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Characterization of four monosialo and a novel disialo Asn N-glycosides from the urine of a patient with aspartylglycosaminuria

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We previously reported for the first time two Japanese patients with aspartylglycosaminuria (AGU). A novel disialo Asn N-glycoside (AG-5) has been isolated from the urine of one of the patients in addition to four known monosialo Asn N-glycosides (AG-1 to AG-4) by gel filtration and anion exchange chromatography in this study. Final purification of AG-5 was achieved by an electrochemical chromatographic method, high performance liquid chromatography with pulsed amperometric detector (HPLC-PAD). The yield of AG-5 was approximately 1 mg l^{-1} urine. The chemical structures of AG-1 to AG-5 were characterized by gas-liquid chromatography, a permethylation study, fast atom bombardment-mass spectrometry (FAB-MS), and nuclear magnetic resonance (NMR). Based on the structural analysis, AG-5 had the following novel structure: NeuAcα2 → 8NeuAcα2 → 3Galβ1 → 4GlcNAcβ1 → Asn.

Keywords: glycoprotein, aspartylglycosaminuria, pulsed amperometric detector

Introduction

Aspartyglycosaminuria (AGU) is a rare inherited disorder of glycoprotein metabolism caused by the defect of the lysosomal enzyme, 1-aspartamido-β-*N*-acetyglucosamineamidohydrolase (aspartyglycosaminidase, AGA, EC3.5.1.26) [1, 2]. Clinically, AGU is characterized by normal development, usually up to age 2– 4 years, followed by a slow regression of motor and intellectual skills. In most cases, the patients show severe mental retardation accompanied by a mild mucopolysaccharidosis-like appearance and coarse facial features (Gargoyle-like face). Most AGU cases are derived from Finnish descent. Recently we diagnosed two Japanese siblings as AGU [3]. These AGU patients showed decreased AGA activity in transformed lymphoblasts and the clinical findings are very similar to those described in classical AGU. However, our patients had the following unique symptoms: myoclonic seizures, angiokeratoma, and longer survival than the cases reported previously [4].

It is very important to detect and characterize accumulated substances in urine in order to diagnose patients with lysosomal storage disorders. In the case of AGU, several unusual Asn N-glycosides including aspartylglucosamine (GlcNAcβ1 → Asn) were shown to be excreted in the urine of the patients [5–10]. The neutral Asn N-glycosides were composed of galactose and mannose attached to GlcNAcβ1 → Asn and the acidic Asn N-glycosides contained *N*-acetylneuraminic acid attached to galactose at the non-reducing terminal of the neutral Asn N-glycosides. Interestingly, they did not have the common core trisaccharide structure found in N-linked type glycoprotein, → Manβ1→ 4 GlcNAcβ1 → 4 GlcNAcβ1 → Asn.

Recently, a new electrochemical method [a chromatographic system consisting of a pulsed amperometric detector (PAD)] has been successfully used for pmol detection of oligosaccharides and glycoproteins [11–14]. By combination of high performance liquid chromatography equipped with

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PAD (HPLC-PAD), oligosaccharides can be separated on the basis of differences in the linkage position of *N*-acetylneuraminic acid. In the present study, we purified five major Asn N-glycosides containing *N*-acetylneuraminic acid from the urine of the AGU Japanese patient using HPLC-PAD. Structural analysis showed that one of them was a novel Asn-N-glycoside containing two residues of *N*-acetylneuraminic acid.

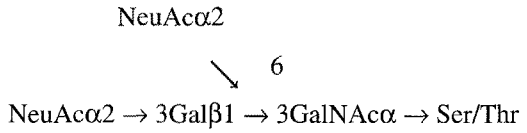
Materials and methods

Urine

Urine from an AGU patient (a 41-year-old Japanese woman) was stored at –20°C before analysis. The clinical features have been reported [3].

Standard compound

Standard oligosaccharide GP-D2 was isolated from the urine of a patient with Kanzaki disease with an α-*N*-acetylgalactosaminidase deficiency [15], as described previously [16]. GP-D2 has the following structure:



High performance thin layer chromatography (HPTLC)

Asn N-glycosides were analysed by HPTLC on precoated silica-gel high performance plates (E. Merck, Darmastadt, FRG). The solvent systems were *n*-propanol:15 M NH_4OH:12 mM $MgCl_2$ (6:1:2, by vol) (solvent A) and *n*-butanol:acetic acid:12 mM $MgCl_2$ (2:1:1, by vol) (solvent B). The spots were detected by resorcinol-HCl reagents.

