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Further Characterization of Allergenicity Active Oligosaccharitols Isolated from a Sea Squirt H-Antigen

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Complete primary structures of five allergenically active oligosaccharitols (HPG- β 2-N5a, -N6, -N7a, -N7b, and -N9) derived from a sea squirt H-antigen were studied. Structural characterization was carried out by a new method in which products of limited periodate oxidation, followed by derivatization with *p*-aminobenzoic acid ethyl ester, were analyzed by a combination of HPLC, fast atom-bombardment mass spectrometry, sequential glycosidase digestion, methylation analysis, and 500-MHz ¹H NMR. Established structures of GalNAc β 1-4 (GalNAc α 1-2Fuca1-3) GlcNAc β 1-3GalNAc-ol, GalNAc β 1-4GlcNAc β 1-3 (GalNAc β 1-4GlcNAc β 1-6)GalNAc-ol, GalNAc β 1-4GlcNAc β 1-3[GalNAc β 1-4 (Fuca1-3) GlcNAc β 1-6]GalNAc-ol, GalNAc β 1-4 (Fuca1-3) GlcNAc β 1-3 [GalNAc β 1-4 (Fuca1-3) GlcNAc β 1-6]GalNAc-ol, and GalNAc β 1-4 (GalNAc α 1-2Fuca1-3) GlcNAc β 1-3 [GalNAc β 1-4 (GalNAc α 1-2Fuca1-3) GlcNAc β 1-6]GalNAc-ol are represented by HPG- β 2-N5a, -N6, -N7a, -N7b, and -N9, respectively. These structures have not been encountered previously. Oligosaccharide units GalNAc α 1-2Fuca1-, GalNAc β 1-4GlcNAc β 1-, and Fuca1-3GlcNAc β 1- are considered to be the allergenically specific epitopes. Partial assignments of 500-MHz ¹H NMR spectra of these novel O-linked oligosaccharitols were attempted. © 1991 Academic Press, Inc.

Sea squirt allergy (1) is an occupational asthmatic disease found in workers on oyster farms of Hiroshima Bay, Japan. At the time of oyster harvesting, when sea squirts (*Styela plicata*) growing on the oyster shells are removed from the sea water, a large volume of body fluid is expelled through the exhalant orifice. The oyster shells become contaminated by the body fluid, which is sprayed in drop-

lets upon the workers engaged in shucking. By repeated inhalation of the sprayed particles, some of the workers become sensitized and develop asthma symptoms. Rhinitis and asthmatic attacks occur only when patients enter a place where substances from the sea squirt can be inhaled. However, if they stay away from work for a few days, their condition improves without special treatment.

Four preparations, namely H (M_r 10⁶ ~ 2 × 10⁷), Gi-rep (M_r 106,000)-, Ei-M (M_r 22,800)-, and DIIIa (M_r 9980)-antigens were isolated from the body fluid of a sea squirt (2-8). These four antigens were all hexosamine-rich acidic glycoproteins, and elicited a markedly positive reaction on asthmatic patients with sea squirt allergy with a concentration as low as 10 μ g/ml (2-5) in the specific allergenicity skin test. Previously, we have shown that these allergenic preparations contain a certain common epitope(s) in their carbohydrate units (6) responsible for the allergenicity. To determine the epitopes specifically responsible for sea squirt allergy, we have investigated structures of oligosaccharides derived from the allergenically active glycopeptides (6-8). In a previous study (8), 11 neutral oligosaccharitols have been isolated from a glycopeptide fraction (HPG) prepared from a pronase digest of H-antigen, and their sugar sequences were determined. However, complete structural elucidation, including the anomeric configurations and patterns of substitution of the branched residue, remains to be clarified.

A new procedure was established for the modification of oligosaccharitol (7). This includes a limited periodate oxidation of *N*-acetylgalactosaminitol (GalNAc-ol)² in the oligosaccharitol and subsequent labeling of the resulting aldehyde with an ultraviolet absorbing compound, *p*-aminobenzoic acid ethyl ester (ABEE), by reductive amina-

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² Abbreviations used: GalNAc-ol, *N*-acetylgalactosaminitol; ABEE, *p*-aminobenzoic acid ethyl ester; FAB, fast atom-bombardment; COSY, ¹H-¹H correlated spectroscopy; ODS, octadecyl silica.

tion. ABEE derivatization of oligosaccharides has not only facilitated their subsequent chromatographic analysis but also renders oligosaccharides more susceptible to digestion with exoglycosidases.

In our present investigation, structures of five allergenically active oligosaccharitols derived from sea squirt H-antigen were determined using a method whereby products of limited periodate oxidation and ABEE derivatization were analyzed by a combination of HPLC, fast atom-bombardment (FAB)-MS, exoglycosidase digestion, methylation analysis, and 500-MHz ^1H NMR.

MATERIALS AND METHODS

Oligosaccharitols. The five allergenically active oligosaccharitols (HPG- β 2-N5a, -N6, -N7a, -N7b, and -N9) examined in this study were the same materials documented in our preceding study (8). They were isolated from a neutral oligosaccharitol fraction (HPG- β 2-N), which had been obtained previously from a sea squirt H-antigen by pronase digestion and subsequent alkaline borohydride treatment. Two allergenically inactive oligosaccharitols, HPG- β 2-N3 (GlcNAc β 1-3GalNAc-ol) and -N4a (GalNAc β 1-4GlcNAc β 1-3GalNAc-ol), were used as reference compounds (8).

Limited periodate oxidation and ABEE derivatization. Limited oxidation of oligosaccharitols was performed according to a previously described method (7) with modifications. Briefly, 0.1 μmol oligosaccharitol was dissolved in 20 μl of 0.1 M sodium acetate buffer (pH 5.6) containing 0.15 M NaCl. To this solution was added an equimolar amount of NaIO_4 dissolved in 20 μl of the same buffer, and the mixture was left to stand for 10 min at 0°C . The reaction was terminated by adding 10 μl of 10 mM ethyleneglycol. The resulting mixture was lyophilized and subjected to ABEE-derivatization as described previously (9, 10). To 40 μl of NaIO_4 -treated oligosaccharitol solution (limited oxidation product), 160 μl of an ABEE labeling reagent (a mixture of 35 mg of ABEE, 3.5 mg of NaBH_3CN , 41 μl of acetic acid, and 350 μl of absolute methanol) was added prior to heating the mixture at 80°C for 45 min. After adding 1 ml of deionized water, the reaction mixture was extracted with diethyl ether to remove excessive ABEE, and the aqueous layer was then applied onto a PRE-SEP C_{18} cartridge (Tessek, CA). After washing with water, the cartridge was eluted stepwise with water:acetonitrile, 19:1, 9:1, 4:1, and 1:1 (v/v) and finally with acetonitrile, monitoring uv-absorption at 304 nm. Fractions with uv-absorbance eluted by water:acetonitrile ratios of 4:1 and/or 1:1 were pooled and evaporated to dryness.

