

N-Acetylgalactosaminyl Disaccharide Terminals Contained in Presumed Carbohydrate Epitopes Specific to Sea-Squirt Allergy¹

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Using the methods of methylation/GLC, MS, and ¹H-NMR analyses, a disaccharide structure of GalNAcα1→2Fuc1→ was identified as one of the major structural units at non-reducing terminals of a highly branched and N-glycosidically linked carbohydrate on an allergenically active glycopeptide, Gp-2 (*M_r* ca. 8,000). Gp-2 was one of the major fractions in the Pronase digest of a sea-squirt antigen, Gi-rep, and is capable of eliciting the skin reaction specific to patients with sea-squirt allergy similarly to Gi-rep. An additional disaccharide structure of GalNAc1→(3/4)HexNAc1→ was also expected as the other major non-reducing terminal unit, though the anomeric configuration of the terminal GalNAc could not be identified. As Gp-2 carbohydrate was found to contain 3 mol each of the two types of terminal GalNAc-containing units, both were nominated as possible components of the IO₄⁻ oxidation-sensitive epitopes which are responsible for the allergenic activity in Gp-2 and its mother antigen, Gi-rep. A few moles of Fuc and Gal were also detected as minor non-reducing terminals in addition to the 6 mol of the GalNAc-containing units.

In the preceding study (1), a sea-squirt antigen preparation termed Gi-rep (2-4) was digested with Pronase E into a large glycopeptide, Gp-1, and a relatively small glycopeptide, Gp-2. On further treatment with alkali in the presence of NaBH₄, Gp-1 gave a significant amount of oligosaccharide alditols containing N-acetylgalactosaminitol as a small molecular size fraction, Gp-1β-b, in addition to a relatively large glycopeptide fraction, Gp-1β-a, which contained no alditol but still some amounts of amino acids. However, an alkali treatment of Gp-2 gave only a glycopeptide fraction, Gp-2β, in which no alditol-containing oligosaccharide was detected.

Therefore, only Gp-1β-b was considered to be composed of the oligosaccharides that have been O-glycosidically linked to and liberated from Gp-1 by the β-elimination reaction (1). However, in the cases of Gp-1β-a and Gp-2β, the carbohydrates have been considered to link to the peptide moieties through an alkali-resistant linkage, presumably N-glycosidic linkage.

All of these glycopeptide and oligosaccharide alditol preparations, as well as Gi-rep as the mother substance, were capable of inducing a remarkable erythema in the skin of patients with sea-squirt allergy at a concentration of less than 10 μg/ml. Furthermore, the allergenic activity in all

of the preparations was eliminated by periodate oxidation treatment (1). Thus, the presumed N-linked carbohydrates in Gp-2β and Gp-1β-a were also considered to contain the oligosaccharide epitope that was equivalent to the allergy-specific epitope residing in the O-linked oligosaccharide giving Gp-1β-b.

In the present study to elucidate the chemical structure of the allergy-specific epitope in Gi-rep, the presumed N-linked carbohydrate of Gp-2 was analyzed for its partial structure which contained the periodate-sensitive sugars.

MATERIALS AND METHODS

Allergenic Glycopeptide, Gp-2—The allergenic sample termed Gp-2 was a hexosamine- and fucose-rich glycopeptide preparation which has been isolated from a Pronase digest of a sea-squirt antigen, Gi-rep, as a relatively small glycopeptide substance (1). The immunological and chemical properties of Gp-2 and Gi-rep were described in previous paper (1-4).

Allergenic Activity—The allergenic activity of a sample was evaluated as previously described by an *in vivo* skin test on asthmatic patients with sea-squirt allergy (5-7).

Antigenic Activity—The immunological reactivity of a sample against a rabbit anti-Gi-rep serum (L 304) was assayed *in vitro* by radioimmunoassay (RIA) (5, 6).

Sugar Composition—For analysis of sugar composition by gas-liquid chromatography (GLC), a carbohydrate sample was hydrolyzed in 4 N trifluoroacetic acid at 100°C for 4 h, and the reaction mixture was evaporated to dryness. The hydrolyzate was reduced with an excess of NaBH₄ at room temperature for 4 h and neutralized with acetic acid, followed by azeotropic distillation with methanol to yield the alditols corresponding to the component sugars. Then the alditols were peracetylated with acetic

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Abbreviations: RIA, radioimmunoassay; LC, liquid chromatography; MS, mass spectrometry; EI, electron impact; FAB, fast atom bombardment; DI, direct inlet; Fuc, fucose; Fuc-ol, fucitol; Gal, galactose; Man, mannose; Me-Pen; methyl pentose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; HexNAc, N-acetylhexosamine; t-, nonreducing terminal; 2-, 2-O-substituted; 3-, 3-O-substituted; 4-, 4-O-substituted; 2,4-, 2,4-di-O-substituted; 2,6-, 2,6-di-O-substituted; 3,4-, 3,4-di-O-substituted; 3,6-, 3,6-di-O-substituted; 2,4,6-, 2,4,6-tri-O-substituted.

anhydride (100°C, 4 h).