Purification of urinary Asn N-glycosides from an AGU patient

On litre of urine was lyophilized to dryness. The lyophilized urine was dissolved in 70 ml of distilled water and centrifuged at 10 000 rpm for 10 min to remove water-insoluble compounds. The water extract was applied to a Bio-gel P-4 column (5 cm × 75 cm) (Bio-Rad Lab., Richmond, CA) and eluted with distilled water. Fractions of 20 ml were collected and analysed by HPTLC. The fractions containing sialylated oligosaccharides were pooled and lyophilized. The lyophilized powder (approximately 1.0 g) was dissolved in 20 ml of distilled water and applied to a Dowex 1-X2 (acetate form, 400 mesh) column (2 cm × 60 cm) which had been equilibrated with distilled water. The acidic oligosaccharides were separated by linear gradient elution of water to 800 mM pyridine/acetic acid buffer, pH 5.0. Fractions of 15 ml were collected and analysed by HPTLC. From the result of HPTLC analysis (Fig. 1), the fractions were pooled into 21 fractions and lyophilized. Five major compounds should be detected in Fr. 3 (AG-3 and AG-4), Fr. 4 (AG-1 and AG-2), and Fr. 11 (AG-5). The powder of each fraction was dissolved in 1 ml of distilled water, and the pH of the solution was adjusted to 7.0 with 0.1 N NaOH solution. Then, each fraction was applied to a Bio-gel P-4 column (1 cm × 120 cm) which had been previously equilibrated with distilled water. Fractions of 1 ml were collected and analysed by

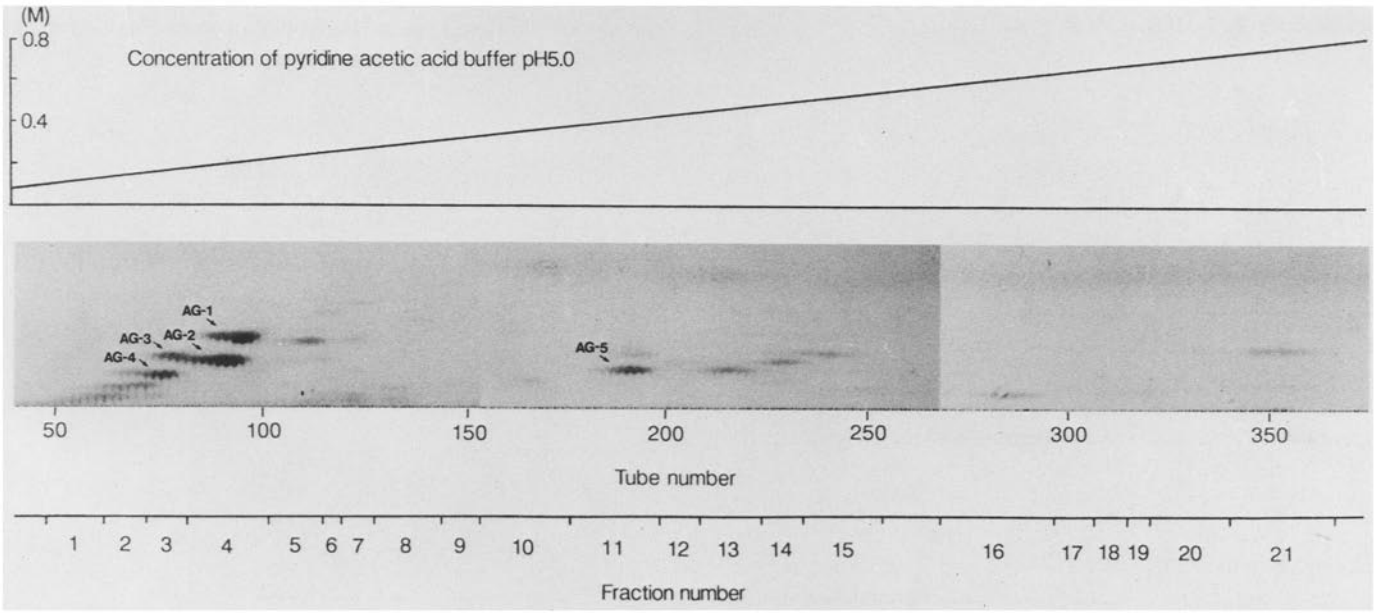


Figure 1. The elution profile of urinary sialo oligosaccharide compounds, from an AGU patient, on Dowex 1-X2 column chromatography. The sialo oligosaccharide compounds were eluted with a linear gradient of pyridine/acetic acid buffer, pH 5.0 as indicated. Fractions (15 ml) of effluent were collected. Sialo oligosaccharide compounds were analysed by HPTLC with solvent A and visualized by spraying with resorcinol/HCl reagent.