Exoglycosidase digestion. The following incubation conditions were used: 0.5 units of jack bean β -N-acetylhexosaminidase (Seikagaku kogyo, Co. Ltd., Tokyo) in 50 μl of 0.1 M citrate phosphate, pH 5.0; 0.5 units of *Acremonium* sp. α -N-acetylgalactosaminidase (Seikagaku kogyo) in 50 μl of 0.1 M citrate phosphate, pH 4.5; and 0.05 units of bovine kidney α -L-fucosidase (Sigma Chemical Co., St. Louis) in 50 μl of 0.05 M citrate phosphate, pH 5.0. All incubations were performed at 37°C for 24 h with a toluene layer placed over the media. Reaction was terminated by heating the mixture at 100°C for 5 min. The mixture was then subjected to chromatography with PRE-SEP C_{18} cartridge in the same manner as described for purification of ABEE-derivatized limited oxidation products.

High-performance liquid chromatography. HPLC analysis was performed with a Shimadzu Model LC-6A liquid chromatograph (Shimadzu Co., Kyoto). Chromatography was carried out on Wakosil 5C18-200 (0.4 $\times$ 25 cm, Wako Pure Chemical Industries, Tokyo) and TSKgel Amide-80 (0.46 $\times$ 25 cm, TOSOH, Tokyo) columns at 40°C . The ODS column was equilibrated with 11% acetonitrile in 100 mM acetic acid. After injection of sample, elution was performed by acetonitrile gradient to 15% in 60 min and then to 19% in 30 min at a flow rate of 0.8 ml/min. The Amide-80 column was equilibrated with acetonitrile:water (86:14, v/v). After injection of a sample, elution was carried out by linear gradient

to acetonitrile:water (58:42, v/v) in 60 min at a flow rate of 0.8 ml/min. Elution was monitored by a uv-detector set at 304 nm (9).

500-MHz proton NMR. Samples were repeatedly exchanged in $^2\text{H}_2\text{O}$ with intermediate lyophilization before finally being redissolved in 0.5 ml $^2\text{H}_2\text{O}$ (99.96 atom % ^2H). ^1H NMR spectra at 500 MHz (JEOL FX 500S FT NMR spectrometer operating in the Fourier-transform mode) were obtained by using quadrature detection with a spectral width of 5000 Hz. Data were collected using 8K data points and were zero-filled twice. The probe temperature was maintained at 25°C . Chemical shifts (ppm), expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, were actually measured with reference to internal acetone ($\delta = 2.225$ ppm).

Other analytical methods. Carbohydrate composition was determined by GC on alditol acetates as described previously (8). Permethylolation of oligosaccharides was carried out by the method of Ciucanu and Kerek (11). Methylated samples were hydrolyzed, reduced, and acetylated by the method of Liang *et al.* (12). Partially methylated alditol acetates were analyzed by GC-MS (8). Negative FAB-MS analysis of ABEE-oligosaccharides was carried out on a JEOL HX-100 mass spectrometer (10).

RESULTS

Limited periodate oxidation and ABEE derivatization. Each of the five allergenic oligosaccharitols, which have previously been characterized with respect to their sugar sequences (8), was subjected to periodate oxidation under controlled conditions to oxidize exocyclic carbon atoms of GalNAc-ol residue in the oligosaccharitol. Methylation analysis (8) demonstrated oligosaccharitols to contain either C-3 monosubstituted GalNAc-ol or C-3 and C-6 disubstituted GalNAc-ol. Therefore, the former would be cleaved by periodate to give an oligosaccharide having 3-acetamido-3-deoxythreose (ThrNAc) at the reducing end, whereas the latter would yield two oligosaccharides having either ThrNAc or glycolaldehyde (GA) at the reducing end. Limited oxidation products were labeled with ABEE (9, 10) and subsequently subjected to structural studies.

Structures of five allergenic oligosaccharitols established on the basis of the following are illustrated in Table I.

Structure of HPG- β 2-N5a. HPG- β 2-N5a with a sugar sequence of GalNAc1-2Fuc1-(GalNAc1-3)4GlcNAc1-3GalNAc-ol (8) produced a single ABEE-derivatized oligosaccharide on limited oxidation/ABEE-derivatization (N5a-A, Fig. 1). In FAB-MS analysis (Table II), the ABEE-derivative (N5a-A) yielded a pseudomolecular ion $[\text{M}-\text{H}]^-$ at m/z 1064 and sequence ions at m/z 861 and 715, characteristics consistent of a pentasaccharide with GalNAc-(GalNAc-Fuc)-GlcNAc-ThrNAc-ABEE structure. This result confirms that the GalNAc-(GalNAc-Fuc)-GlcNAc- sequence was linked to the C-3 position of GalNAc-ol.

To determine anomeric configurations of the constituent monosaccharides, the ABEE derivative was subjected to the sequential exoglycosidase digestion, and the product at each step was analyzed by HPLC and FAB-MS. Digestion of the ABEE-derivative with β -N-acetylhexosamin-

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[illegible]

idase yielded a single product with a pseudomolecular ion $[M-H]^-$ at m/z 861, corresponding to GalNAc-Fuc-GlcNAc-ThrNAc-ABEE (Fig. 2). This indicates that one of the nonreducing terminal GalNAc residues was in a β -configuration. The β -N-acetylhexosaminidase digest was susceptible to degradation with α -N-acetylgalactosaminidase but resistant to α -L-fucosidase (Fig. 2). The α -N-acetylgalactosaminidase digest showed a pseudomolecular ion $[M-H]^-$ at m/z 658 corresponding to Fuc-GlcNAc-ThrNAc-ABEE, indicating the other nonreducing terminal GalNAc was linked to Fuc via an α -glycosidic linkage. The resulting trisaccharide-ABEE was susceptible to digestion with α -L-fucosidase. The digest was eluted at the position of GlcNAc1-2ThrNAc-ABEE prepared from HPG- β 2-N3 (Fig. 2). Finally, cleavage of the α -L-fucosidase digest with β -N-acetylhexosaminidase gave a product corresponding to ThrNAc-ABEE (Fig. 2).