Amino Acid Composition—Amino acids were analyzed with an amino acid analyzer (KLA-5, Hitachi, Tokyo) after hydrolysis of a sample in 6 N HCl at 110°C for 24 h.

Periodate Oxidation—Periodate oxidation of carbohydrate samples was carried out according to the procedure used in the preceding study (1) under monitoring with an isotachophoretic analyzer (IP-2A, Shimadzu, Kyoto) (1, 8).

Liquid Chromatography (LC)—LC was on a liquid chromatograph apparatus (HLC-803C, Toso, Shin-Nanyo) equipped with a differential refractometer (RI-8, Toso) and a spectrophotometer (UV-8, Toso). The columns used were of TSK Gel SW 2000 (Toso) and ODS AL-312 YMC (YMC, Osaka). Sephadex G-25 (Pharmacia, Uppsala) and Ultrogel AcA 54 (LKB, Bromma) were used for preparative gel chromatography. Dowex 1×4 (Dow Chemical, Midland) was used for anion exchange chromatography.

Methylation—Carbohydrate samples were methylated according to the method of Hakomori-Stellner (9), and for purification the product was chromatographed on a Sephadex LH-20 column (1×25 cm) with a mixture of equal volumes of chloroform and methanol (10).

For GLC analysis of a methylated sample, the sample was subjected to solvolysis in 0.5 ml of 93% acetic acid containing 0.5 N sulfuric acid at 80°C for 16 h and cooled to room temperature. Subsequently, 0.5 ml of deionized water was added to the product, and the mixture was heated at 80°C for an additional 5 h for hydrolysis (11). The hydrolyzate was then passed through a Dowex 1×4 column (acetate form, 0.6×5 cm) and washed with methanol. After the effluent and the washings were pooled and evaporated, the oily residue was reduced with NaBH₄ to alditol, followed by peracetylation with acetic anhydride at 100°C for 4 h.

Gas Liquid Chromatography (GLC)—GLC analysis was on a gas chromatograph apparatus equipped with an FID detector (JEOL-20K, JEOL, Tokyo). Alditol acetates of neutral sugars were analyzed on a glass column (2 mm i.d.×100 cm) packed with 3% ECNSS-M on Gaschrom Q at 180°C. Alditol acetates of amino sugars and partially methylated alditol acetates were analyzed on a glass column (2 mm i.d.×100 cm) packed with 2% OV-17 on Gaschrom Q at 200°C and by increasing the column temperature from 150°C up to 240°C at a rate of 2°C/min (12), respectively.

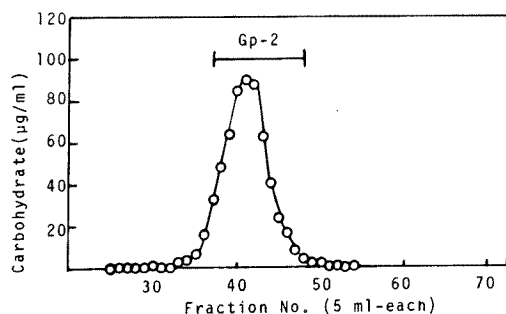


Fig. 1. Gel chromatogram for Gp-2. A 240-mg portion of Gp-2 (1) was chromatographed on a column of Ultrogel AcA 54 (1.6×86 cm) with 1% NaCl at a flow rate of 30 ml/h into 5-ml fractions. The carbohydrate content in each fraction was determined by the phenol-sulfuric acid method and expressed as glucose equivalent.

For GLC-mass spectrometry (GC-MS), partially methylated alditol acetates were chromatographed on a glass column (2 mm i.d.×100 cm) packed with 2% OV-17 on Gaschrom Q by increasing the column temperature from 150°C up to 250°C at a rate of 2°C/min using an MS-GCG05 gas chromatograph (JEOL); the separated components were subjected to electron-impact MS (EI-MS) analysis on a DX-300 mass-spectrometric analyzer (JEOL).

Mass Spectrometry (MS)—For the EI-MS analysis, the ionization potential was 70 eV, the accelerating voltage was 3.0 kV, and the ionization current was 300 µA. Direct inlet (DI)/EI-MS analysis was performed on the same MS-analyzer by introducing a probe through a sample applicator for in-beam MS analysis (13).

¹H-Nuclear Magnetic Resonance Spectrometry (NMR)—NMR analysis was on an FX-500s FT NMR apparatus (500 MHz, JEOL) equipped with a data recorder operating in the Fourier-transform mode at 25°C. Samples were repeatedly freeze-dried from a solution in 99.8% D₂O to exchange hydrogen in the sample with deuterium before analysis. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate was used as an internal standard for chemical shifts.