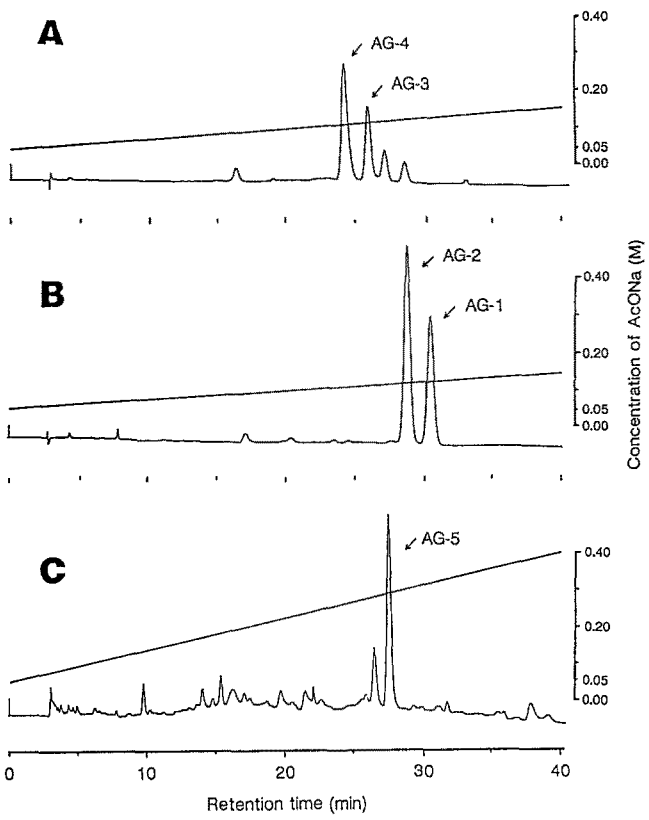


Figure 2. The elution profiles of urinary Asn N-glycosides, from an AGU patient, on HPLC-PAD. The Asn N-glycosides obtained from Dowex 1-X2 column chromatography were further subjected to HPLC-PAD and eluted with a linear gradient of sodium acetate solution as indicated in the Figure. Details are described under Materials and Methods. A, fr. 3; B, fr. 4; C, fr. 11.

HPTLC. The fractions containing sialylated oligosaccharides were pooled and lyophilized. Final purification of five major sialo-compounds was carried out by means of HPLC-PAD. HPLC-PAD consisted of a Dionex Bio LC gradient pump, model PAD 2 detector, and a Carbopac PA-1 column (acetate form) (1.84 cm × 25.0 cm) (DIONEX, Sunnyvale, CA). Urinary Asn N-glycosides were eluted with linear gradient systems of 50–200 mM sodium acetate in 100 mM NaOH for 60 min for Fr. 3 and Fr. 4, and 50–400 mM sodium acetate in 100 mM NaOH for 40 min for Fr. 11. The flow rate was 2.0 ml min^{-1}. For the detection of Asn N-glycosides, the following pulse potentials and durations were used: $E_1 = 0.1$ V ($t_1 = 120$ ms), $E_2 = 0.6$ V ($t_2 = 120$ ms), $E_3 = -0.8$ V (t_3=300 ms) [11]. The Asn N-glycosides purified by HPLC-PAD were desalted by Micro Acilyzer (Asahikasei, Tokyo, Japan) and lyophilized.

Structural analysis

Amino acids were determined on a JLC-5AH automatic amino acid analyser (Japan Electron Optics Lab., Tokyo, Japan) after the samples were hydrolysed with 6 N HCl at 110°C for 24 h. Sugar composition was determined by gas-liquid chromatography after methanolysis, *N*-acetylation, and trimethylsilylation as described by Bhatti *et al.* [17]. The Asn N-glycosides were permethylated by the method of Hakomori [18]. The partially methylated alditol acetates were prepared as described previously [19, 20] and analysed by gas-liquid chromatography equipped with a capillary column of 3% OV-101 (0.2 mm × 25 m, Shimadzu, Kyoto, Japan). Negative ion FAB-MS was carried out with a Jeol JMS-DX304 mass spectrometer (Jeol Ltd, Tokyo, Japan) equipped with a JMA-DA5000 data system. The sample, 2 μg, was dissolved in 2 μl of 50% ethylene glycol solution. Proton NMR spectra were obtained with a

