Determination of the Absolute Configuration of Sialic Acids in Gangliosides from the Sea Cucumber *Cucumaria echinata*

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Received March 15, 2007; accepted April 6, 2007

Enantiomeric pairs of sialic acid, D- and L-NeuAc (*N*-acetylneuraminic acid), were converted to D- and L-arabinose, respectively, by chemical degradation. Using this method, the absolute configuration of the sialic acid residues, NeuAc and NeuGc (*N*-glycolylneuraminic acid), in the gangliosides from the sea cucumber *Cucumaria echinata* was determined to be the D-form. Although naturally occurring sialic acids have been believed to be the D-form on the basis of biosynthetic evidence, this is the first report of the determination of the absolute configuration of the sialic acid residues in gangliosides using chemical methods.

Key words sialic acid; absolute configuration; ganglioside; sea cucumber; *Cucumaria echinata*

Gangliosides, an important group of glycosphingolipids due to their biological functions,[1] are characterized by the presence of one or more moles of sialic acid, *N*-acetylneuraminic acid (NeuAc) or *N*-glycolylneuraminic acid (NeuGc), in their carbohydrate parts. In general, naturally occurring sialic acids have been believed to be the D-form on the basis of biosynthetic evidence, *i.e.*, NeuAc is synthesized from pyruvic acid and *N*-acetyl-D-mannosamine enzymatically.[2] However, there has been no report on the determination of the absolute configuration of the sialic acid residues in gangliosides. We attempted to determine the absolute configuration (D or L) of the sialic acid residues in the gangliosides[3,4] from the sea cucumber *Cucumaria echinata*.

For the determination of the absolute configuration of aldoses in gangliosides, we have used the Hara method.[5] However, this method cannot be applied to sialic acids directly. Therefore we prepared aldose from sialic acid, preserving the absolute configuration of the parent sialic acid, and then applied the Hara method to the aldose. As shown in Fig. 1, D(L)-arabinose should be obtained from D(L)-sialic acid in the process. First, this assumption was proved.

D-NeuAc (**1**) was acetylated to give pentaacetate (**2**), which was methanolyzed with HCl–MeOH to yield methyl 2-*O*-methylneuraminate (**3**). Protection of the diol in the side chain of **3** with acetone afforded an isopropyridene derivative (**4**). Oxidation of **4** with $NaIO_4$ gave a dial derivative (**5**). Finally, compound **5** was hydrolyzed to separate arabinose (**6**). The absolute configuration of **6** was verified as being the D-form using the Hara method. On the other hand, L-NeuAc (**7**), which was synthesized from L-glucose using the authentic method,[6] gave L-arabinose in the same manner as **1**. Thus the fact that D(L)-sialic acid could be converted to D(L)-arabinose was confirmed, as shown in Fig. 1.

Next, this method was applied to the gangliosides SJG-1, CG-1, CEG-3, CEG-4, CEG-5, CEG-6, HLG-3, CEG-8, and CEG-9[3,4] from the sea cucumber *C. echinata*. The nine gangliosides were each methanolyzed and the sialic acid derivatives (**3**) produced were converted to ketals (**4**). Periodic acid oxidation of **4** followed by acidic hydrolysis of the oxidation products **5** gave arabinoses (**6**). Compound **6**, obtained from each ganglioside, was determined to be the D-form using the Hara method, as shown in Fig. 2. Accordingly, the sialic acid residues (NeuAc and NeuGc) in the gangliosides from *C. echinata* must be the D-form.

To the best of our knowledge, this is the first report of the determination of the absolute configuration of the sialic acid