RESULTS

Composition and Allergic and Antigenic Activities of Gp-2—A 240-mg portion of Gp-2 was rechromatographed on a column of Ultrogel AcA 54 for further purification as shown in Fig. 1. As the Gp-2 seemed to be substantially homogeneous, the fractions from No. 37 to 48 were pooled, dialyzed and freeze-dried to yield 140 mg of purified Gp-2.

Table I shows that the allergenic activity of the purified Gp-2 was comparable to that of Gi-rep as examined *in vivo* by skin test, whereas the antigenic activity was 16% of that of Gi-rep as assayed *in vitro* by RIA. Table II shows the chemical composition of the purified Gp-2. The immunological activities and the chemical properties of the purified Gp-2 were essentially the same as those before the purification (1). Thus, the purified preparation was simply termed Gp-2 and subjected to the following studies.

The molecular weight of Gp-2 was measured to be

TABLE I. Allergenic and antigenic activities of Gp-2 before and after periodate oxidation. To examine the allergenic activity *in vivo*, a 20-µl portion of a 50 µg/ml solution of each sample in 0.9% NaCl was subjected to direct skin test against patients (a-f) with sea-squirt allergy as previously described (5-7). When after 15 min erythema was 0 to 10, 10 to 20, 20 to 30, 30 to 40, or >40 mm in diameter, the symbol -, ±, +, #, or ## was assigned, respectively. The antigenic activity *in vitro* was assayed against a rabbit anti-Gi-rep serum by the RIA method (1) and represented with specific activity assayed as Gi-rep equivalent.

Sample	Allergenic activity detected by skin test ^a						Specific activity assayed by RIA as Gi-rep equivalent (µg/µg)
	a	b	c	d	e	f	
Gi-rep	+	+	#	+	+	+	1.00
Purified Gp-2	+	+	±	#	#	+	0.16
IO ₄ -oxidized Gp-2	-	-	-	-	-	-	<0.01

^aSince the response to the skin test varied with the allergic sensitivity of the patient, the sample concentration for the test was empirically fixed at 50 µg/ml as adequate but minimal for Gi-rep to induce a detectable erythema on the skin of most of the patients with sea-squirt allergy; no skin reaction due to Gi-rep has ever been detected in healthy cases even when the antigen concentration was elevated to 1 mg/ml (5-7).

TABLE II. Chemical compositions of Gp-2 before and after periodate oxidation. In the case of Gp-2, the molar contents in parentheses are based on the molecular weight of Gp-2, which was estimated to be 8,000 by gel chromatography (see text). In the case of IO_4^- -oxidized Gp-2, the molar contents of residual sugars were calculated on the assumption that the 9 mol of GlcNAc in Gp-2 were mostly conserved even after IO_4^- -oxidation (see text).

Component	Intact Gp-2		IO_4^- -oxidized Gp-2		Loss by oxidation (mol/mol)
	(mmol/g)	(mol/mol)	(mmol/g)	(mol/mol)	
Fuc	0.59	(4.7)	0.21	(1.2)	(3.5)
Gal	0.43	(3.4)	0.19	(1.1)	(1.3)
Man	0.53	(4.2)	0.44	(2.5)	(1.7)
GalNAc	1.19	(9.5)	0.80	(4.6)	(4.9)
GlcNAc	1.12	(9.0)	1.56	(9.0)	(0)
H_2SO_4	0.19	(1.5)	0.15	(0.9)	(0.6)
Asx	0.18	(1.4)			
Thr	0.13	(1.0)			
Ser	0.09	(0.7)			
Glx	0.09	(0.7)			
Gly	0.12	(1.0)			
Ala	0.08	(0.6)			
Val	0.04	(0.3)			
Ile	0.04	(0.3)			
Leu	0.03	(0.2)			

TABLE III. Methylation/GLC analysis of Gp-2. A peracetylated sample of the partially methylated alditols from permethylated Gp-2 was analyzed by GLC on a glass column (ϕ 2 mm $\times$ 200 cm) packed with 2% OV 17-coated Gaschrom Q. The column temperature was kept at 150°C for 5 min, then elevated to 240°C at a rate of 2°C/min.

