Study on Food Components: The Structure of *N*-Linked Asialo Carbohydrate from the Edible Bird's Nest Built by *Collocalia fuciphaga*

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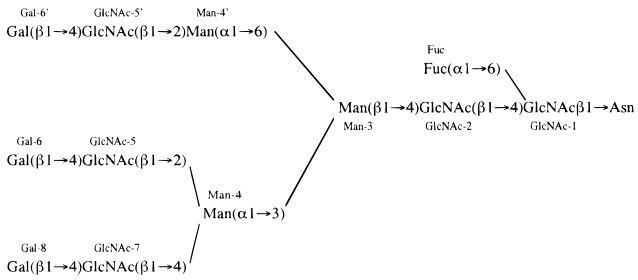
The *N*-linked asialo carbohydrate, released by digestion treatment of the glycoprotein of the edible bird's nest of *Collocalia fuciphaga*, was identified as L-asparagine-linked fucose-containing triantennary oligosaccharide, composed of Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3-(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcβ1, on the basis of chemical and spectroscopic methods, in addition to GLC and HPLC analyses on achiral columns.

Keywords: *Collocalia fuciphaga; edible bird's nest; nest-cementing substance; pronase digestion; desialylation; N-linked fucose-containing triantennary oligosaccharide*

INTRODUCTION

The Oriental swiftlet (*Collocalia* species) living in southeastern Asia and the islands of the Indian and Pacific Oceans is known to make an edible bird's nest. The nest-cementing substance of the swiftlet was found to originate predominantly from the salivary glands, which, under endocrine control, reach maximum secretory activity during the breeding and nesting season (Howe et al., 1961). The nest referred to as "Enso" is highly esteemed as a quasi-medicinal food (Medwey, 1969) and has a high reputation as one of the materials in high-class Chinese cuisine called "yànchá o". The chemical constituents of the nest-cementing substance of the swiftlet have been reported for the investigations of the biological activity of mucoid (Howe et al., 1961), the nature of protein (Wang, 1920), and the compositions of amino acids and carbohydrates (Kathan and Weeks, 1969; Houdret et al., 1975), in addition to the isolation (Pozsgay et al., 1987) and the quantitative estimation (Aminoff, 1961) of *N*-acetylneuraminic acids. Further, Hanisch and Uhlenbruck (1984) had reported that salivary sialomucins from the edible bird's nest of Chinese swallows contain both *N*- and *O*-glycosidically linked saccharides and elucidate the structures of *O*-glycosidically linked carbohydrate moieties on salivary glycoproteins. In the process of structural elucidation, they reported that the sialomucins of the edible bird's nest are characterized by the *O*-glycosidically linked saccharides, accompanied by *N*-glycosidically linked glycans, the contents of which are less than half of the total carbohydrate.

In connection with the study on food components, we also examined the carbohydrate and amino acid compositions of the mucoid obtained from the nest-cementing substance of *Collocalia fuciphaga* Apodidae ("Shokuyo-anatsubame" in Japanese) (Oda, 1975, 1983). Our further investigation of the nest-cementing substance has now isolated an L-asparagine-linked asialo carbohydrate unit, which was obtained from pronase digestion, followed by desialylation and gel filtration chromatographies, of the hot water extract of the nest. In this paper, we report the evidence of the structural elucidation of *N*-linked asialo carbohydrate with the configurational assignments of sugars and asparagine by a combination of chemical and spectroscopic methods together with GLC and HPLC analyses on achiral columns.



MATERIALS AND METHODS

Materials. The nest of *C. fuciphaga* was purchased in Malaysia in 1992. It was broken into pieces measuring 6.7−8.2 cm in width and 2.1−3.2 cm in depth and then dried in air.

Absorbance Determination. The absorbances for eluates in each gel filtration were monitored at both 490 nm for the carbohydrate moiety with the phenol−sulfuric acid method and 280 nm for the glycoprotein moiety on a Shimadzu UV-240 spectrophotometer. IR absorption bands were obtained on a JASCO A-100 spectrophotometer.