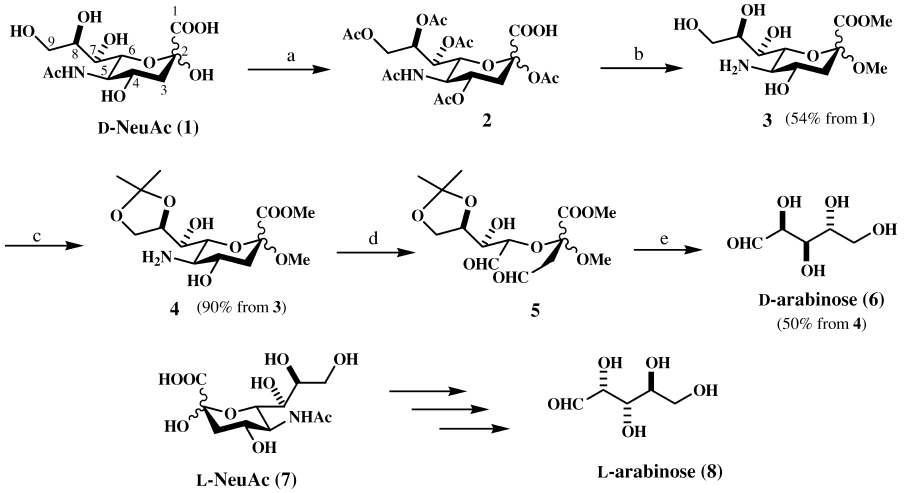

Fig. 1. Preparation of D(L)-Arabinose from D(L)-NeuAc

Reagents and conditions: (a) Ac_2O, pyridine; (b) 10% HCl–MeOH, 80 °C, 18 h; (c) DMF, acetone, 10% HCl–MeOH; (d) $NaIO_4$, H_2O; (e) 90% HCOOH–10% CF_3COOH (1/1), 100 °C, 12 h.

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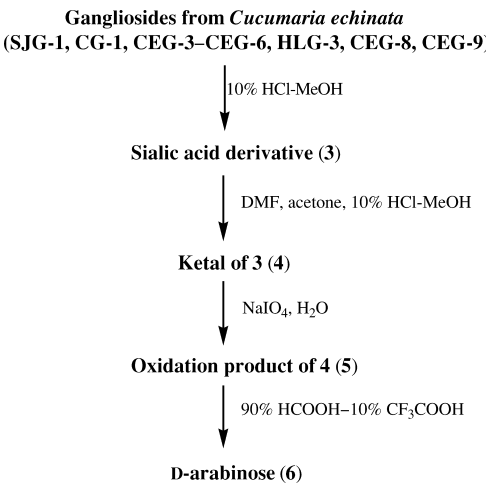
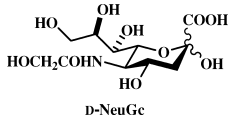


Fig. 2. Determination of the Absolute Configuration of Sialic Acid Residues of Gangliosides from *Cucumaria echinata* by Conversion to Arabinose

SJG-1: **NeuGc**α2→6Glcβ1→1 ceramide
CG-1: 8-*O*-sulfo-**NeuGc**α2→6Glcβ1→1 ceramide
CEG-3: 4-*O*-acetyl-Fucα1→11**NeuGc**α2→6Glcβ1→1 ceramide
CEG-4: Fucα1→11**NeuGc**α2→6Glcβ1→1 ceramide
CEG-5: Fucα1→11**NeuGc**α2→6Glcβ1→1 ceramide
CEG-6: Fucα1→11**NeuGc**α2→4**NeuAc**α2→6Glcβ1→1 ceramide
HLG-3: Fucα1→11**NeuGc**α2→4**NeuAc**α2→6Glcβ1→1 ceramide
CEG-8: **NeuGc**2→11**NeuGc**2→4**NeuAc**2→6Glc1→1 ceramide
CEG-9: **NeuGc**2→11**NeuGc**2→4**NeuAc**2→6Glc1→1 ceramide

Bold: sialic acid



D-NeuGc

residues in gangliosides using chemical methods and thus is noteworthy.

Experimental

Optical rotations were measured with a Jasco Dip-370 digital polarimeter at 23 °C. ^{1}H-NMR spectra were recorded on a Jeol GX-270 spectrometer (270 MHz) or a Varian Unity-500 spectrometer (500 MHz). Positive- and negative-ion FAB-MS spectra were acquired with a Jeol JMS-SX-102 mass spectrometer [xenon atom beam; matrix, *m*-nitrobenzylalcohol (positive-ion mode) and triethanolamine (negative-ion mode)]. Gas chromatographs were recorded with a Shimadzu QP-5050A [EI mode; ionizing potential, 120 eV; column, Neutra Bond-5 (0.25 mm×30 m, GL Science Inc.); carrier gas, He].