Correspondence	Component	Retention time (min)	Content (mol/mol)
1 (t-Fuc)	1,5-di- <i>O</i> -acetyl-2,3,4-tri- <i>O</i> -methylfucitol	5.1	1.5
2 (2-Fuc)	1,2,5-tri- <i>O</i> -acetyl-3,4-di- <i>O</i> -methylfucitol	8.6	3.0
3 (t-Gal)	1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylgalactitol	9.9	1.3
4 (4-Gal)	1,4,5-tri- <i>O</i> -acetyl-2,3,6-tri- <i>O</i> -methylgalactitol	13.5	0.8
5 (2,4=Gal)	1,2,4,5-tetra- <i>O</i> -acetyl-3,6-di- <i>O</i> -methylgalactitol	17.6	0.2
6 (2,6=Gal)	1,2,5,6-tetra- <i>O</i> -acetyl-3,4-di- <i>O</i> -methylgalactitol	18.0	0.8
7 (3,6=Gal)	1,3,5,6-tetra- <i>O</i> -acetyl-2,4-di- <i>O</i> -methylgalactitol	19.3	0.6
8 (2-Man)	1,2,5-tri- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methylmannitol	12.5	2.1
9 (2,4,6=Man)	1,2,4,5,6-penta- <i>O</i> -acetyl-3- <i>O</i> -methylmannitol	22.8	2.6
10 (t-GalNAc)	1,5-di- <i>O</i> -acetyl-3,4,6-tri- <i>O</i> -methyl-2- <i>N</i> -methylacetamido-2-deoxygalactitol	24.3	5.8
11 (3-GalNAc)	1,3,5-tri- <i>O</i> -acetyl-4,6-di- <i>O</i> -methyl-2- <i>N</i> -methylacetamido-2-deoxygalactitol	28.7	2.0
12 (4-GlcNAc)	1,4,5-tri- <i>O</i> -acetyl-3,6-di- <i>O</i> -methyl-2- <i>N</i> -methylacetamido-2-deoxyglucitol	26.6	5.0
13 (3,4-GlcNAc)	1,3,4,5-tetra- <i>O</i> -acetyl-6- <i>O</i> -methyl-2- <i>N</i> -methylacetamido-2-deoxyglucitol	30.4	4.2

approximately 8,000 by gel chromatography on a TSK Gel SW 2000 column, based on the elution behavior of Gi-rep (M_r 106,000), Ei-M (M_r 22,800), and DIIIa (M_r 9,980) as

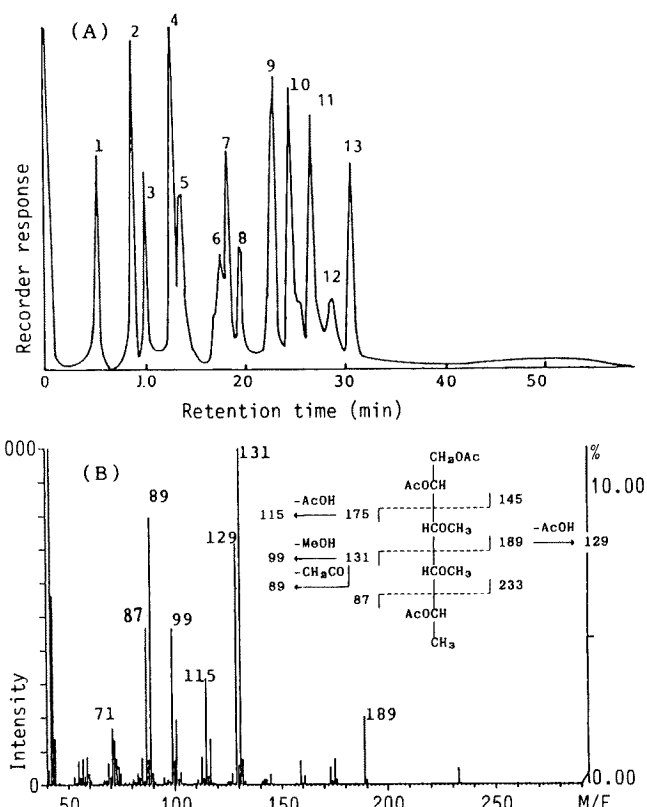


Fig. 2. GLC/EI-MS spectrum for the presumed 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylfucitol derived from permethylated Gp-2 (A). The peak component eluted at a retention time of 8.6 min (peak 2) in the GLC was subjected to EI-MS analysis (B).

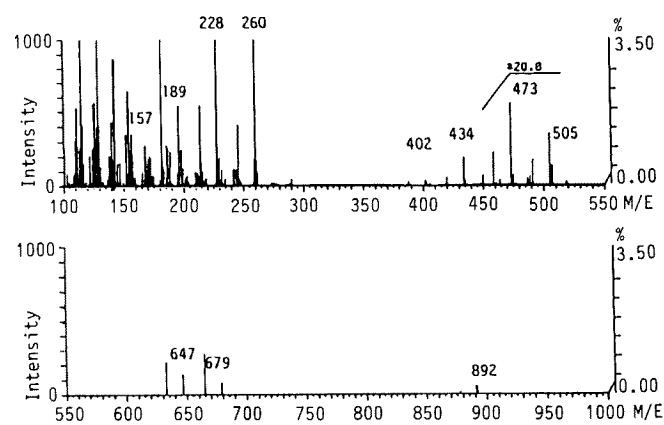


Fig. 3. DI/EI-MS spectrum for permethylated Gp-2.

standards (data not shown).