Methylation analyses on N5a-A and its β -N-acetylhexosaminidase digest (Table III) demonstrated that C-3,4-disubstituted GlcNAc in the substrate was converted to C-3-monosubstituted GlcNAc by β -GalNAc elimination. These results indicate the structure of N5a-A was GalNAc β 1-4 (GalNAc α 1-2Fuc α 1-3) GlcNAc β 1-2ThrNAc-ABEE (Table II).

The structure of HPG- β 2-N5a was thus established (Table I).

Structure of HPG- β 2-N6. HPG- β 2-N6 with a sugar sequence of GalNAc1 - 4GlcNAc1 - 3 (GalNAc1 - 4GlcNAc1-6)GalNAc-ol yielded two ABEE-derivatized limited oxidation products (N6-A and N6-B) in a ratio of 1:1 (Fig. 1). FAB-MS on N6-A and N6-B indicated a pseudomolecular ion $[M-H]^-$ and fragment ions consistent with GalNAc-GlcNAc-ThrNAc-ABEE and GalNAc-GlcNAc-GA-ABEE (Table II), respectively. These results confirm that GalNAc-ol was disubstituted at C-3 and C-6 positions with two units of GalNAc-GlcNAc-.

β -N-Acetylhexosaminidase digestion converted the N6-A and -B to products corresponding to ThrNAc-ABEE and GA-ABEE, respectively (Fig. 2).

Therefore, the structure of HPG- β 2-N6 was established as shown in Table I.

Structure of HPG- β 2-N7a. Similarly, HPG- β 2-N7a with a sugar sequence of GalNAc1-(Fuc1-),3,4GlcNAc1-

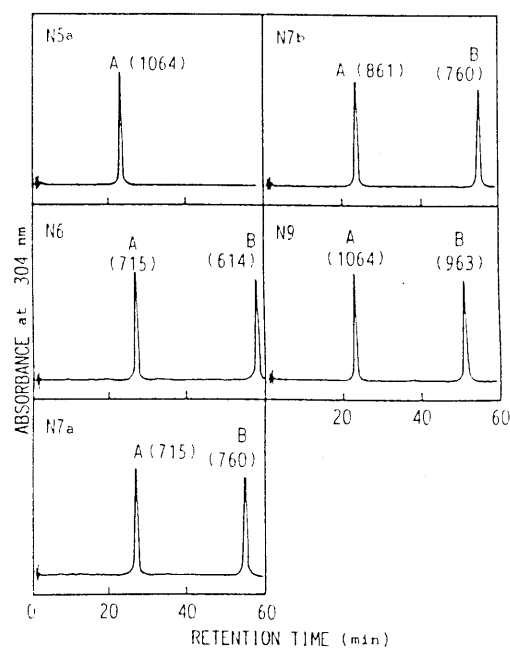


FIG. 1. Reverse-phase HPLC analysis of the ABEE-derivatized limited periodate oxidation products of oligosaccharitols, HPG- β -2-N5a, -N6, -N7a, -N7b, and -N9. A Wakosil 5C18-200 column was used and the elution was performed as described under Materials and Methods. Numbers in parentheses indicate the m/z values of $[M-H]^-$ ion in negative FAB-MS.

TABLE II
Structures and m/z Values of Pseudomolecular $[M-H]^-$ Ion and Sequence Ions in Negative FAB MS
Analysis of ABEE-Derivatized Limited Periodate Oxidation Products

Preparation	m/z of $[M-H]^-$	Sequence ions	Structure
1. HPG- β 2-N5a-A	1064	861, 715	GalNAc α 1-2ThrNAc-ABEE
2. HPG- β 2-N6-A HPG- β 2-N6-B	715 614	512, 309 411	GalNAc α 1-2Fuc α 1
3. HPG- β 2-N7a-A HPG- β 2-N7a-B	715 760	512, 309 614, 557	GalNAc β 1-4GlcNAc β 1-2ThrNAc-ABEE GalNAc β 1-4GlcNAc β 1-2GA-ABEE GalNAc β 1-4GlcNAc β 1-2ThrNAc-ABEE GalNAc β 1-4GlcNAc β 1-2GA-ABEE
4. HPG- β 2-N7b-A HPG- β 2-N7b-B	861 760	715, 658 614, 557	Fuc α 1 GalNAc β 1-4GlcNAc β 1-2ThrNAc-ABEE Fuc α 1 GalNAc β 1-4GlcNAc β 1-2GA-ABEE
5. HPG- β 2-N9-A HPG- β 2-N9-B	1064 963	861, 715 760, 614	Fuc α 1 GalNAc β 1-4GlcNAc β 1-2ThrNAc-ABEE GalNAc α 1-2Fuc α 1 GalNAc β 1-4GlcNAc β 1-2GA-ABEE GalNAc α 1-2Fuc α 1

(GalNAc1-4GlcNAc1-3,6GalNAc-ol (8) yielded two ABEE-derivatized limited oxidation products (N7a-A and N7a-B, Fig. 1). Results of FAB-MS analysis and β -N-acetylhexosaminidase digestion of one of the ABEE-derivatives (N7a-A) were consistent to those for GalNAc β 1-4GlcNAc β 1-2ThrNAc-ABEE. Hence, the GalNAc β 1-4GlcNAc β 1-unit was probably linked to the C-3 position of GalNAc-ol.

The other remaining ABEE-derivatized limited oxidation product (N7a-B) provided a pseudomolecular ion $[M-H]^-$ and fragment ions (Table II) consistent with the structure of GalNAc-(Fuc-)GlcNAc-GA-ABEE. When the ABEE-derivative was sequentially digested with β -N-acetylhexosaminidase, α -L-fucosidase and β -N-acetylhexosaminidase, a product coeluting with GA-ABEE was obtained (Fig. 2; N7a-B). Methylation analyses on N7a-B and its β -N-acetylhexosaminidase digest showed that 3,4-*O*-disubstituted GlcNAc in the substrate was converted to C-3 monosubstituted GlcNAc by β -GalNAc

elimination, indicating that the nonreducing terminal β -GalNAc was linked to the C-4 position of the internal GlcNAc. Thus, the structure of N7a-B was concluded to be GalNAc β 1-4(Fuc α 1-3)GlcNAc β 1-2GA-ABEE (Table II).