***N*-Acetyl-2,4,7,8,9-penta-*O*-acetyl-D-neuraminic Acid (2)** D-NeuAc (**1**) (20 mg) was heated with Ac_2O (0.5 ml) and pyridine (0.5 ml) at 60 °C for 2 h. The reaction mixture was concentrated *in vacuo* to give **2** (33.4 mg). $[\alpha]_D$ −10.4° (c=1.0, H_2O). Negative-ion FAB-MS *m/z*: 518 $[M-H]^-$. ^{1}H-NMR (D_2O) δ: 5.43 (1H, dd, J=7.7, 1.8 Hz, 7-H), 5.29 (1H, ddd, J=11.5, 10.3, 5.1 Hz, 4-H), 5.17 (1H, ddd, J=7.7, 4.1, 3.0 Hz, 8-H), 4.45 (2H, dd, J=12.8, 3.0 Hz, 9-H_2), 4.10 (1H, dd, J=10.6, 1.8 Hz, 6-H), 3.94 (1H, t, J=10.4 Hz, 5-H), 2.45 (1H, dd, J=13.6, 11.5 Hz, 3-H), 1.87 (1H, dd, J=13.6, 5.1 Hz, 3-H), 1.99, 2.02, 2.03, 2.03, 2.12, 2.14 (each 3H, s, $COCH_3$×6).

Methyl 2-*O*-Methyl-D-neuraminate (3) Compound **2** (33.4 mg) was heated with 10% HCl in MeOH (0.5 ml) at 80 °C for 18 h. The reaction mixture was concentrated *in vacuo* and the residue was chromatographed on silica gel (solvent $CHCl_3$–MeOH–H_2O, 6 : 4 : 1) to give **3** (10.3 mg, 54% from **1**). $[\alpha]_D$ −23.2° (c=1.0, H_2O). Positive-ion FAB-MS *m/z*: 296 $[M+H]^+$. ^{1}H-NMR (D_2O) δ: 4.42 (1H, m, 4-H), 4.19 (1H, m, 7-H), 4.05 (1H, m, 8-H), 3.95 (2H, m, 9-H_2), 3.92 (3H, s, $COOCH_3$), 3.53 (1H, m, 6-H), 3.30 (3H, s, OCH_3), 2.84 (1H, m, 5-H), 2.68 (1H, dd, J=13.2, 12.0 Hz, 3-H), 1.78 (1H, dd, J=13.2, 4.5 Hz, 3-H).

Methyl 8,9-*O*-Isopropylidene-2-*O*-methyl-D-neuraminate (4) Compound **3** (10.3 mg) was dissolved in DMF (0.5 ml), and acetone (0.5 ml) and 10% HCl in MeOH (20 μl) were added. The mixture was heated at 60 °C for 4 h, neutralized with Ag_2CO_3, centrifuged, and the clear supernatant solution was concentrated *in vacuo*. The residue was chromatographed on silica gel (solvent $CHCl_3$–MeOH, 8 : 2) to give **4** (10.5 mg, 90%). $[\alpha]_D$ −17.1° (c=1.0, H_2O). Positive-ion FAB-MS *m/z*: 336 $[M+H]^+$. ^{1}H-NMR (D_2O) δ: 4.44 (1H, m, 4-H), 4.34 (1H, m, 7-H), 4.14 (1H, m, 8-H), 4.12 (1H, m, 9-H), 3.90 (3H, s, $COOCH_3$), 3.77 (1H, m, 9-H), 3.56 (1H, m, 6-H), 3.30 (3H, s, OCH_3), 2.80 (1H, m, 5-H), 2.47 (1H, dd, J=12.9, 11.5 Hz, 3-H), 1.85 (1H, dd, J=12.9, 5.1 Hz, 3-H), 1.45, 1.40 [each 3H, s, $C(CH_3)_2$].

Preparation of D-Arabinose (6) from 4 To the solution of **4** (10.5 mg) in H_2O (0.5 ml), $NaIO_4$ (1.0 mg) was added and stirred at room temperature for 1 h. The reaction mixture was diluted with H_2O (5 ml) and a small amount of ethylene glycol, and extracted with *n*-BuOH (3 ml×3). The organic layer was washed with H_2O and concentrated *in vacuo* to give **5** as a crude product. Compound **5** was heated with 90% HCOOH–10% CF_3COOH (1 : 1) (0.5 ml) at 100 °C for 12 h, and the reaction mixture was concentrated *in vacuo*. The residue was purified on silica gel TLC (solvent $CHCl_3$–MeOH–H_2O, 7 : 3 : 0.5) to give **6** (2.3 mg, 50% from **4**).