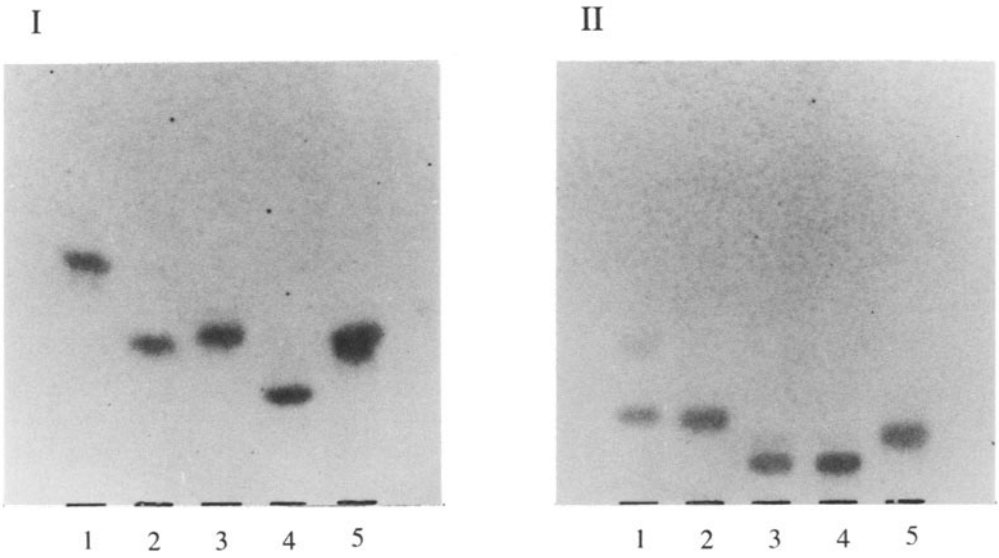
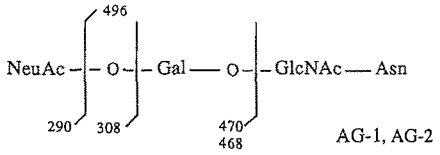


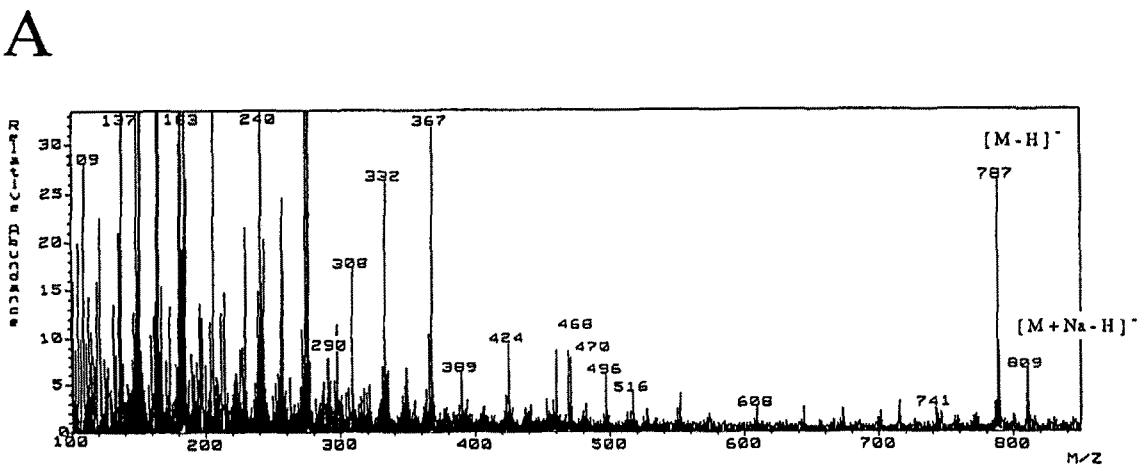
Figure 3. HPTLC of purified urinary Asn N-glycosides. The plate was developed with solvent A (I) and solvent B (II). Lane 1, AG-1; lane 2, AG-2; lane 3, AG-3; lane 4, AG-4; lane 5, AG-5. Asn N-glycosides were visualized by spraying with resorcinol/HCl reagent.

Table 1. Molar ratio of partially methylated alditol acetates obtained from Asn N-glycosides.

Alditol acetates	*Relative molar ratio*				
	AG-1	*AG-2*	*AG-3*	*AG-4*	*AG-5*
2,4,6-*O*-Me-1,3,5-*O*-Ac-galactitol[a]	1.00	–	1.00	1.00	1.00
2,3,4-*O*-Me-1,5,6-*O*-Ac-galactitol	–	1.00	–	1.03	–
3,6-*O*-Me,*N*-Ac-Me-1,4,5-*O*-Ac-glucosaminitol	0.97	0.99	0.96	1.97	0.96

Gas liquid chromatography was carried out on a capillary column of 3% OV-101 (0.2 mm × 25 m) at 160–240°C at a rate of 2°C per min. Values are expressed as relative molar ratio to 2,4,6-*O*-Me-1,3,5-*O*-Ac-galactitol or 2,3,4-*O*-Me-1,5,6-*O*-Ac-galactitol of AG-1.
[a]Me, methyl; Ac, acetyl.