Periodate Oxidation—The carbohydrate moiety of Gp-2 was examined for its contribution to the allergenic and antigenic activities by the periodate oxidation treatment. A 10-mg portion of Gp-2 was incubated in 2 ml of 10 mM NaIO_4 at 25°C for 24 h and the residual IO_4^- was inactivated with ethyleneglycol. The product was then dialyzed against deionized water and freeze-dried to yield 7 mg of IO_4^- -oxidized Gp-2.

As shown in Table I, periodate treatment apparently inactivated both of the *in vivo* and *in vitro* activities in

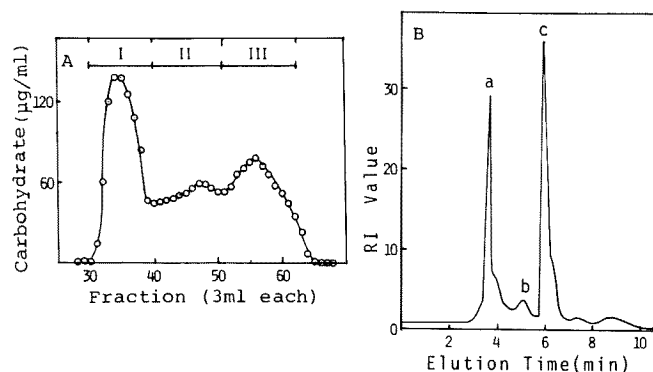


Fig. 4. Isolation of a disaccharide as one of the major units of the non-reducing terminals in the carbohydrate chain of Gp-2. (A) A 10-mg portion of Gp-2 was hydrolyzed in 20 mM H_2SO_4 at 100°C for 90 min. The reaction mixture was applied onto a column of Dowex 1×4 (OH^- form, 1.6×12 cm) and the column was washed with deionized water. The substance adsorbed on the column was eluted with 1 N HCl as the acid-hydrolyzed product (Gp-2a) and freeze-dried. Gp-2a was then chromatographed on a column of Sephadex G-25 (1.5×100 cm) with deionized water into 3-ml fractions. Fractions No. 52 to 62 were pooled as the smallest component in the hydrolyzate (Gp-2a-III). (B) Gp-2a-III was reduced with NaBH_4 to an alditol Gp-2a-III-ol, and the reduced sample was chromatographed on a column of ODS-AL-312 with deionized water into three fractions under monitoring with a refractometer. Most of the carbohydrate was recovered in the third peak fractions (fraction c), while the first and second peak fractions (fractions a and b) contained only a trace amount of and no carbohydrate, respectively, as examined by the phenol-sulfuric acid method. Thus, fraction c was pooled and freeze-dried (Gp-2a-III-ol-c).

Gp-2, supporting the previous suggestion that a certain structure in the carbohydrate chains of Gp-2 was responsible for both of the activities (I).

The IO_4^- -oxidized Gp-2 is compared with the intact one in sugar composition in Table II. A marked loss was detected in fucose (Fuc), galactose (Gal), mannose (Man), and *N*-acetylgalactosamine (GalNAc), whereas only *N*-acetylglucosamine (GlcNAc) seemed to be mostly resistant to the oxidation. These IO_4^- -sensitive sugars are expected to contribute to both the allergenic and antigenic activity.

Methylation/GLC Analysis—Permethylated Gp-2 was analyzed for the composition of partially methylated sugar residues: the results are summarized in Table III. It was recognized that the carbohydrate moiety of Gp-2 had a highly branched chain and contained 6 mol of non-reducing terminal GalNAc (t-GalNAc), 1–2 mol of terminal Fuc (t-Fuc), and 1 mol of terminal Gal (t-Gal). In addition to 8–9 mol of the terminal sugars, 3 mol of 2-*O*-substituted Fuc (2-Fuc), less than 1 mol each of 4-*O*-substituted Gal (4-Gal), and 2,6-di-*O*-substituted Gal (2,6-Gal), and 2 mol of 2-*O*-substituted Man (2-Man) also corresponded to the IO_4^- -sensitive residues (Table II). By assuming that all GlcNAc residues in Gp-2 were conserved even after IO_4^- -treatment, the molecular contents of the other sugars in IO_4^- -oxidized Gp-2 were calculated to be as shown in Table II.

The 2-Fuc-containing structure is considered to be quite unusual in naturally occurring carbohydrate. However, as shown in Fig. 2, the component expected to be the 2-Fuc derivative (the peak component, at the retention time of 8.6 min in Table III) gave essentially the same EI-MS