NMR Spectroscopy. NMR spectra were recorded on a JEOL GSX-500 spectrometer operating in the Fourier trans-

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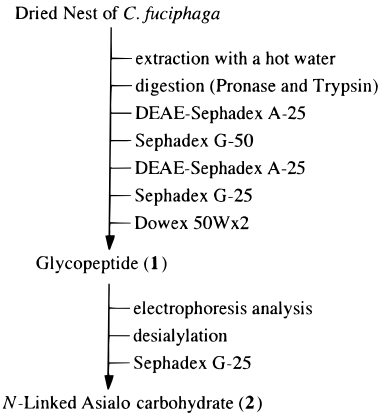

Figure 1. Scheme for the isolation of *N*-linked asialo carbohydrate **2**.

form mode. The measurements of ^{1}H and ^{13}C were performed at 500 and 125 MHz, respectively. Samples were dissolved in 99.8% D_2O, lyophilized, and dissolved again in 99.8% D_2O. ^{1}H NMR chemical shifts are expressed in parts per million downfield from internal DSS, measured by reference to internal acetone. The acetone in reference to internal DSS was determined to be 2.216 ppm in D_2O at 27 °C. *J* values are given in hertz. ^{13}C NMR chemical shifts are expressed from external tetramethylsilane. A spectral width of approximately 16 000 Hz was employed with the CHCOSY spectra acquired as 1024 data points in t_2 for 256 data points in t_1 increments and zero-filled to 2048 and 512 points in t_2 and t_1, respectively, prior to Fourier transformation. NOESY experiments were performed with mixing times of 0.75 s. Multiplicities of the ^{13}C signals were determined by DEPT experiments.

MS Spectrometry. FABMS were performed on a JEOL JMS−SX102A mass spectrometer with a mixture of glycerol and thioglycerol (1:1) as a matrix in the positive and negative modes. EIMS spectra were obtained with a Hitachi M80B spectrometer at 70 eV with an ion-source temperature of 200 °C.

GLC Analysis. GLC analysis of the methyl trifluoroacetyl derivatives of the sugars was carried out on a Shimadzu GC-4CM instrument with a flame ionization detector (FID) using a glass column (3 mm × 2 m) packed with 3% SE−30 on Chromosorb W (60−80 mesh) under the following conditions: carrier gas, N_2; temperature from 90 to 200 °C (programmed at 2 °C/min). GLC analyses of the TMS ethers of the MBA alditol derivatives of the sugars were run on a Shimadzu GC-14A instrument equipped with FID under the following conditions: 0.53 mm × 25 m fused-silica capillary column (HiCap CBP10−W25−100); carrier gas, He; split ratio, *ca.* 100:1; injection temperature, 260 °C; column temperatures, 170 °C for the TMS ethers of the MBA alditol derivative of fucose, 190 °C for the derivatives of mannose and galactose, and 245 °C for the derivative of *N*-acetylglucosamine, respectively.

HPLC Analysis. HPLC analysis of an enantiomeric aspartic acid was carried out on a Shimadzu LC-9A instrument equipped with a Shimadzu SPD-6A UV spectrophotometric detector under the following conditions: 3.9 × 150 mm packed column (Waters, Opti Pak CE); wavelength, 200 nm; eluent, aqueous $HClO_4$ (pH 1); flow rate, 0.2 mL/min; temperature, 18 °C.

RESULTS

The present method for isolation of *N*-linked asialo carbohydrate (**2**) from the dried nest of *C. fuciphaga* is represented schematically in Figure 1.

