0	62.4
F3-4	Gal*p*	104.7	71.9	73.4	69.0	75.4	61.3
	Gal*p*	103.2	71.9	73.3	**77.8**	74.7	60.9
	Glc-PA	44.9	70.4	71.9	**79.8**	71.0	62.5
F3-5	Gal*p*	104.4	71.3	72.8	68.9	75.3	61.2
	Gal*p*	130.0	71.7	**82.2**	68.5	74.9	60.9
	Glc-PA	44.5	70.8	70.5	**80.4**	70.5	62.4

[a] Bold indicates signals shifted downfield markedly as compared with the corresponding signals in PA-D-glucitol or methyl β-D-galactopyranoside.

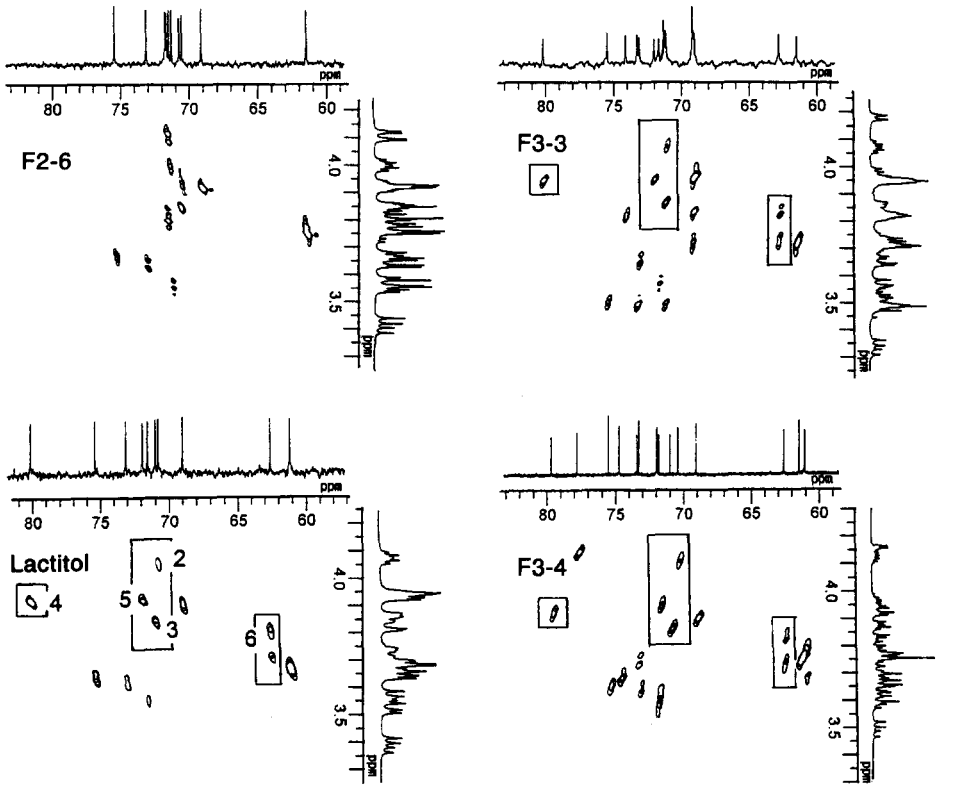

Fig. 3. Partial two-dimensional CH-COSY spectra of PA-lactitol and three PA-galacto-oligosaccharides, F2-6, F3-3, and F3-4. The enclosed areas show the signals characteristic of the PA-lactitol structure.

CH-COSY experiments. In the spectrum of F2-1, the C-6 signal of the PA-galactitol residue was observed to be markedly shifted downfield (δ 72.0) as compared with that of PA-D-galactitol. Thus, this component was identified as β-D-Gal*p*-(1 → 6)-D-Gal-PA. Further, F2-3 showing the C-3 signal shifted similarly (δ 78.9) was identified as β-D-Gal*p*-(1 → 3)-D-Gal-PA. Full assignments of the signals are shown in Table 2.

Trisaccharides.—Signal assignments in the ^{13}C-NMR spectra of F3-1 to F3-5 are shown in Table 3.

The CH-COSY spectra of F3-3, F3-4, and F3-5 showed a set of characteristic cross-peak signals which were also present in the spectrum of PA-lactitol. These patterns for F3-3, F3-4, and PA-lactitol are shown in Fig. 3, which also shows the absence of the characteristic signals in the spectrum of F2-6. Thus, the above three PA trisaccharides F3-3–F3-5 were presumed to contain the PA-lactitol residue at the reducing end. This view was substantiated by the higher δ values (around 80) for the C-4 signals of the PA-glucitol residues in these components.

The linkage sites of the terminal galactosyl group were clarified by the downfield shifts (δ 68.6 for C-6 in F3-3, 77.8 for C-4 in F3-4, and 82.2 for C-3 in F3-5) of carbon

signals of the galactose moiety of the PA-lactose residue. In this manner the structures of F3-3, F3-4, and F3-5 were determined as β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 4)-D-Glc-PA, β-D-Gal*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-D-Glc-PA, and β-D-Gal*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc-PA, respectively.