Preparation of L-Arabinose (8) from L-NeuAc (7) In the same manner as for **1**, compound **7** (8.4 mg) afforded **8** (1.8 mg, 44.1%).

Determination of the Absolute Configuration of 6 and 8 (Hara Method) Compounds **6** (1 mg) and **8** (1 mg) were each heated with L-cysteine methyl ester hydrochloride (1.5 mg) and pyridine (0.2 ml) at 70 °C for 1 h. Then, 0.1 ml of 1-(trimethylsilyl) imidazole was added and the mixture was heated at 70 °C for a further 15 min. The reaction mixture was diluted with 50% MeOH (0.8 ml), extracted with *n*-hexane (0.5 ml×3), and the *n*-hexane layer was concentrated *in vacuo* to yield trimethylsilyl ether of the methyl (4*R*)-thiazolidine-4-carboxylate derivative. Each derivative was analyzed using gas chromatography [column temperature: 180—300 °C (rate of temperature increase 2.5 °C/min)]; t_R [min]=23.5 (derivative of **6**), 22.1 (derivative of **8**) (derivative of D-arabinose, 23.5, L-arabinose, 22.1).

Determination of the Absolute Configuration of Sialic Acid Residues of Gangliosides from *C. echinata* Each ganglioside (2 mg) shown in Fig. 2 was heated with 10% HCl in MeOH (0.5 ml) at 80 °C for 18 h and concentrated *in vacuo*. The residue was dissolved in DMF (0.3 ml), and acetone (0.3 ml) and 10% HCl in MeOH (10 μl) were added. The mixture was heated at 60 °C for 4 h, neutralized with Ag_2CO_3, centrifuged, and the clear supernatant solution was concentrated *in vacuo*. To the solution of the product in H_2O (0.5 ml), $NaIO_4$ (1.0 mg) was added and stirred at room temperature for 1 h. The reaction mixture was diluted with H_2O (5 ml) and a small amount of ethylene glycol, and extracted with *n*-BuOH (3 ml×3). The organic layer was concentrated *in vacuo*, the reaction product was heated with 90% HCOOH–10% CF_3COOH (1 : 1) (0.5 ml) at 100 °C for 12 h, and the reaction mixture was concentrated *in vacuo*. The arabinose from sialic acid, contained in the reaction product, was induced to the thiazolidine derivative, and the derivative was analyzed using gas chromatography in the same manner as in **6**; t_R [min] (original ganglioside)=23.5 (SJG-1), 23.5 (CG-1), 23.5 (CEG-3), 23.4 (CEG-4), 23.4 (CEG-5), 23.5 (CEG-6), 23.5 (HLG-3), 23.5 (CEG-8), 23.4 (CEG-9) (derivative of D-arabinose, 23.5, L-arabinose, 22.1).

Acknowledgments We thank Mr. Y. Tanaka and Ms. Y. Soeda of the Faculty of Pharmaceutical Sciences, Kyushu University, for the NMR measurements. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 13024260, Priority Area A) from the Ministry of Education, Culture, Science, Sports and Technology, Japan, and a grant (No. 16510163, 18510187) from the Japan Society for the Promotion of Science, which are gratefully acknowledged.

References and Notes

1) Hakomori S., Igarashi Y., *J. Biochem.* (Tokyo), **118**, 1091—1103 (1995).
2) Comb D. G., Roseman S., *J. Biol. Chem.*, **235**, 2529—2537 (1960).
3) Kisa F., Yamada K., Miyamoto T., Inagaki M., Higuchi R., *Chem. Pharm. Bull.*, **54**, 982—987 (2006).
4) Kisa F., Yamada K., Miyamoto T., Inagaki M., Higuchi R., *Chem. Pharm. Bull.*, **54**, 1293—1298 (2006).
5) The Hara method is a well-known method using determination of the absolute configuration of aldoses in which trimethylsilyl ethers of the methyl (4*R*)-thiazolidine-4-carboxylate derivative of aldose enantiomers can be separated by GLC; Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **35**, 501—506 (1987).
6) Kozlov I. A., Mao S., Xu Y., Huang X., Lee L., Sears P. S., Gao C., Coyle A. R., Janda K. D., Wong C.-H., *ChemBioChem*, **2**, 741—746 (2001).