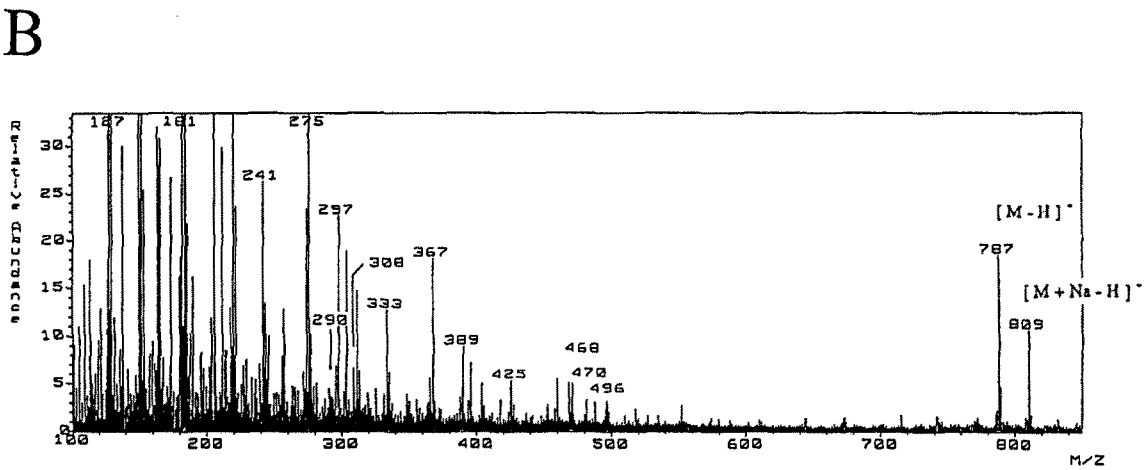
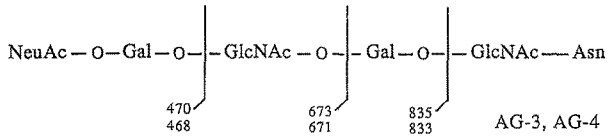


Figure 4. Negative FAB-MS spectra of Asn N-glycosides. The negative spectra were taken as described under Materials and methods. A, AG-1; B, AG-2; C, AG-3; D, AG-4; E, AG-5.



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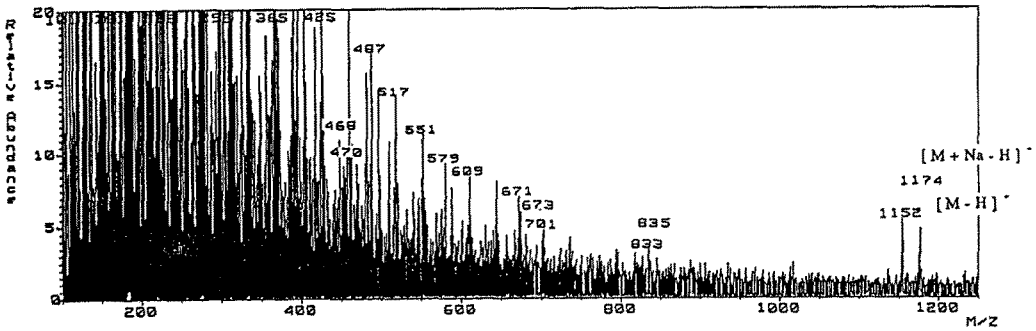

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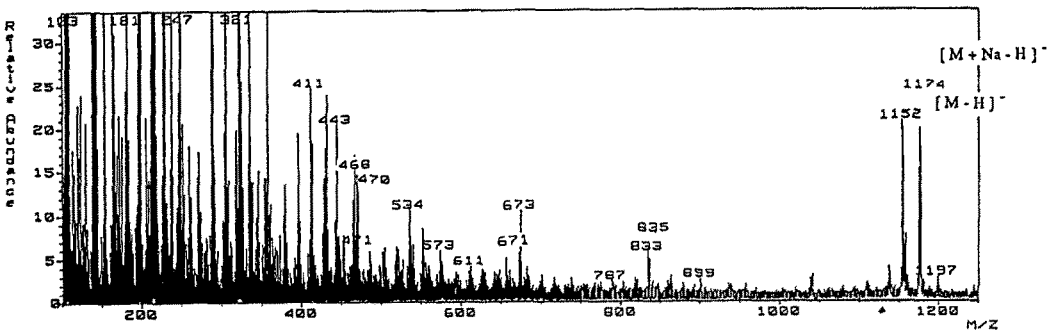