On the basis of these results, the structure of HPG- β 2-N7a in Table I was established.

Structure of HPG- β 2-N7b. HPG- β 2-N7b with a sugar sequence of [GalNAc1-(Fuc1-3,4GlcNAc1-2)₂3,6GalNAc-ol (8) similarly yielded two ABEE-derivatized limited periodate oxidation products (N7b-A and N7b-B, Fig. 1). FAB-MS on N7b-A and N7b-B produced $[M-H]^-$ ion and fragment ions corresponding to GalNAc-(Fuc-)GlcNAc-ThrNAc-ABEE and GalNAc-(Fuc-)GlcNAc-GA-ABEE (Table II), respectively.

N7b-A was converted to a product coeluting with Fuc α 1-3GlcNAc β 1-2ThrNAc-ABEE when subjected to β -N-acetylhexosaminidase digestion (compare N7b-A with N5a-A in Fig. 2). Sequential digestion of the resulting

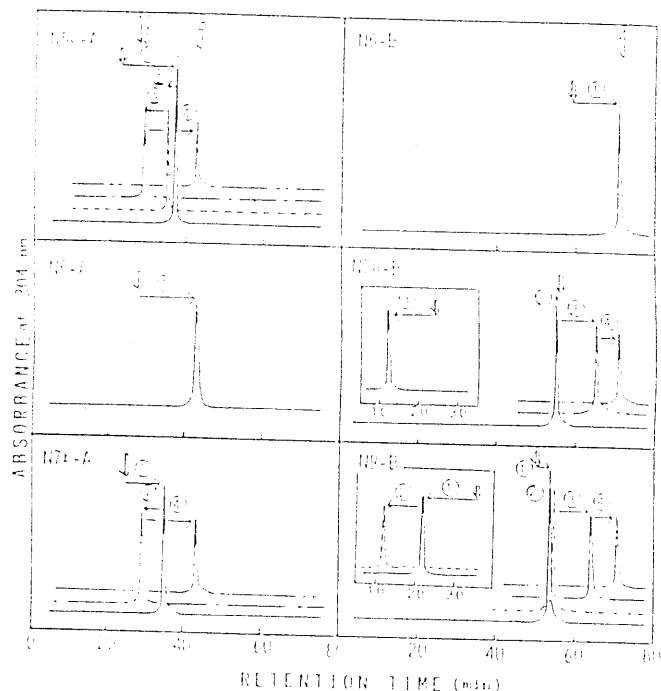


FIG. 2. HPLC analysis of the products obtained from ABEE-oligosaccharides by sequential exoglycosidase digestion. HPG- β 2-N5a-A, -N6-A, -N6-B, -N7a-B, -N7b-A, and -N9-B indicate the peak numbers in Fig. 1. ABEE-oligosaccharides were sequentially digested with ① (jack bean β -N-acetylhexosaminidase), ② (*Acronium* sp. α -N-acetylgalactosaminidase), ③ (bovine kidney α -L-fucosidase), and ④ (jack bean β -N-acetylhexosaminidase). Hydrolysates were analyzed by ODS-HPLC under similar conditions to those described in the legend to Fig. 1. Arrows (•) indicate the elution positions of 1 (GalNAc β 1-4GlcNAc β 1-2ThrNAc-ABEE), 2 (GlcNAc β 1-2ThrNAc-ABEE), 3 (ThrNAc-ABEE), and 4 (GA-ABEE). These standard ABEE-oligosaccharides were prepared by ABEE derivatization of limited oxidation products of HPG- β 2-N3 and -N4a (8). Open arrows indicate the elution positions of native ABEE-oligosaccharides. The internal small figures show the elution profile of the ABEE-oligosaccharides by Amide-80 HPLC. These products had not been efficiently separated by previous ODS-HPLC. The Amide-80 column was eluted with a linear acetonitrile:water gradient (86:14 up to 58:42, v/v in 60 min) at 40°C.

trisaccharide-ABEE with α -L-fucosidase and β -N-acetylhexosaminidase produced a derivative corresponding to ThrNAc-ABEE (Fig. 2). Methylation analyses of N7b-A and its β -N-acetylhexosaminidase digest (Table III) showed that C-3,4-disubstituted GlcNAc in the substrate was converted to C-3-substituted GlcNAc by such treatment. These results indicate that a GalNAc β 1-4(Fuca1-3)GlcNAc β 1- unit was linked to the C-3 position of GalNAc-ol.

The other ABEE-oligosaccharide (N7b-B) derived from HPG- β 2-N7b coincided with N7a-B not only in FAB-MS (Table II) but also in response to exoglycosidases (data not shown). Furthermore, its ^1H NMR spectrum was identical to that of N7a-B (see below). Thus, HPG- β 2-N7b was considered to have a GalNAc β 1-4(Fuca1-3)GlcNAc β 1- unit at the C-6 position of GalNAc-ol.

From the above, the structure of HPG- β 2-N7b (Table I) was established.

Structure of HPG- β 2-N9. In the present study, sugar composition analysis showed the previously purified HPG- β 2-N9 (8) to contain monosaccharides Fuc, GalNAc, GlcNAc, and GalNAc-ol in the molar ratio of 2:4:2:1. Negative FAB-MS showed a pseudomolecular ion $[\text{M}-\text{H}]^-$ at m/z 1732 and fragment ions corresponding to M-HexNAc, M-HexNAc-Fuc, and M-HexNAc-Fuc-HexNAc $_2$ at m/z 1529, 1383, and 977, respectively. Furthermore, methylation analysis indicated that HPG- β 2-N9 contained nonreducing terminal GalNAc (four residues), C-2-substituted Fuc (two residues), C-3,4-disubstituted GlcNAc (two residues) and C-3,6-disubstituted GalNAc-ol (one residue) (Table III). These findings suggest that the nonasaccharitol probably corresponded to a structure of [GalNAc1-2Fuc1-(GalNAc1-3,4GlcNAc1-)] $_2$ 3,6GalNAc-ol.

Limited periodate oxidation and subsequent ABEE derivatization of HPG- β 2-N9 provided two peaks on ODS-HPLC (N9-A and N9-B, Fig. 1). One of the ABEE-derivatives (N9-A) showed chromatographic behavior in HPLC on ODS column (Fig. 1), negative FAB-MS (Table II), ^1H NMR spectrum (see below), and response to exoglycosidases similar to that of GalNAc β 1-4(GalNAc1-2Fuca1-3)GlcNAc β 1-2ThrNAc-ABEE (see N5a-A).