Isolation of the Glycopeptide 1. The dried nest of *C. fuciphaga* was ground in a mortar to give a slightly brownish powder (15 g). This powder was immersed in distilled water (1.5 L) overnight and then heated at 80 °C for 5 h, followed by filtration with a duplicate thin cloth to give a hot water soluble fraction. This fraction was filtered through a nylon cloth (No. 160) overnight at room temperature. The filtrate obtained was lyophilized to give a white powder (6.1 g), which was suspended in distilled water. The suspension was subjected to digestion with 2% pronase P (Kaken Kagaku Co. Ltd.) in 0.1 M borate buffer solution (pH 8.0) for 3 days at 39 °C, and then 1% trypsin (Sigma) was added to the solution, followed by further digestion for 24 h. The digested solution was transferred into a cellulose tube (tubing size, 27/32; Sanko Junyaku Co. Ltd.) for dialysis against distilled water. The dialysis was continued for 24 h at room temperature. The solution inside the tube was lyophilized to give a white powder (2.8 g). An aliquot (825 mg) of the white powder was dissolved in distilled water (16 mL) and then applied to a DEAE−Sephadex A-25 (OH^-) (Pharmacia Fine Chemicals AB Uppsala Sweden) column (1.8 × 40 cm). The elution was performed successively with distilled water (100 mL), 0.05 M NaCl (100 mL), 0.13 M NaCl (200 mL), 0.22 M NaCl (210 mL), and 0.3 M NaCl (100 mL) each at a flow rate of 5 mL/30 min, monitoring an aliquot (0.1 mL) in each 5 mL of effluents at wavelengths of 280 and 490 nm. The eluate fractions with 0.13 M NaCl gave absorption maxima at both wavelengths. The fractions were pooled and dialyzed for 12 h in a cellulose tube (tubing size, 30/32), followed by lyophilization to give a white solid (579 mg). The solid was dissolved in 0.15 M KCl (35 mL) and then applied to a Sephadex G-50 (fine) (Pharmacia Fine Chemicals Co. Ltd.) column (2.6 × 100 cm) with 0.15 M KCl as the eluente at a flow rate of 5 mL/25 min. The fractions with absorption maxima at both wavelengths were collected, dialyzed, and lyophilized to give a white solid (282 mg). The solid, after dissolution in distilled water (5.5 mL), was applied to a DEAE−Sephadex A-25 (OH^-) column (1.8 × 40 cm) successively with distilled water (125 mL), 0.05 M NaCl (90 mL), 0.1 M NaCl (100 mL), 0.22 M NaCl (200 mL), and 0.3 M NaCl (90 mL) as the eluents each at a flow rate of 5 mL/35 min. Absorption maxima at both wavelengths were observed in the eluate fractions with 0.22 M NaCl. The fractions were collected and then successively reapplied to a Sephadex G-25 (fine) (Pharmacia Fine Chemicals Co. Ltd.) column (2.6 × 90 cm) with distilled water as the eluent at a flow rate of 5 mL/30 min and then a Dowex 50Wx2 (200−400 mesh; pyridine form) (Dow Chemicals Co. Ltd.) column (1.8 × 41 cm) with 0.001 M pyridine−formate buffer solution (pH 3.0) as the eluent at a flow rate of 1 mL/40 min, monitoring at both wavelengths of 280 and 490 nm, respectively. The eluates with absorption maxima at both wavelengths were collected, dialyzed, and lyophilized to give a glycopeptide (**1**) as a white powder (141 mg).

The white powder was subjected to an electrophoresis analysis performed on a Zyoko System Type 238 electrophoresis instrument with a cellulose acetate strip (Separax, Fuji Film Co. Ltd.) in 0.1 M barium acetate for 2 h at pH 5.6 using a potential gradient of 0.5 mA/cm. Glycopeptide **1** gave one spot, which was stained with 0.5% toluidine blue reagent.

Identification of the Sugars in 1. The methanolysis of **1** (5 mg) was carried out with 5% HCl−MeOH (1 mL) at 85 °C for 24 h under nitrogen atmosphere. The reaction mixture was neutralized with aqueous Ag_2CO_3. After centrifuging, the supernatant was evaporated

Table 1. ^{1}H NMR Chemical Shifts (δ, D_2O): Assignments for *N*-Asialo Carbohydrate 2

1.16 (3H, d, $J = 9$ Hz)	Fuc C_6-H_3
2.04 (6H, s)	$-COCH_3 \times 2$
2.07 (9H, s)	$-COCH_3 \times 3$
2.70 (2H, m)	β-methylene protons in Asn
3.68−3.71 (5H, m)	GlcNAc $C_4-H \times 5$
3.98 (1H, t-like)	α-methine proton in Asn
4.10 (1H, br)	Fuc C_5-H
4.46 (3H, each d, $J = 7.5$ Hz)	Gal anomeric $H \times 3$
4.54 (1H, d, $J = 8.5$ Hz)	GlcNAc-5 anomeric *H*
4.56 (1H, d, $J = 8.5$ Hz)	GlcNAc-5′ anomeric *H*
4.57 (1H, d, $J = 8.5$ Hz)	GlcNAc-7 anomeric *H*
4.64 (1H, d, $J = 7.5$ Hz)	GlcNAc-2 anomeric *H*
4.74 (1H, s)	Man-3 anomeric *H*
4.83 (1H, d, $J = 4.0$ Hz)	Fuc anomeric *H*
4.91 (1H, s)	Man-4′ anomeric *H*
5.06 (1H, d, $J = 10$ Hz)	GlcNAc-1 anomeric *H*
5.11 (1H, s)	Man-4 anomeric *H*

Table 2. ^{13}C NMR Chemical Shifts (δ, D_2O): Assignments for *N*-Asialo Carbohydrate 2