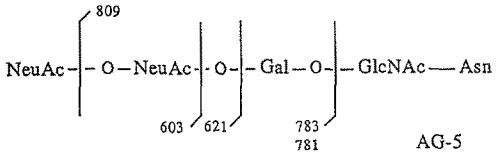

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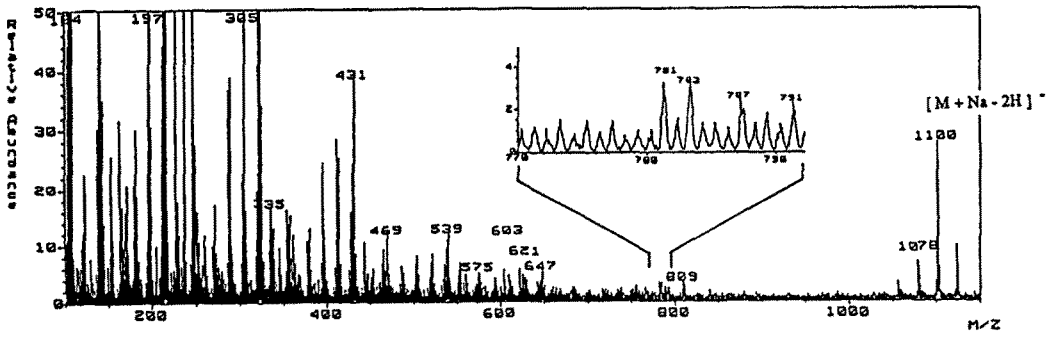

Figure 4. Negative FAB-MS spectra of Asn N-glycosides. The negative spectra were taken as described under Materials and methods. A, AG-1; B, AG-2; C, AG-3; D, AG-4; E, AG-5.

400-MHz NMR spectrometer (Jeol GX-400). The sample (1 mg) was dissolved in 0.5 ml of D_2O containing tetramethylsilane. The operating temperature was 40°C.

Results

Purification of Asn N-glycosides from urine of AGU patient

As shown in Fig. 1, sialo compounds were separated into 21 fractions by Dowex 1-X2 anion exchange column chromatography. Five major sialo compounds that were termed AG-1, AG-2, AG-3, AG-4, and AG-5 were further purified by HPLC-PAD as shown in Fig. 2. HPTLC analysis showed that AG-1 to AG-5 were purified to homogeneity (Fig. 3).

Chemical structures of AG-1 to AG-5

Carbohydrate and amino acid compositions of the five major Asn N-glycosides (AG-1 to AG-5) were as follows. AG-1 to AG-5 were composed of galactose, *N*-acetylglucosamine, *N*-acetylneuraminic acid and asparagine. Relative molar ratios (Gal = 1.0) of GlcNAc, NeuAc and Asn for each Asn N-glycosides as determined by gas-liquid chromatography were 0.82:0.82:1.12 (AG-1), 1.03:0.97:1.20(AG-2), 0.98:0.46:0.60 (AG-3), 0.92:0.44:0.58 (AG-4), 1.10:2.15:1.06 (AG-5). Accordingly, the integral molar ratios (Gal:GlcNAc:NeuAc:Asn) were 1:1:1:1 (AG-1 and AG-2), 2:2:1:1 (AG-3 and AG-4) and 1:1:2:1 (AG-5). This result indicated that AG-5 was an Asn N-glycoside containing two *N*-acetylneuraminic acid residues.

The partially methylated alditol acetates were analysed by gas liquid chromatography to determine the linkage positions of the carbohydrates. The data are listed in Table 1. This result indicated that none of the Asn N-glycosides contained branching structures. Also, the composition of methylated alditol acetates of AG-5 was the same as that of AG-1. Accordingly, two *N*-acetylneuraminic acids of AG-5 were thought to be linked serially.