As regards the other remaining ABEE-derivatized limited periodate oxidation product (N9-B), a pseudomolecular ion $[\text{M}-\text{H}]^-$ and fragment ions corresponding to GalNAc-Fuc-(GalNAc-1)GlcNAc-GA-ABEE were obtained (Table II). Digestion of the derivative with β -N-acetylhexosaminidase yielded a product (Fig. 2) which had a pseudomolecular ion $[\text{M}-\text{H}]^-$ and fragment ions consistent with GalNAc-Fuc-GlcNAc-GA-ABEE (data not shown). Subsequent digestion of the resulting tetrasaccharide-ABEE with α -N-acetylgalactosaminidase produced a compound that eluted at the position of Fuca1-3GlcNAc β 1-2GA-ABEE (compare N9-B with N7a-B in Fig. 2). Sequential digestions of the compound corresponding to Fuc1-3GlcNAc1-2GA-ABEE with α -L-fucosidase and β -N-acetylhexosaminidase provided a product coeluting with GA-ABEE (Fig. 2). Comparison of the results of the methylation analyses of N9-B and its β -N-acetylhexosaminidase digest demonstrate that C-3,4-disubstituted GlcNAc in the substrate was converted to C-3-substituted GlcNAc by enzymatic elimination of a β -GalNAc residue (Table III). Therefore, N9-B probably corresponded to GalNAc β 1-4(GalNAc1-2Fuca1-3)GlcNAc β 1-2GA-ABEE (Table II).

The structure of HPG- β 2-N9 was hence established (Table I).

^1H NMR of oligosaccharitols. Features of the 500-MHz ^1H NMR spectra of the novel oligosaccharitols of the HPG- β 2-N series were examined in order to extend the database of chemical shifts of oligosaccharitols. ^1H NMR

TABLE II
Methylation Analysis of Native Oligosaccharitols N4a and ABEE-Derivatized Limited Periodate
Oxidation Products and Their β -N-Acetylhexosaminidase Digest

Oxidation Products and Their β -N-Acetylhexosaminidase Digest									
Partially methylated ^a alditol acetate	ABEE-derivatized limited periodate oxidation products, HPG- β 2-					β -N-Acetylhexosaminidase digest of HPG- β 2-			
	N9	N5a-A	N7a-B	N7b-A	N9-B	N5a-A	N7a-B	N7b-A	N9-B
	mol/mol oligosaccharide ^b								
Fucitol	—	—	—	—	—	—	—	—	—
2,3,4-Tri- <i>O</i> -methyl (1,5-di- <i>O</i> -acetyl)	—	—	1.0	—	—	—	—	—	—
3,4-Di- <i>O</i> -methyl (1,2,5-tri- <i>O</i> -acetyl)	1.8	1.0	—	1.0	—	—	—	—	—
2- <i>N</i> -Methylacetamido-2-deoxyglucitol	—	—	—	—	1.0	1.0	1.0	1.0	—
3,6-Di- <i>O</i> -methyl (1,4,5-tri- <i>O</i> -acetyl)	—	—	—	—	—	—	—	—	—
4,6-Di- <i>O</i> -methyl (1,3,5-tri- <i>O</i> -acetyl)	—	—	—	—	—	—	—	—	1.0
6-Mono- <i>O</i> -methyl (1,3,4,5-tetra- <i>O</i> - acetyl)	—	—	—	—	—	—	—	—	—
2- <i>N</i> -Methylacetamido-2-deoxygalactitol	2.2	0.9	1.1	0.9	1.2	1.1	1.1	1.1	1.2
3,4,6-Tri- <i>O</i> -methyl (1,5-di- <i>O</i> -acetyl)	4.2	2.1	1.0	0.9	—	—	—	—	—
1,4,5-Tri- <i>O</i> -methyl (3,6-di- <i>O</i> -acetyl)	1.0	—	—	—	2.2	1.2	—	—	—
	—	—	—	—	—	—	—	—	1.1
	—	—	—	—	—	—	—	—	—

^a Partially methylated ABEE-monosaccharide at the reducing end could not be detected by the method.

^b Molar contents of partially methylated alditol acetate were calculated from the molar contents of the corresponding oligosaccharide.

^a Partially methylated ABEE-monosaccharide at the reducing end could not be detected by the present method.

^b Molar contents of partially methylated alditol acetate were calculated by assuming *N*-acetylglactosaminitol or fucitol as 1.0.

spectra of HPG- β 2-N5a, -N6, and -N7b are shown in Fig. 3. Chemical shifts of some structural reporter-group protons of five allergenically active oligosaccharitols are summarized in Table IV.

In the NMR spectrum of HPG- β 2-N5a (Fig. 3 and Table IV), two α - and two β -anomeric proton signals were observed at $\delta = 5.3$ –5.4 and $\delta = 4.4$ –4.6 ppm, respectively. Assignments of these anomeric proton signals were difficult due to the lack of appropriate reference oligosaccharitols. Thus, to establish some general rules for defining the influences of introducing Fuc α 1–3 and GalNAc α 1–2Fuc α 1–3 on the chemical shifts of the GalNAc β 1–4GlcNAc β 1–unit, NMR spectra of the ABEE-derivatives of limited periodate oxidation products of oligosaccharitols were examined (Fig. 4 and Table V). When α 1–3-linked Fuc was attached to GlcNAc² (steps from HPG- β 2-N4a-A to -N7b-A and from HPG- β 2-N6-B to -N7a-B), H-1 of GlcNAc² showed a slight upfield shift ($\Delta\delta \approx -0.005$ ppm), whereas that of GalNAc^{4,2} indicated a relatively large upfield shift ($\Delta\delta \approx -0.05 \sim -0.06$ ppm). When α 1–2-linked GalNAc was attached to the Fuc α 1–residue (steps HPG- β 2-N7b-A $\rightarrow$ -N5a-A and HPG- β 2-N7a-B $\rightarrow$ -N9-B, Table V), H-1 of GlcNAc² shifted upfield ($\Delta\delta \approx -0.04 \sim -0.05$ ppm), whereas H-1 of both GalNAc^{4,2} ($\Delta\delta \approx -0.01 \sim -0.03$ ppm) and Fuc^{3,2} ($\Delta\delta \approx +0.2$ ppm) shifted downfield.