15.2	Fuc *C*-6
21.9, 22.0, 22.1, and 22.2 × 2	$-COCH_3 \times 5$
54.6, 54.7, 54.8, and 54.9 × 2	GlcNAc *C*-2 × 5
59.8	GlcNAc-2,-5,-5′,-7 *C*-6
60.8	Gal *C*-6 × 3
60.2, 61.0, and 61.4	Man-3,-4,-4′ *C*-6
65.5	GlcNAc-1 *C*-6
67.2	Fuc *C*-5
68.0	Fuc *C*-4
68.3	Gal *C*-2 × 3 and *C*-4 × 3
69.3	Fuc *C*-2 and *C*-3
69.9	Man-3, *C*-2
72.3	Gal *C*-3 × 3
75.1	Gal *C*-5 × 3
75.8	Man-4 *C*-2
76.2	Man-4′ *C*-2
78.0	GlcNAc *C*-4 × 5
96.9	GlcNAc-1 anomeric *C* and Man-4′ anomeric *C*
99.1	Fuc anomeric *C*
99.2	Man-4 anomeric *C*
100.2	Man-3 anomeric *C*
100.2, 100.8, and 101.3 × 2	GlcNAc-2,-5,-5′,-7 anomeric *C*
102.7	Gal anomeric *C* × 3
174.3 and 174.4	>*C*=O

under vacuum, coevaporated with MeOH (1 mL), and then dried over P_2O_5 in a vacuum desiccator overnight to give a dried material. Following the method by Arakawa et al. (1976), the dried material was trifluoroacetylated with trifluoroacetic anhydride (TFAA) (0.1 mL) in EtOAc (0.1 mL) at room temperature for 2 h under occasional shaking. The reaction mixture was injected directly into a gas chromatograph. Each peak on the gas chromatogram was identified as the methyl trifluoroacetyl derivatives of fucose, mannose, galactose, *N*-acetylglucosamine, and sialic acid, respectively, by co-GLC with the corresponding methyl trifluoroacetyl derivatives prepared from authentic sugars.

Desialylation of 1. A mixture of **1** (59 mg) dissolved in 0.05 M H_2SO_4 (120 mL) was heated at 80 °C for 1 h. The reaction mixture was neutralized with aqueous NaOH, followed by dialysis in a cellulose tube against distilled water for 24 h at room temperature. The outer liquid was lyophilized and then extracted with MeOH. The MeOH extract showed the presence of sodium salt of sialic acid, which was identified by the thiobarbituric acid reaction (Warren, 1959), the flame reaction for sodium element, and a cellulose TLC (Merck 5716) analysis. Co-cellulose TLC of the MeOH extract with an authentic sodium *N*-acetylneuraminate was carried out using a developing solvent mixture of EtOH−AcOH−H_2O (7:2:2 v/v/v), and each spot was visualized at R_f 0.42 by spraying with *p*-(dimethylamino)benzaldehyde (DMAB)−trichloroacetic acid reagent (Reissig et al., 1955). On the other hand, the inner liquid containing a desialylated glycopeptide was lyophilized and then dissolved in a small amount of distilled water. The solution was applied to a Sephadex G-25 column (1.8 × 77 cm) with distilled water as the eluent at a flow rate of 5 mL/40 min, monitoring at both wavelengths of 280 and 490 nm at room temperature. The absorption maxima fractions at both wavelengths were collected and lyophilized to give a desialylated glycopeptide **2** (24.6 mg). FABMS: *m/z* 2266 $[M + H]^+$ and *m/z* 2288 $[M + Na]^+$ in the positive ion FABMS and *m/z* 2264 $[M - H]^-$ in the negative ion FABMS. IR: ν_{max} (Nujol, cm^{-1}) 3305 (OH). The assignments of the ^{1}H and ^{13}C NMR signals were performed on the basis of DEPT, CHCOSY, and NOESY spectral data. ^{1}H and ^{13}C NMR data for **2** are represented in Tables 1 and 2, respectively.