Negative FAB-MS spectra of AG-1 to AG-5 are shown in Fig. 4. Molecular ion pairs, $(M + Na–H)^-$ and $(M–H)^-$ were obtained in all cases, that is, *m/z* 809 and 787 for AG-1 and AG-2, *m/z* 1174 and 1152 for AG-3 and AG-4, and *m/z* 1100 and 1078 for AG-5. These results were consistent with the results of compositional analysis of carbohydrates and amino acids. Combined with the methylation analysis, AG-1 and AG-2 were determined to have the same core structure, Galβ1 → 4GlcNAcβ1 → Asn. The only difference was that the former possessed an α2-3 linked *N*-acetylneuraminic acid at the non-reducing end while the latter had an α2-6 linked *N*-acetylneuraminic acid. AG-3 and AG-4 also had the same core structure: Galβ1 → 4GlcNAcβ1 → 3 Galβ1 → 4GlcNAcβ1 → Asn. Like AG-1 and AG-2, AG-3 contains a NeuAcα2 → 3Gal residue while AG-4 has a NeuAcα2 → 6Gal residue. Characteristic ions for AG-5, $(NeuAc\text{-}NeuAc+Na\text{-}2H)^-$ and $(NeuAc\text{-}NeuAc+Na\text{-}H_2O\text{-}2H)^-$, were observed at *m/z* 621 and 603, respectively.

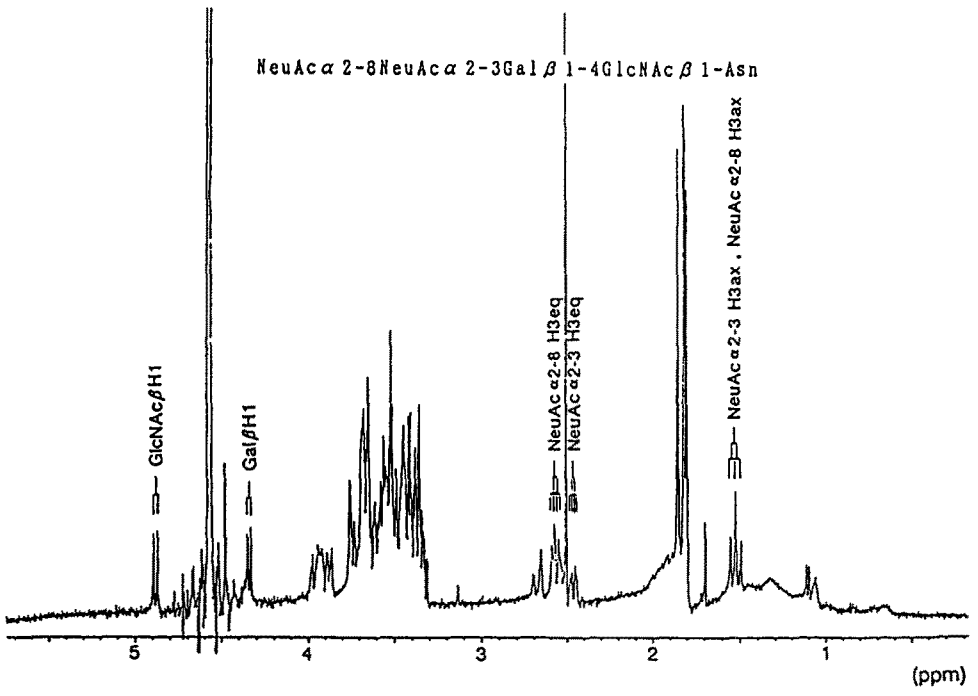

Figure 5. Four hundred MHz proton NMR spectra of AG-5. The spectra were taken at 40°C.

Table 2. Proton chemical shifts (ppm) and coupling constants (Hz) of Asn N-glycosides.

Residue	*Reporter group*	*AG-1*	*AG-2*	*AG-3*	*AG-4*	*AG-5*
Chemical shift						
Gal (2)[a]	H–1	4.52	4.41	4.43	4.42	4.42
Gal (4)	H–1	–	–	4.52	4.42	–
GlcNAc (1)	H–1	5.06	5.09	5.06	4.91	4.89
GlcNAc (3)	H–1	–	–	ND[b]	4.57	–
NeuAc (α2-3)	H–3eq	2.72	–	2.72	–	2.48
	H–3ax	1.76	–	1.76	–	1.54
NeuAc (α2-6)	H–3eq	–	2.64	–	2.46	–
	H-3ax	–	1.67	–	1.67	–
NeuAc (α2-8)	H-3eq	–	–	–	–	2.58
	H-3ax	–	–	–	–	1.54
J value						
Gal (2)	J1,2	7.7	8.1	8.1	7.7	6.2
Gal (4)	J1,2	–	–	7.7	7.3	–
GlcNAc (1)	J1,2	9.5	9.5	9.5	9.9	9.5
GlcNAc (3)	J1,2	–	–	ND	5.5	–
NeuAc (α2-3)	J3eq,3ax	– 12.5	–	– 12.5	–	– 12.1
	J3eq,4	4.8	–	5.1	–	4.0
	J3ax,4	12.1	–	12.1	–	11.7
NeuAc (α2-6)	J3eq,3ax	–	–12.8	–	– 12.5	–
	J3eq,4	–	5.1	–	4.8	–
	J3ax,4	–	12.1	–	12.1	
NeuAc (α2-8)	J3eq,3ax	–	–	–	–	– 12.8
	J3eq,4	–	–	–	–	5.5
	J3ax,4	–	–	–	–	11.7

[a] Values in parentheses indicate.
AG-1, AG-2, NeuAc-Gal(2)-GlcNAc(1)-Asn;
AG-3, AG-4, NeuAc-Gal(4)-GlcNAc(3)-Gal(2)-GlcNAc(1)-Asn;
AG-5, NeuAc-NeuAc-Gal(2)-GlcNAc(1)-Asn.
[b] ND, not determined.