On the basis of these findings, anomeric proton signals at $\delta = 4.570$ ppm ($J = 8.55$ Hz) and $\delta = 4.477$ ppm ($J = 8.24$ Hz) in the spectrum of HPG- β 2-N5a could be assigned respectively to H-1 of β -GlcNAc³ and β -GalNAc^{4,3}, as compared with HPG- β 2-N4a. The other anomeric proton signals at $\delta = 5.333$ ppm ($J = 3.36$ Hz) and $\delta = 5.311$ ppm ($J = 3.66$ Hz) were assigned to H-1 of α -

Fuc^{3,3} and α -GalNAc^{2,3,3}, respectively, by 2D-COSY NMR analysis (data not shown). It should be noted that the signals of H-2 and H-5 of GalNAc-ol showed large upfield shifts with the attachment of the GalNAc α 1–2Fuc α 1–unit to GlcNAc³, as compared with N4a. Similar upfield shifts of H-2 and H-5 of GalNAc-ol were observed in the step from HPG- β 2-N6 to HPG- β 2-N9.

Spectra of HPG- β 2-N6, -N7a, and -N7b contained the multiplets for H-2 ($\delta = 4.28$ ppm) and H-5 ($\delta = 4.23$ ppm) of GalNAc-ol. The presence of GlcNAc β 1–3(GlcNAc β 1–6)GalNAc-ol core structure in these oligosaccharitols was therefore indicated (13). The signals at $\delta = 4.599$ ppm and $\delta = 4.508 \sim 9$ ppm in the spectra of HPG- β 2-N6 and -N7a (Table IV, Fig. 3) could be assigned to H-1 of GlcNAc³ and GalNAc^{4,3}, respectively (as compared with HPG- β 2-N4a). The other two β -anomeric doublet signals could be assigned to H-1 of GlcNAc⁶ and GalNAc^{4,6} (Table IV) based on the aforementioned shift effects by the attachment of α 1–3 Fuc to GlcNAc. A similar upfield shift was observed for H-1 of GalNAc^{4,3} in the step from HPG- β 2-N7a to -N7b (Table IV).

In the NMR spectrum of HPG- β 2-N9, characteristic signals of H-2 ($\delta = 4.240$ ppm) and H-5 ($\delta = 4.189$ ppm) indicated the presence of a GalNAc α 1–2Fuc α 1–unit in the saccharitol. As mentioned before (steps for HPG- β 2-N7b-A to -N5a-A and HPG- β 2-N7a-B to -N9-B), an upfield shift of approximately 0.05 ppm was expected on the chemical shifts of H-1 of GlcNAc³ and GlcNAc⁶ by the extension of HPG- β 2-N7b with GalNAc in α 1–2 linkage to Fuc residues. Therefore, signals at $\delta = 4.544$ ppm and $\delta = 4.490$ ppm could be assigned to H-1 of GlcNAc³ and GlcNAc⁶, respectively. Similarly, based on the aforementioned downfield shift of H-1 of Fuc by the attachment

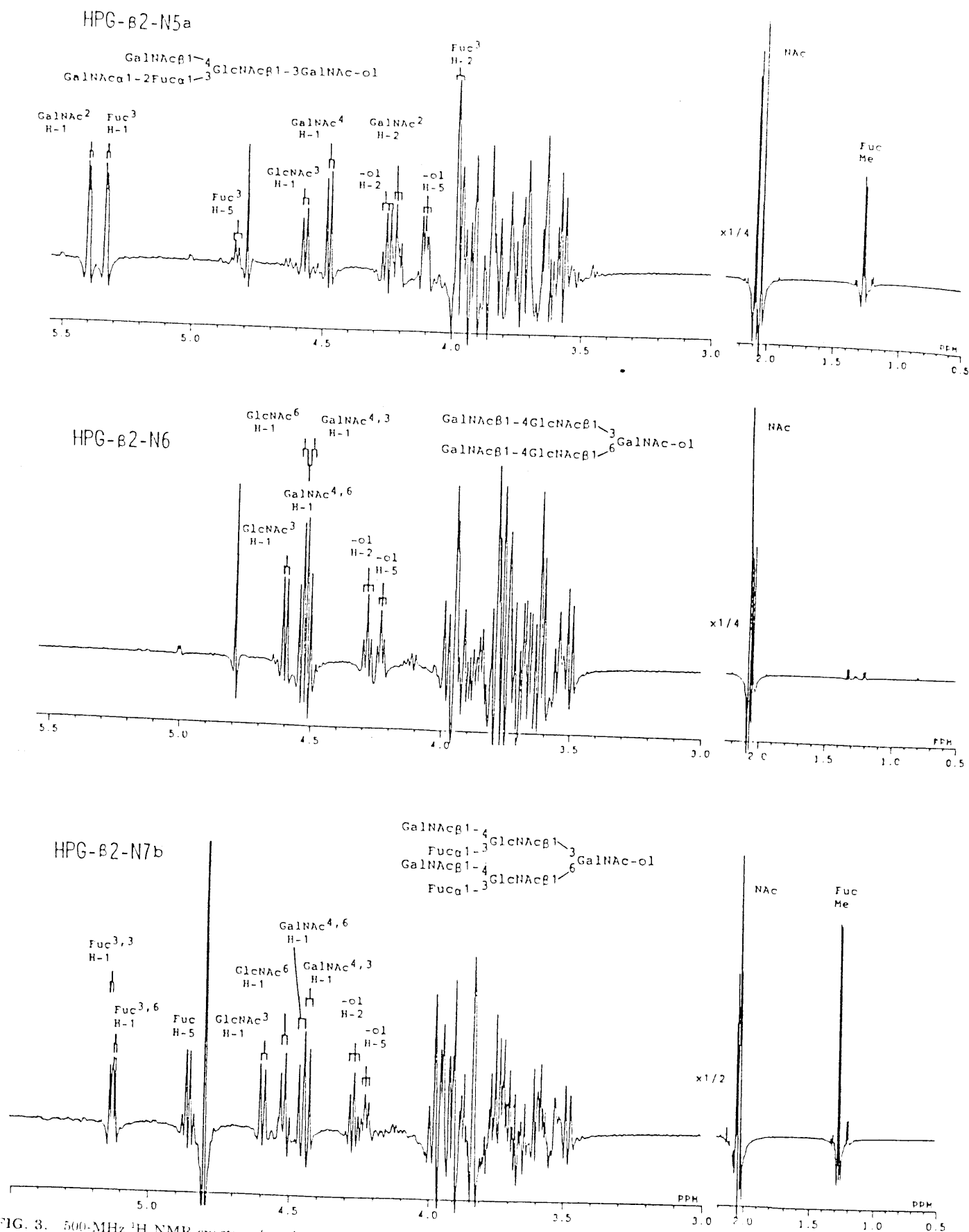


FIG. 3. 500-MHz ^1H NMR spectra of native oligosaccharitols, HPG-B2-N5a, -N6, and -N7b from H-antigen in $^2\text{H}_2\text{O}$ at 25°C . The relative intensity scale of the N-acetyl and methyl proton region deviates from the other part of the spectrum as indicated.