Identification of the Enantiomeric Sugars in 2. Following the method by Oshima et al. (1983), the enantiomeric sugars in **2** were identified by GLC analysis of their corresponding TMS ethers of the MBA alditols. **2** (4 mg) was hydrolyzed with a solution of 3.5% HCl/50% dioxane (1 mL) at 80 °C for 3 h, followed by neutralization with Amberlite MB-3 (H^+ and OH^- forms) and evaporated to give a hydrolysate as a syrup (2 mg). A mixture of a solution of the syrup in distilled water (0.1 mL) and a solution of L-(−)-α-methylbenzylamine (14 mg) and $NaBH_3CN$ (0.8 mg) in EtOH (0.1 mL) was kept at 40 °C for 3 h. Several drops of AcOH were added to the reaction mixture. The mixture was evaporated and further coevaporated with MeOH (1 mL). The oily residue obtained was dried over P_2O_5 in a vacuum desiccator overnight and then mixed with dry CH_3CN (0.2 mL) and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) (0.05 mL). After the mixture stood for 15 min at room temperature in a stoppered tube, a small amount of hexane was added to the reaction mixture to extract the TMS ethers of the MBA alditols. The hexane extract was subjected to GLC analyses, and each peak on the gas chromatograms was identified as the TMS ethers of the MBA alditols of L-fucose, D-mannose, D-galactose, and *N*-acetyl-D-glucosamine, respectively, by co-GLC with the TMS ethers of the MBA-alditols derived from authentic enantiomeric sugars.

Identification of Amino Acid 2. A mixture of **2** (0.8 mg) and 6 M HCl (1.0 mL) was heated at 110 °C for 18 h in an evacuated glass tube. The reaction mixture was subjected to TLC (Merck GF_{254}, 0.25 mm thickness) using two different developing solvent systems: *n*-BuOH−AcOH−H_2O (4:1:2) and pyridine−acetone−NH_4OH−H_2O (10:6:1:4). After the mixture was air-dried, each spot was visualized at R_f 0.17 and R_f 0.60, respectively, on each plate by spraying with the ninhydrin reagent and heating on a hot plate. Each spot was identified as aspartic acid by co-TLC with an authentic specimen under the same conditions as described above. Further, the reaction mixture was evaporated to dryness under reduced pressure and then dissolved in 0.1 M HCl. This solution was subjected to HPLC analysis. Aspartic acid was identified as the L form by co-HPLC analysis with the authentic D and L forms of aspartic acid on a chiral column. It is apparent that L-aspartic acid was derived from L-asparagine by acid hydrolysis, as is well-known. These findings indicated the presence of L-asparagine (Asn) in the amino acid moiety of **2**.

Table 3. EIMS Fragmentation Patterns of Seven Acetyl Derivatives of the Partially Methylated Alditols

acetylated and methylated alditols	*m/z* (% relative abundance)[a]
1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol	175 (6.8), 131 (25), 117 (63), 115 (73), 101 (100), 89 (16), 72 (44), 59 (14)
1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol	323 (0.2), 205 (70), 173 (6.8), 161 (82), 145 (88), 129 (90), 117 (97), 113 (43), 101 (100), 99 (21), 87 (77), 71 (63), 59 (7.9)
1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol	189 (9.0), 161 (19), 145 (3.4), 129 (100), 113 (4.6), 101 (19), 99 (17), 87 (38), 71 (14), 59 (1.2)
1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol	233 (9.0), 189 (24), 129 (100), 113 (32), 99 (25), 87 (57), 71 (11), 60 (0.2), 59 (0.3)
1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol	233 (4.6), 201 (1.2), 189 (14), 173 (1.9), 139 (9.0), 129 (61), 117 (100), 101 (11), 99 (9.0), 87 (32), 71 (5.6), 58 (0.1)
1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol	391 (2.4), 233 (56), 202 (13), 173 (4.4), 170 (11), 158 (96), 142 (49), 131 (13), 129 (31), 125 (13), 116 (100), 98 (47), 87 (42), 74 (72)
1,4,5,6-tetra-*O*-acetyl-3-mono-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol	261 (8.0), 202 (2.0), 158 (60), 142 (16), 124 (8.9), 116 (100), 98 (8.9), 87 (6.7), 86 (3.3), 74 (36)

[a] Relative abundance was calculated over the fragment of *m/z* 50.

Permethylation of 2. Permethylation of **2** was carried out following the method developed by Hakomori (1964). A solution of **2** (8.0 mg) in DMSO (0.5 mL) was added to a homogenized mixture of NaH (35 mg) and DMSO (0.7 mL). The reaction mixture was stirring overnight at room temperature under nitrogen atmosphere. An excess of CH_3I (0.5 mL) was added to the reaction mixture with homogenizing by ultrasonication for 30 min, and the solution was left standing for 8 h. After removal of the solvent by evaporation under vacuum, the reaction mixture was subjected to preparative TLC (Merck GF_{254}, 0.50 mm thickness) with $CHCl_3$−MeOH (9:1) to give the permethylated product **3** (10 mg); the IR absorption band due to a hydroxyl group at 3305 cm^{-1} observed in the case of **2** disappeared.