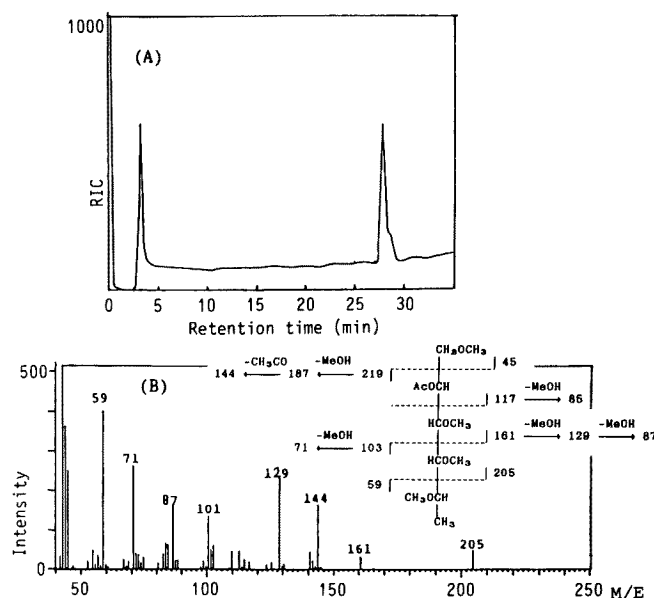


Fig. 5. CLC/EI-MS spectrum for the presumed 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-Fuc-ol derivative derived from permethylated Gp-2a-III-ol-c. Peracetylated derivatives of the acid-hydrolyzate of permethylated Gp-2a-III-ol-c gave two peaks in the GLC analysis at retention times of 3.4 and 27.8 min under the conditions that the temperature of the glass column packed with 2% OV-17/Gaschrom Q (2 mm i.d. $\times$ 100 cm) was kept at 140°C during the initial 4 min, and then elevated to 240°C at a rate of $2^\circ\text{C}/\text{min}$. Although the peak at 27.8 min was identified as 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxy-galactitol, the peak at 3.4 min was unidentified due to the lack of corresponding literature data. The component of the unidentified peak was therefore subjected to EI-MS analysis.

spectrum as that reported for 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-6-deoxyhexitol by Jansson *et al.* (14). The EI-MS signals at m/z 87, 89, 99, 115, 129, 131, and 189 are all characteristic of the 6-deoxyhexitol derivative, whereas the signal at m/z 117 characteristic to 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-6-deoxyhexitol and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-6-deoxyhexitol (14) was quite weak. Although the signals at m/z 129, 87, and 189 are also characteristic of 1,2,4-tri-*O*-acetyl-3,5-di-*O*-methylpentitol, the signals observed at m/z 89, 99, and 131 contradict the correspondence of the component to the 2-*O*-substituted pentitol derivative. Thus, the component was identified as 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylfucitol corresponding to 2-Fuc.

DI/EI-MS Analysis—As shown in Fig. 3, in the direct inlet DI/EI-MS spectrum for permethylated Gp-2, the signal at m/z 892 (dcbaA_1) correlates to a methylated terminal tetrasaccharide composed of 3 mol of *N*-acetylhexosamine (HexNAc) and 1 mol of methyl pentose (Me-Pen). m/z 679 (cbaA_1) and m/z 647 (cbaA_2) both correspond to a methylated terminal trisaccharide composed of 2 HexNAcs and 1 Me-Pen. The signals at m/z 505 (baA_1) and m/z 473 (baA_2) both correspond to a methylated terminal disaccharide composed of 2 mol of HexNAc. The signals at m/z 434 (baA_1) and 402 (baA_2) are both ascribed to a methylated terminal disaccharide composed of a HexNAc and a Me-Pen. The signals at m/z 260 (aA_1) and m/z 189 (aA_1) correspond to a methylated terminal HexNAc (t-HexNAc) and a methylated t-Fuc, respectively.

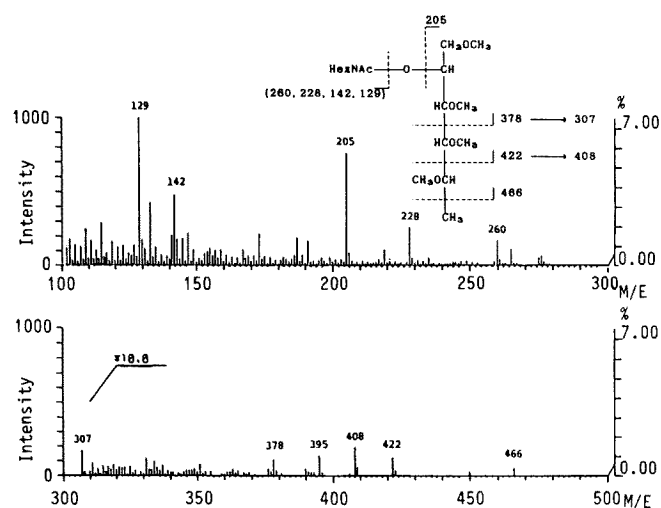


Fig. 6. DI/EI-MS spectrum for permethylated Gp-2a-III-ol-c that was presumed to be derived from one of the major non-reducing terminal regions of carbohydrate chain of Gp-2.