In order to elucidate the anomeric configurations of galactose and *N*-acetylglucosamine in AG-1 to AG-5, the five intact Asn N-glycosides were analysed by 400 MHz proton NMR spectrometry. The NMR spectrum of AG-5 is shown in Fig. 5. The signals were assigned according to the data of Brockhausen *et al.* [21], Shimamura and Inoue [22], and ourselves [23] (Table 2). The configuration of galactose and *N*-acetylglucosamine was assigned as β in AG-1 to AG-5. The signals appearing in the range of 2.4 and 2.8 ppm were assigned as the protons with equatorial configuration at the C-3 of *N*-acetylneuraminic acid. Also, the protons with axial configuration at the C-3 of *N*-acetylneuraminic acid were detected in the range of 1.5 to 1.8 ppm. The signals at 1.76 ppm, 1.67 ppm, and 1.54 ppm were assigned to the axial H-3 proton of *N*-acetylneuraminic acid attached to the α2-3, α2-6, and α2-8 linkage, respectively, according to the literature [16, 24–26].

Based on the results described above, the chemical structures of AG-1 to AG-5 are proposed as follows:

AG-1: NeuAcα2 → 3Galβ1 → 4GlcNAcβ1 → Asn
AG-2: NeuAcα2 → 6Galβ1 → 4GlcNAcβ1 → Asn
AG-3: NeuAcα2 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → Asn
AG-4: NeuAcα2 → 6Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → Asn
AG-5: NeuAcα2 → 8NeuAcα2 → Galβ1 → 4GlcNAcβ1 → Asn.

Discussion

In this study, we purified five Asn N-glycosides from the urine of an AGU patient using HPLC-PAD. Using this method, urinary compounds can be separated according to the small structural differences in the linkage position of *N*-acetylneuraminic acid without any modifications. In addition to AG-1 to AG-4 which had been previously reported, a novel disialylated Asn N-glycosides, AG-5, was isolated.

This is the first report of the NeuAcα2 → 8NeuAcα2 → 3 Gal structure in N-linked type urinary oligosaccharides. There have been few reports showing the natural occurrence of the disialyl structure in oligosaccharides from glycoproteins and glycopeptides. Fukuda *et al.* reported the presence of the disialyl structure in O-linked type oligosaccharide from human erythrocyte glycophorins [27]. Therefore, although the origin of the Asn N-glycoside is unknown, there is a possibility that the accumulation of the unique compound is associated with AGU.

It is unlikely that AG-1 to AG-5 are derived from naturally occurring N-linked type glycoprotein since Galβ1→4 GlcNAcβ1→Asn structure differs from the core structure of the N-linked type glycoprotein, Manβ1→4GlcNAcβ1→ 4GlcNAcβ1→Asn. In this study, we have also detected GlcNAc-Asn as a major storage compound in the neutral oligosaccharide fraction of the urine (data not shown). The accumulated GlcNAc-Asn is assumed to serve as the precursor for the synthesis of disialyl Asn N-glycoside, AG-5, *in vivo*; Gal and GlcNAc are sequentially transferred to GlcNAc-Asn and the resultant compounds are additionally sialylated by the action of sialyltransferase. Interestingly, we could not detect the positional isomer of AG-5, NeuAcα2→8NeuAcα2→6Galβ1→4GlcNAcβ →Asn. This indicates that α2-8 sialyltransferase may act preferentially on NeuAcα2 → 3Gal, but not NeuAcα2 → 6Gal.

Molecular genetic analysis of the two Japanese AGU patients has shown that a new splicing defect in the aspartylglycosaminidase gene cause the enzyme deficiency in these patients [28]. This mutation is quite unique to the present cases, compared with those found in Norwegian and Swedish patients. The relationship between the gene mutations, phenotypic expression, and heterogeneity of urinary oligosaccharides is not clear at present. The combination of urinary oligosaccharide analysis by HPLC-PAD with molecular analysis of the aspartylglycosaminidase gene in AGU patients should answer this question.

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