EIMS Analysis of the Hydrolysate of 3. The permethylate **3** was hydrolyzed with 25% trifluoroacetic acid (TFA) (0.5 mL) for 2 h at 110 °C under nitrogen atmosphere. After concentration of the solvent in vacuo, $NaBH_4$ (10 mg) was added to a solution of the hydrolysate dissolved in distilled water (1.0 mL) and then the mixture was stirred overnight at room temperature. The reaction mixture was, after an addition of a few drops of AcOH and then MeOH, evaporated under reduced pressure to dryness to give a reduction product. The reduction product was acetylated with acetic anhydride and dry pyridine for 4 h at 55 °C to give a mixture of partially methylated acetates, which was subjected to GC−MS analysis to obtain EIMS spectra of each peak on a gas chromatogram. The gas chromatogram exhibited the presence of seven acetyl derivatives of partially methylated alditols with the relative intensity ratios 1:3:1:1:1:4:1. The EIMS fragmentation patterns of these seven acetyl derivatives of partially methylated alditols are summarized in Table 3. On the basis of the EIMS fragmentation patterns of the authentic specimens illustrated in the literature (Jansson et al., 1976; Tai et al., 1975), these seven acetylated and methylated alditols are identified as 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol, 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol, 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol, 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol, 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol, 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol, and 1,4,5,6-tetra-*O*-acetyl-3-mono-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol, respectively.

These findings indicated that the asialo carbohydrate moiety of **2** is composed of three terminal galactose moieties (Gal-6,-6′,-8), one terminal fucose moiety (Fuc), four 1,4-disubstituted GlcNAc moieties (GlcNAc-2,-5,-5′,-7), one 1,2-disubstituted mannose moiety (Man-4′), one 1,2,4-trisubstituted mannose moiety (Man-4), one 1,3,6-trisubstituted mannose moiety (Man-3), and one 1,4,6-trisubstituted GlcNAc moiety (GlcNAc-1).

DISCUSSION

Compositions of the Sugar and Amino Acid Moieties in 2. The molecular weight of **2** was found to be 2265 on the basis of the appearance of the ion peaks at *m/z* 2266 $[M + H]^+$ and *m/z* 2288 $[M + Na]^+$ in the positive ion FABMS and the ion peak at *m/z* 2264 $[M - H]^-$ in the negative ion FABMS. The ^1H and ^{13}C NMR spectral data of **2**, as given in Tables 1 and 2, indicated its asialo carbohydrate moiety to be composed of three units of β-galactopyranose, five units of 2-acetamide-2-deoxy-β-glucopyranose, two units of α-mannopyranose, one unit of β-mannopyranose, and one unit of α-fucopyranose. This indication was confirmed both by the interpretation of the CHCOSY spectra and by the similarity of the chemical shifts and coupling constants of 12 anomeric protons with the intensity ratios 3:1:1:1:1:1:1:1:1:1:1 from 4.46 to 5.11 ppm with those of glycopeptide (class B: GPII-5) derived from human plasma α_1-acid glycoprotein (Fournet et al., 1978), $Man_3GlcNAc_2$−Asn isolated from riboflavin-binding protein (Japanese quail egg white) by means of pronase digestion (Koyama et al., 1986), glycopeptides (E3, D3 C3B, and A3) prepared from hen ovalbumin (Carver et al., 1981), and Fucα1→6GlcNAcβ1→Asn isolated from urine of a patient with fucosidosis (Dorland et al., 1977).

The configurations of sugars in the asialo carbohydrate moiety of **2** were confirmed by GLC separation of the sugar enantiomers as diastereoisomeric MBA alditols on an achiral fused-silica capillary column. **2** was hydrolyzed with hydrochloric acid to give a mixed sugar portion. Following the method by Oshima et al. (1983), the sugar portion was converted to the TMS ethers of the MBA alditols and then subjected to GLC and co-GLC analyses with the TMS ethers of the MBA alditols derived from authentic enantiomeric sugars. On the basis of these GLC and co-GLC analyses, the sugar portion of **2** was found to be composed of L-fucose, D-mannose, D-galactose, and 2-acetamide-2-deoxy-D-glucopyranose.

On the other hand, HPLC and co-HPLC analyses of the acid hydrolysate of **2** showed the presence of L-aspartic acid on a chiral column in comparison with the authentic D and L forms of aspartic acid. Thus, the amino acid moiety of **2** was found to be composed of one unit of L-asparagine on the basis of both the result of the HPLC analysis of the acid hydrolysate of **2** and the estimation of the molecular weight of **2**.