In the ^{13}C-NMR spectra of F3-1 and F3-2, the C-6 signals of the PA-glucitol residues were found to be shifted downfield (δ 69.9 and 71.8, respectively) just as for F2-6. On the other hand, the C-4 signal of this residue in F3-1 was also shifted downfield (δ 79.1). Thus, F3-1 was identified as a branched-chain trisaccharide, β-D-Gal*p*-(1 → 4)-[β-D-Gal*p*-(1 → 6)]-D-Glc-PA. Characteristic paired signals located quite close to each other were observed for several carbons in the spectrum of F3-1, characterizing both of the galactose residues as nonreducing termini.

In a similar way, F3-2 was identified as β-D-Gal*p*-(1 → 6)-β-D-Gal*p*-(1 → 6)-D-Glc-PA by recognizing the foregoing C-6 signal and also the analogous C-6 signal (δ 69.1) of one galactose residue. In the linear trisaccharides, it may be noted that the δ values for nonreducing terminal galactose residues are almost the same regardless of the position of galactosylation.

Tetrasaccharides and pentasaccharides.—The ^{13}C signals of the tetra- and penta-saccharides could be assigned only partially because of the considerable complexity of the spectra. However, the structures for F4-2 and F5-1 were determined by assigning two and three signals at δ 68.8–69.3, respectively, to the glycosylated C-6 carbons in the galactose residues, and the structure for F4-9 was determined by assigning two overlapping signals at δ 82.3 to the analogous C-3 carbons. These determinations were made easy by the detection of PA-lactitol residues in these sugars from the signal pattern in their CH-COSY spectra. The results are shown in Table 4, together with the structures of the di- and tri-saccharides.

In conclusion, it may be generally of interest that the conversion of the galacto-oligosaccharides into their PA derivatives facilitated the separation of these sugars from

Table 4
Structures of the galacto-oligosaccharide components

Component	Structure
F2-1	β-Gal*p*-(1 → 6)-Gal
F2-2	β-Gal*p*-(1 → 4)-Glc
F2-3	β-Gal*p*-(1 → 3)-Gal
F2-4	β-Gal*p*-(1 → 3)-Glc
F2-5	β-Gal*p*-(1 → 3)-Glc
F2-6	β-Gal*p*-(1 → 6)-Glc
F3-1	β-Gal*p*-(1 → 4)-[β-Gal*p*-(1 → 6)]-Glc
F3-2	β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 6)-Glc
F3-3	β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 4)-Glc
F3-4	β-Gal*p*-(1 → 4)-β-Gal*p*-(1 → 4)-Glc
F3-5	β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 4)-Glc
F4-2	β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 4)-Glc
F4-9	β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 3)-β-Gal*p*-(1 → 4)-Glc
F5-1	β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 6)-β-Gal*p*-(1 → 4)-Glc

each other. Furthermore, it is worth noting that pyridylamination was also helpful in the analysis of the reducing-end sugar residues and the determination of the complete structures of the oligosaccharides, especially of the di- and tri-saccharides, by giving simplified NMR spectra, free of complications due to reducing-end anomers.

Acknowledgments

The authors thank Dr K. Izumi of Kyoto Institute of Technology and Dr H. Yamada of Yakult Central Institute for their guidance during the preparation of the manuscript. We thank Dr T. Osawa, Mr T. Watanabe, Mr T. Makino, and Mr M. Nagaoka for valuable discussions, and Mr K. Matsuura of JEOL Co. for obtaining the mass spectra.

References

[1] R. Tanaka, H. Takayama, M. Morotomi, T. Kuroshima, S. Ueyama, K. Matsumoto, A. Kuroda, and M. Mutai, *Bifidobact. Microflora*, 2 (1983) 17–24.
[2] M. Ito, Y. Deguchi, A. Miyamori, K. Matsumoto, H. Kikuchi, K. Matsumoto, Y. Kobayashi, T. Yajima, and T. Kan, *Microb. Ecol. Health Dis.*, 3 (1990) 285–292.
[3] S. Zarate and M.H. Lopetz-Leiva, *J. Food Prot.*, 53 (1990) 262–268.
[4] K. Matsumoto, Y. Kobayashi, N. Tamura, T. Watanabe, and T. Kan, *Denpun Kagaku*, 36 (1989) 123–130.
[5] T. Toba, A. Yokota, and S. Adachi, *Food Chem.*, 16 (1985) 147–162.
[6] B.S. Prakash, K. Suyama, T. Itoh, and S. Adachi, *J. Agric. Food Chem.*, 37 (1989) 334–337.
[7] S. Hase, T. Ibuki, and T. Ikenaka, *J. Biochem. (Tokyo)*, 95 (1984) 197–203.
[8] A. Kondo, J. Suzuki, N. Kuraya, S. Hase, I. Kato, and T. Ikenaka, *Agric. Biol. Chem.*, 54 (1990) 2169–2170.
[9] J. Suzuki, A. Kondo, I. Kato, S. Hase, and T. Ikenaka, *Agric. Biol. Chem.*, 55 (1991) 283–284.
[10] H. Takemoto, S. Hase, and T. Ikenaka, *Anal. Biochem.*, 145 (1985) 245–250.
[11] G. Bodenhausen and D.J. Ruben, *Chem. Phys. Lett.*, 69 (1980) 185–189.
[12] M. Ikura and K. Hikichi, *Carbohydr. Res.*, 163 (1987) 1–8.
[13] J.D. Allen and J.A. Thoma, *Biochemistry*, 17 (1978) 2338– 2344.
[14] S. Yanahira, T. Suguri, T. Yakabe, Y. Ikeuchi, G. Hanagata, and E. Deya, *Carbohydr. Res.*, 223 (1992) 151–159.