By referring to the results of the methylation/GLC analysis (Table III), we suggest that Gp-2 carbohydrate contains 3 mol each of the two types of non-reducing terminal disaccharide units, GalNAc1 $\rightarrow$ 2Fuc1 $\rightarrow$ and GalNAc1 $\rightarrow$ (3/4)HexNAc1 $\rightarrow$, as the major non-reducing terminals.

Fragments from Non-Reducing Terminals—To examine the structure of the non-reducing terminal region, 10 mg of Gp-2 was partially hydrolyzed in 20 mM H₂SO₄ at 100°C for 1.5 h and applied onto a column of Dowex 1 $\times$ 4. After washing the column with deionized water, the acid-treated product (Gp-2a) was eluted with 1 N HCl and freeze-dried. The freeze-dried Gp-2a was then chromatographed on a column of Sephadex G-25 into three fractions (I, II, and III) as shown in Fig. 4A.

The fractions covering the third peak (No. 52 to 64) were pooled and freeze-dried to yield 1.5 mg of Gp-2a-III as the smallest fragment. It contained approximately equimolar GalNAc and Fuc as examined by GLC analysis of its acid-hydrolyzate. About 35% of the GalNAc and 66% of the Fuc in Gp-2 were recovered into Gp-2a-III.

For structural analysis, Gp-2a-III was reduced with NaBH₄ to the corresponding alditol (Gp-2a-III-ol) and chromatographed on a column of ODS-AL-312 with deionized water into three fractions (peaks a, b, and c) as shown in Fig. 4B. Most of the carbohydrate was recovered in peak c (Gp-2a-III-ol-c), whereas no carbohydrate was detected in peak a, and only a trace amount of HexNAc alditol (HexNAc-ol) was detected in peak b by GLC analysis.

On methylation/GLC analysis, the peracetylated derivative of the hydrolyzate of permethylated Gp-2a-III-ol-c gave two signals with approximately equal peak area at retention times of 3.4 and 27.8 min. The second peak was identified as 1,5-di-*O*-acetyl-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxy-galactitol from the retention time and EI-MS spectrum (data not shown), and corresponded to t-GalNAc, whereas the first peak could not be identified as no literature data was available for the GLC behavior.

However, as shown in Fig. 5, most of the major signals in the EI-MS spectrum for the unidentified component corre-

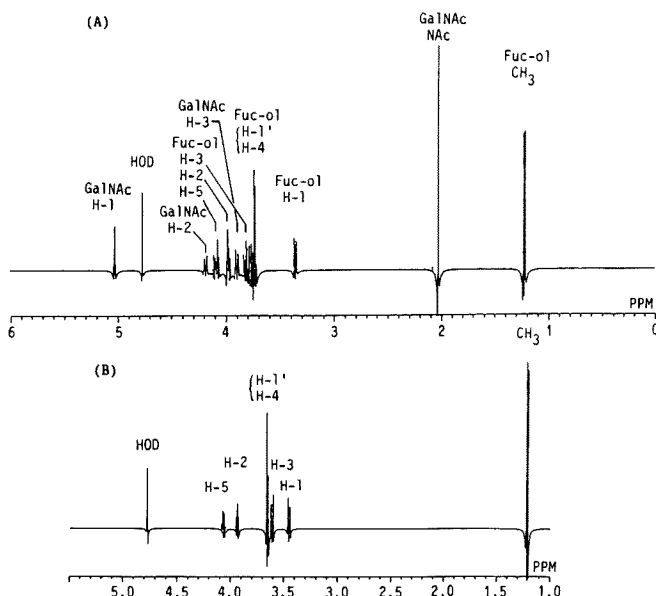


Fig. 7. ¹H-NMR spectra of Gp-2a-III-ol-c (A) and fucitol (B).

sponded to those for the proposed structure of 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-Fuc-ol and its possible fragments. This assignment was consistent with the results of methylation/GLC analysis, which indicated that Gp-2 and Gp-2a-III-ol-c contained 2-Fuc and 2-Fuc-ol, respectively (Table III). Thus Gp-2a-III-ol-c was identified as GalNAc1 $\rightarrow$ 2Fuc-ol.

DI/EI-MS analysis of permethylated Gp-2a-III-ol-c also supported this identification. As shown in Fig. 6, the characteristic DI/EI-MS signals at *m/z* 466 (M-15), 422 (M-58), and 378 (M-103) supported this disaccharide alditol structure. The signal at *m/z* 205 was apparently attributable to 2-Fuc-ol, and those at *m/z* 260 (aA₁), 228 (aA₂), 142 (F₁, G₁), and 129 (H₁) were all ascribed to t-GalNAc.

Gp-2a-III-ol-c was also subjected to NMR analysis to examine the anomeric configuration of t-GalNAc. As shown in Fig. 7A, the chemical shift of 5.021 ppm was ascribable to t-GalNAc residue. In conjunction with the data reported by Vliegthart *et al.* (15) and Feeney *et al.* (16), the coupling constant of 4.27 Hz observed in the signal indicated that the t-GalNAc was attached to 2-Fuc-ol through α -glycosidic linkage. Additional chemical shifts of 4.188, 3.902, and 2.023 ppm were also ascribed to H-2, H-3, and *N*-acetyl protons of t-GalNAc, respectively (16).