These findings indicated clearly that **2** possesses one unit of L-asparagine linking to an oligosaccharide composed of three units of β-D-galactopyranose (Gal-6,-6′,-8), five units of 2-acetamide-2-deoxy-β-D-glucopyranose (GLcNAc-1,-2,-5,-5′,-7), two units of α-D-mannopyranose (Man-4 and Man-4′), one unit of β-D-mannopyranose (Man-3), and one unit of α-L-fucopyranose (Fuc).

Glycosidic Linkage Positions and Sequence of the Carbohydrate Moiety to Asn. The glycosidic linkage positions and sequence of 12 sugars in the asialo carbohydrate moiety of **2** were determined by the EIMS fragmentation patterns of 7 acetyl derivatives of the partially methylated alditols with the relative intensity ratios 1:3:1:1:1:4:1 on the gas chromatogram, together with the coincidence of the chemical shifts and coupling constants of the anomeric protons in the ^{1}H NMR spectrum of **2** with those of the literature data (Fournet et al., 1978; Koyama et al., 1986; Carver et al., 1981; Dorland et al., 1977). The presence of three terminal Gal-6,-6′,-8 [δ 4.46 (3H, each d, J = 7.5 Hz, 3 C_1-H)] was supported by the EIMS fragments due to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol obtained from reduced 1-monosubstituted Gal-6,-6′,-8. Each C-1 of Gal-6,-6′,-8 was found to link to the corresponding C-4 of GlcNAc-5,-5′,-7 on the basis of the appearance of the EIMS fragments of 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol obtained from reduced 1,4-disubstituted GlcNAc-5,-5′,-7. The anomeric proton signal at d 4.56 (1H, d, J = 8.5 Hz, C_1-H) of GlcNAc-5′ indicated that its C-1 links to C-2 of Man-4′. This indication was supported by the appearance of the EIMS fragments due to 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylhexitol obtained from reduced 1,2-disubstituted Man-4′. On the other hand, the appearance of anomeric proton signals at δ 4.54 (1H, d, J = 8.5 Hz, C_1-H) and δ 4.57 (1H, d, J = 8.5 Hz, C_1-H) assignable to those of GlcNAc-5 and GlcNAc-7 suggested that each C-1 of GlcNAc-5 and GlcNAc-7 links to C-4 and C-2 of Man-4, respectively. These suggestions were supported by the appearance of the EIMS fragments due to 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol obtained from reduced 1,2,4-trisubstituted Man-4. Further, it was proved that each C-1 of Man-4′ [δ 4.91 (1H, s, C_1-H)] and Man-4 [δ 5.11 (1H, s, C_1-H)] links to C-6 and C-3 of Man-3, respectively, on the basis of the appearance of the EIMS fragments corresponding to 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methylhexitol obtained from reduced 1,3,6-trisubstituted Man-3. These findings indicated clearly that the carbohydrate moiety of **2** possesses two oligosaccharide chains composed of Galβ1→4GlcNAcβ1→2Manα1 and Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1 linking to C-6 and C-3 of an inner branching Man-3, respectively.

Further, the glycosidic linkage positions and sequence among Man-3, GlcNAc-1, GlcNAc-2, and Fuc and the linkage mode of the sugar chain to the Asn moiety were elucidated by the EIMS fragmentation patterns of three acetylated and methylated alditols obtained from reduced Fuc, GlcNAc-2, and GlcNAc-1, together with the NMR spectroscopic studies of **2**. The appearance of the EIMS fragments due to 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methylhexitol, 1,4,5-tri-*O*-acetyl-3,6-di-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol, and 1,4,5,6-tetra-*O*-acetyl-3-mono-*O*-methyl-2-*N*-methylacetamide-2-deoxyhexitol suggested the presence of 1-monosubstituted Fuc, 1,4-disubstituted GlcNAc-2, and 1,4,6-trisubstituted GlcNAc-1, respectively. These suggestions were confirmed both by the coincidence of the chemical shifts and coupling constants of anomeric protons of Man-3 [δ 4.74 (1H, s, C_1-H)], GlcNAc-2 [δ 4.64 (1H, d, J = 7.5 Hz, C_1-H)], GlcNAc-1 [δ 5.06 (1H, d, J = 10 Hz, C_1-H)], and Fuc [4.83 (1H, d, J = 4.0 Hz, C_1-H)] with those of the literature data (Fournet et al., 1978; Koyama et al., 1986; Carver et al., 1981; Dorland et al., 1977; Carver and Grey, 1981) and by the interpretation of the CHCOSY spectra.