TABLE IV
¹H Chemical Shift of Major Structural Reporter-Group Protons of Constituent Monosaccharides
 for Neutral Oligosaccharitols Obtained from H-Antigen

		Chemical shift in compounds: HPG- β 2-						
Residue	Reporter group	N3 ^a 	N4a ^a 	N5a 	N6 	N7a 	N7b 	N9 
		ppm						
GalNAc-ol	H-2	4.291	4.291	4.237	4.283	4.283	4.283	4.240
GlcNAc ^b	H-5	4.146	4.140	4.103	4.232	4.231	4.237	4.189
GalNAc ^b	H-1	4.606	4.612	4.570	4.599	4.599	4.599	4.544
Fuc ^{3,6}	H-1	—	4.511	4.477	4.508	4.509	4.439	4.462
	H-2	—	—	5.333 ^c	—	—	5.136	5.338
	H-5	—	—	3.96	—	—	ND ^d	ND
GalNAc ^{4,6}	Me	—	—	4.837	—	—	ND ^d	ND
	H-1	—	—	1.268	—	—	4.866	4.837
GlcNAc ^b	H-2	—	—	5.401 ^d	—	—	1.268	1.261
GalNAc ^{4,6}	H-1	—	—	4.216	—	—	—	5.394
Fuc ^{3,6}	H-1	—	—	—	4.537	4.529	4.527	4.212
	H-2	—	—	—	4.524	4.462	4.458	4.490
	H-5	—	—	—	—	5.126	5.126	4.439 ^e
	Me	—	—	—	—	ND ^d	ND ^d	5.328
GalNAc ^{2,3,6}	H-1	—	—	—	—	4.867	4.866	ND ^d
	H-2	—	—	—	—	1.268	1.268	4.837
	NAc ^f	2.040 (1) ^g	2.038 (1)	2.043 (2)	2.043 (1)	2.047 (1)	2.042 (1)	2.039 (3)
		2.087 (1)	2.071 (1)	2.080 (1)	2.059 (1)	2.049 (1)	2.053 (2)	2.073 (2)
			2.081 (1)	2.092 (1)	2.075 (3)	2.076 (3)	2.071 (1)	2.082 (2)
							2.080 (1)	

Note. Chemical shifts are given relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (using internal acetone at $\delta = 2.225$ ppm) in ²H₂O at 25°C. For complete structures of compounds, see Table I. In the heading, the structures are represented as follows: $\diamond$, GalNAc-ol; $\bullet$, GlcNAc; $\blacksquare$, GalNAc β 1-4; $\square$, GalNAc α 1-2; $\blacktriangle$, Fuc α 1-3.

NMR characteristics matched those documented previously (13-15) for this structure.

Signals were assigned by comparison with the spectrum of HPG- β 2-N3.

^a This signal was connected to the signal at $\delta = 3.96$ ppm that is assignable to H-2 of Fuc (16-19) in a 2D-COSY NMR.

^b This signal was connected to the signal of H-2 of α -GalNAc at $\delta = 4.216$ ppm (18-20).

^c Assignments are interchangeable.

^d Value could not be determined.

^e Acetyl methyl protons could not be assigned due to lack of reference oligosaccharitols.

^f Numbers in parentheses indicate the number of monosaccharide residues.

of GalNAc in α 1-2 linkage to Fuc ($\Delta\delta \approx +0.2$ ppm), two signals at $\delta = 5.328$ ppm and $\delta = 5.338$ ppm could be assigned to H-1 of Fuc^{3,6} and Fuc^{3,3}, respectively (as compared with HPG- β 2-N7b). The signal at $\delta = 5.394$ ppm corresponding to two protons was assigned to H-1 of α -GalNAc at the nonreducing terminal as compared with HPG- β 2-N5a. Two other β -anomeric proton signals could be ascribed to either GalNAc^{4,3} or GalNAc^{4,6}.

DISCUSSION

The sea squirt H-antigen is a large ($M_r 10^6 \sim 2 \times 10^7$) acidic glycoprotein capable of inducing a remarkable pos-

itive reaction on the skin of patients with asthma from sea squirt allergy. The structures of allergenically active oligosaccharitols derived from H-antigen depicted two types of core-region structure commonly found in mucus glycoproteins (Table I); namely, GlcNAc β 1-3GalNAc-ol and GlcNAc β 1-3(GlcNAc β 1-6)GalNAc-ol (13-15, 21). However, it is remarkable that all oligosaccharitols from H-antigen contained the GalNAc β 1-4GlcNAc β 1- backbone sequence, since most of oligosaccharitols from mucus glycoproteins contain the Gal β 1-3 or -4GlcNAc β 1- sequence (13-15). The more striking structural feature is the presence of the oligosaccharide sequence containing internally linked Fuc residue, GalNAc β 1-4(GalNAc α 1-