By comparing Fig. 7A with Fig. 7B for unsubstituted fucitol, the chemical shifts of 3.350, 4.068, 1.217 ppm were ascribable to H-1, H-5, and methyl protons of 2-Fuc-ol. The additional chemical shifts of 3.975 and 3.808 were also ascribed to H-2 and H-3 of 2-Fuc-ol, although the values are 0.06 and 0.20 ppm higher than the corresponding values of unsubstituted fucitol. The multiple signals observed around 3.7 ppm might contain the signals of H-1' and H-4 of 2-Fuc-ol. The structure of Gp-2a-III-ol-c was thus identified as GalNAc α 1 $\rightarrow$ 2Fuc-ol.

DISCUSSION

The results of the periodate oxidation experiments have reaffirmed previous suggestion that the allergenic and

antigenic activities of Gp-2 depend on certain structural units residing in its carbohydrate moiety (Table II), which has been presumed to link *N*-glycosidically to the peptide moiety (1).

Methylation/GLC analysis of the intact and IO_4^- -oxidized Gp-2 revealed that the Gp-2 carbohydrate chains were highly branched and contained 6 mol of GalNAc and fewer moles each of Fuc and Gal as non-reducing terminals (Table III). DI/EI-MS analysis of permethylated Gp-2 suggested that Gp-2 contained two types of disaccharide units at the non-reducing terminals, *i.e.*, GalNAc→Fuc→ and GalNAc→HexNAc→ (Fig. 3).

Although the internal Fuc-containing structure seemed quite unusual for a naturally occurring carbohydrate, a disaccharide fragment composed of equimolar GalNAc and Fuc, Gp-2a-III, was isolated from a partial acid-hydrolyzate of Gp-2 as one of the major fragments (Fig. 4A), and the fragment was reduced to the corresponding alditol, Gp-2a-III-ol-c (Fig. 4B).

GC-MS analysis detected 1,5-diacetyl-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-2-deoxygalactitol and the presumed 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-Fuc-ol in the peracetylated hydrolyzate of permethylated Gp-2a-III-ol-c as the peaks at GLC-retention times of 27.8 and 3.4 min, respectively. Although no literature data was available for the Fuc-ol derivative, most signals appeared in EI-MS spectrum (Fig. 5) for the component were consistent with the structure of 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-Fuc-ol and its fragments. Therefore, the Fuc-ol derivative was correlated to 2-Fuc-ol and Gp-2a-III-ol-c was identified as GalNAc1→2Fuc-ol. DI/EI-MS analysis of the permethylated disaccharide alditol also supported the structural correspondence of GalNAc1→2Fuc-ol to Gp-2a-III-ol-c (Fig. 6).

$^1\text{H-NMR}$ analysis revealed that the linkage of t-GalNAc to 2-Fuc-ol was in α -configuration (Fig. 7). Therefore, the 3 mol of 2-Fuc detected in methylation/GLC analysis (Table III) were considered to form 3 mol of GalNAc α 1→2Fuc1→ units at the non-reducing terminals. We have recently confirmed that Gp-2a-III-ol-c liberated GalNAc by the action of α -*N*-acetylgalactosaminidase (α -GalNAcase) purified from internal organs of sea-squirt, though the acidic glycopeptide, Gp-2, was still tolerant to the α -GalNAcase (unpublished data). The details will be reported elsewhere.

On the other hand, the other disaccharide unit of HexNAc→HexNAc→ was also detected in DI/EI-MS analysis of permethylated Gp-2 (Fig. 3). From the results of methylation/GLC analysis (Table III), the non-reducing terminal

HexNAc corresponded to t-GalNAc and the second HexNAc could be attributed to 3-GalNAc or 4-GlcNAc. Therefore, 3 mol of GalNAc1→(3/4)HexNAc1→ units were considered to form additional non-reducing terminals in Gp-2 saccharide.

The 3 mol each of GalNAc1→2Fuc1→ and GalNAc1→(3/4)HexNAc1→ are apparently IO_4^- -sensitive and the majority in the 8-9 mol of non-reducing terminals of Gp-2 saccharide. Accordingly, the two types of t-GalNAc-containing disaccharide units are both nominated as possible components of the epitopes specific to sea-squirt allergy in Gp-2 and its mother antigen, Gi-rep.

However, t-Fuc and t-Gal as the minor non-reducing terminals in Gp-2 are also IO_4^- -oxidation sensitive. Thus, not only the t-GalNAc-containing terminals but also the minor terminals should be further examined as possible components of the allergy-specific epitope because of the influence of the treatments with suitable glycosidases on the allergenic activity of Gp-2.

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