The ^{1}H NMR spectrum of **2** further exhibited the presence of three proton signals at δ 2.70 (2H, m) and δ 3.98 (1H, t-like), which were assignable to the β-methylene protons and α-methine proton, respectively, in the Asn moiety. The exhibition suggested that C-1 of GlcNAc-1 links to the *N*-4-L-aspartyl residue of the Asn moiety. The *N*-linkage mode was finally confirmed by direct comparisons of the ^{1}H NMR spectrum and its spectral data with those of 2-acetamido-6-*O*-(α-L-fucopyranosyl)-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine (Dorland et al., 1977). The comparisons were simultaneously indicative of the linkage of C-1 of Fuc to C-6 of GlcNAc-1. It is well-known that fucosyl residues are in two different linkages in Asn-linked glycopeptides, α(1,6)-linked to the Asn−GlcNAc of the core and α(1,3)-linked to an arm GlcNAc, and that the mean chemical shift values are 4.783 ± 0.007 (C_1-H), 4.128 ± 0.004 (C_5-H), and 1.203 ± 0.006 (C_6-H_3) ppm in the case of the α(1,6)-linked fucose, while the mean chemical shift values are 5.111 ± 0.004 (C_1-H), 4.829 ± 0.011 (C_5-H), and 1.174 ± 0.004 (C_6-H_3) ppm in the case of the α(1,3)-linked fucose (Carver and Grey, 1981). Our results for the chemical shift values of C_1-H [4.83 (1H, d, J = 4.0 Hz)], C_5-H [δ 4.10 (1H, br)], and C_6-H_3 [δ 1.16 (3H, d, J = 9 Hz)] were in fair agreement with those in the case of the α(1,6)-linked fucose to the GlcNAc−Asn of the core. These findings indicated clearly that **2** possesses the core structure of an asparagine-linked fucose-containing chain composed of Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcβ1→Asn. Thus, **2** was found to possess the asparagine-linked fucose-containing triantennary oligosaccharide structure at C-1, C-3, and C-6 of the inner branching Man-3.

Conclusion. Consequently, the structure of **2** was identified as L-asparagine-linked fucose-containing triantennary oligosaccharide, composed of Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAcβ1.

Hanisch and Uhlenbruck (1984) elucidated the structures of *O*-glycosidically linked oligosaccharides, as major components, on salivary glycoproteins from the edible bird's nest of Chinese swallows and indicated the presence of *N*-glycosidically linked glycans (molar ratio Man/GlcNAc/Gal, 3:4:8) as minor components. So far, the *N*-linked triantennary oligosaccharides have been found widespread as the fundamental structures of the asialo carbohydrate units derived from human plasma α_1-acid glycoprotein (Fournet et al., 1978), the glyco-

peptides from riboflavin-binding protein (Koyama et al., 1986), the glycopeptides from hen ovalbumin (Carver et al., 1981), and the glycopeptides from fetal calf fetuin (Takasaki and Kobata, 1986) and bovine fetuin (Townsend et al., 1986). In addition, the *N*-linked fucose-containing triantennary structure of **2** is also well-known as one of the primary structures of the unit B-type oligosaccharides of porcine thyroglobulin (Yamamoto et al., 1981) and the oligosaccharides of urinary and recombinant erythropoietins (Tsuda et al., 1988).

Although the molar ratio and composition of sugars in **2** are different from those proposed by Hanisch and Uhlenbruck (1984), it is interesting that the occurrence of such a *N*-linked fucose-containing triantennary oligosaccharide was demonstrated in the nest-cementing substance of the edible bird's nest, highly esteemed as a quasi-medicinal food and reputed as one of the materials in high-class Chinese cuisine.

ABBREVIATIONS USED

Fuc, L-fucopyranose; Gal, D-galactopyranose; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; Man, D-mannopyranose; Asn, L-asparagine; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; TMS ethers, trimethylsilyl ethers; MBA alditols, 1-(L-α-methylbenzylamino)-1-deoxyalditols; CHCOSY, $^{13}C-^{1}H$ correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; DEPT, distortionless enhancement by polarization transfer; FABMS, fast atom bombardment-mass spectrometry; EIMS, electron impact-mass spectrometry. Abbreviations for $Man_3GlcNAc_2-Asn$ (Koyama et al., 1986) and Fucα1→6GlcNAcβ1→Asn (Dorland et al., 1977) cited in this paper were quoted as they are in the literature.

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