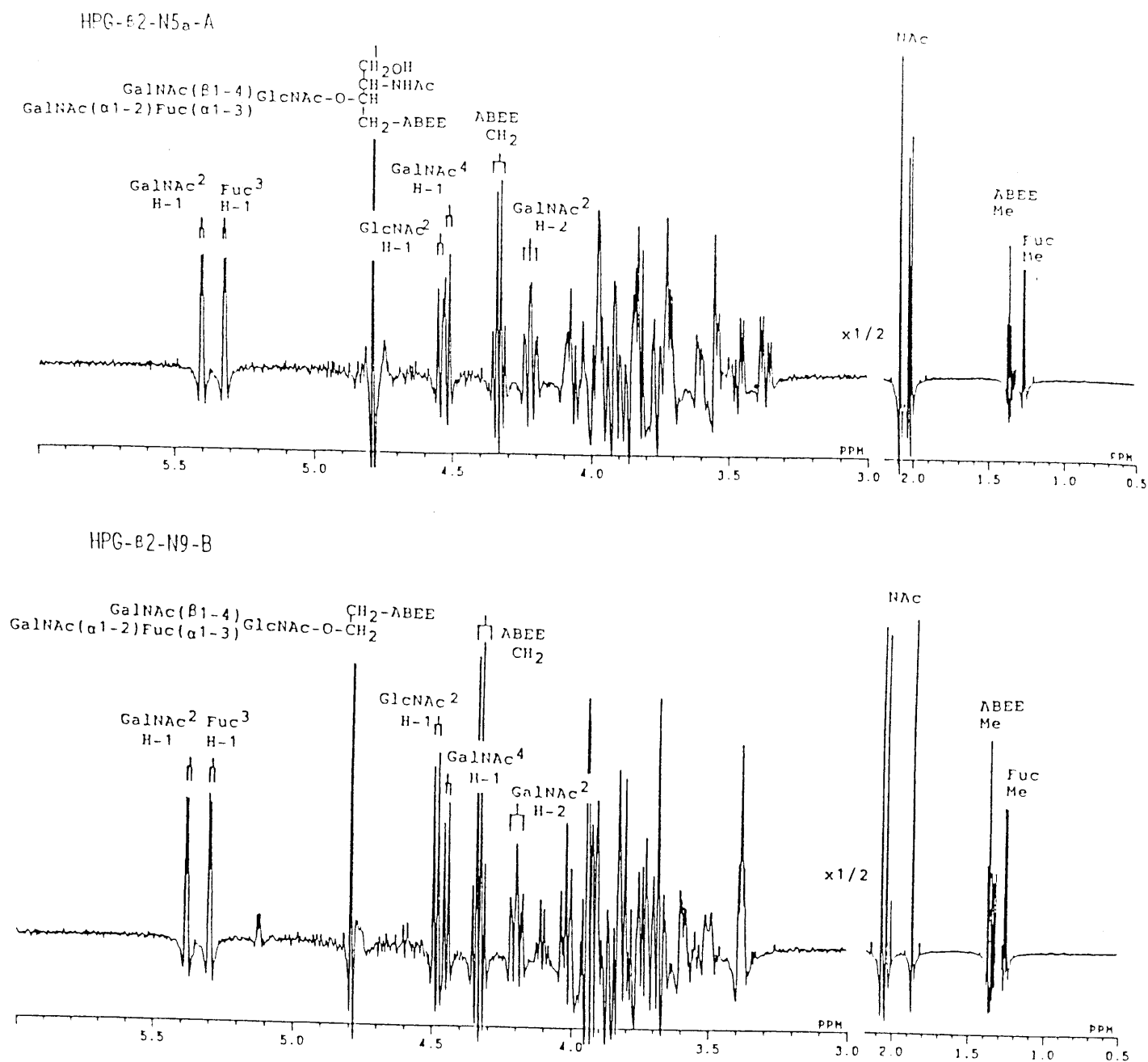


FIG. 4. 500-MHz ^1H NMR spectra of the ABEE-derivatized limited periodate oxidation products. The relative intensity scale of the *N*-acetyl and methyl proton region deviates from the other part of the spectrum as indicated.

2Fuc α 1-3)GlcNAc β 1-. Oligosaccharides containing internally linked Fuc residues have been reported in glycosphingolipids from the fresh water bivalve *Hyriopsis schlegelli* (22, 23), but not in glycoproteins. All structures described in our present study have not been previously reported.

As the allergic reaction in skin depends on the cross-linkage of IgE antibody with each allergenically active oligosaccharitol, the disaccharide units GalNAc α 1-2Fuc α 1-, GalNAc β 1-4GlcNAc β 1- and Fuc α 1-3GlcNAc β 1- can be nominated as the allergy-specific epitopes in this sea

squirt antigen. The smallest allergenically active oligosaccharitol, HPG- β 2-N5a, has also been found in another sea squirt antigen, Gi-rep (7).

In our present study, oligosaccharitols were subjected to limited periodate oxidation, a process where the C4-C5 and/or C5-C6 glycols of the GalNAc-ol residue in oligosaccharitols are selectively oxidized, and the resulting aldehyde(s) is labeled with an ultraviolet absorbing compound, ABEE, by reductive amination. This method generated ABEE-oligosaccharides with ThrNAc at their reducing end from oligosaccharitols having the GlcNAc β 1-

TABLE V
¹H-Chemical Shift of Major Structural Reporter-Group Protons of the ABEE-Derivatized Limited Periodate Oxidation Products

Residue	Reporter group	Chemical shift ^a in compounds: HP ₉ -32 ^b						
		N3-A ●-R ^c	N4a-A ■-●-R	N7b-A ■-●-R	N5a-A ■-●-R	N6-A ■-●-R	N7a-B ■-●-R	N9-B ■-●-R
ThrNAc	NAc	2.013	2.011	2.000	2.002	—	—	—
GlcNAc ^{1,2}	H-1	4.595	4.596	4.592	4.542	—	—	—
	NAc	—	—	—	—	—	—	—
GalNAc ^{1,2}	H-1	2.073	2.074	2.055	2.076 ^c	4.527	4.522	4.484
	NAc	—	4.536	4.483	4.516	1.832	1.815	1.862
Fuc ^{1,2}	H-1	—	2.065	2.035	2.076	4.511	4.448	4.456
	Me	—	—	5.114	5.315	2.065	2.035	2.065
GalNAc ^{2,3,2'}	H-1	—	—	1.264	1.258	—	5.073	5.295
	H-2	—	—	—	5.402	—	1.251	1.248
	NAc	—	—	—	4.144	—	—	5.387
		—	—	—	2.023	—	—	4.196
		—	—	—	—	—	—	2.031

Note. For complete structure of compounds, see Table II. Details are as in Table IV.
^a Data were obtained at 500 MHz in ²H₂O solution at 25°C.
^b R = ThrNAc-ABEE, R' = GA-ABEE.
^c Assignments are interchangeable.

3GalNAc-ol core, whereas ABEE-oligosaccharides with ThrNAc and glycolaldehyde were derived from oligosaccharitols having a branched GlcNAcβ1-3(GlcNAcβ1-3)GalNAc-ol core. The present method was a very convenient and highly sensitive technique for the analysis of O-glycosidically linked oligosaccharides.

Partial assignments of 500-MHz ¹H NMR spectra of the above new types of oligosaccharitols have been attempted in our present study. Follow-up studies on complete assignments of the spectra are now in progress using a combination of 1D and 2D NMR techniques in our laboratories.

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