Studies on Sialic Acids. XIII. Isolation of 3-Deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic Acid (KDN) from Chum Salmon, *Oncorhynchus keta*

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A method for analysis of 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (KDN) by high-performance liquid chromatography (HPLC) with a strongly basic anion-exchange resin was developed. The method was applied to detect free KDN in water used to rinse of fertilized eggs of chum salmon, *Oncorhynchus keta*. Furthermore, KDN was isolated from the water as a fully protected derivative.

Keywords sialic acid; 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid; KDN; HPLC; isolation; chum salmon

A deaminated sialic acid, 3-deoxy-D-*glycero*-D-*galacto*-2-nonulopyranosonic acid (**1**, KDN), has been found in glycoprotein of unfertilized egg of salmoid fishes, and several types of glycosidically linked **1** have been identified.[1-3] Inoue *et al.*[1] analyzed **1** as a protected derivative by gas-liquid chromatography, but there has been no report on the useful analysis of unprotected **1**. We have developed a method for analysis of **1** by high-performance liquid chromatography (HPLC) with a strongly basic anion-exchange resin. It is also useful for the separation of *N*-acetyl-D-neuraminic acid (NeuAc) and **1**. Using this method, we could detect free **1** in water used for washing away the excess sperm of chum salmon just after fertilization of eggs. Furthermore, we isolated **1** from the water as a protected derivative. Authentic **1** was prepared by the method of reported in our recent papers.[4,5]

Analysis of 1 by HPLC This method was based on that employed in the analysis of NeuAc and its derivative.[6]

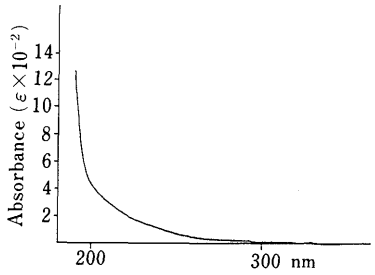


Fig. 1. UV Absorption of KDN

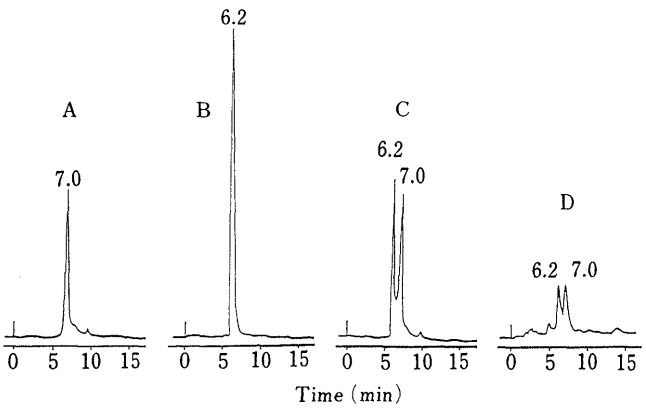


Fig. 2. High-Performance Liquid Chromatograms of (A) KDN, (B) NeuAc, (C) KDN+NeuAc, (D) Extracted Sample

Analysis of **1** could be performed on a small column (4.6 × 150 mm) filled with the strongly basic anion-exchange resin Hitachi GEL 3013-N, using aqueous 5 mM sodium sulfate solution as the mobile phase. The column effluent could be monitored for **1** at wavelength between 200 and 250 nm, and we selected 210 nm. The ultraviolet (UV) absorption spectrum of **1** is shown in Fig. 1. Figure 2 shows chromatograms of authentic **1** (A), NeuAc (B), and a mixture (C). The retention times of **1** and NeuAc were 7.0 and 6.2 min, respectively. In Fig. 2C, it can be seen that the peak of **1** was separated from the peak of NeuAc.

Detection of 1 by HPLC from Chum Salmon The water used for washing away the excess sperm of chum salmon, *Oncorhynchus keta*, was collected and evaporated at room temperature under reduced pressure, and the residue was freeze-dried. The freeze-dried powder[7] (100 mg) was suspended in water, and the suspension was stirred at room temperature, and filtered through Celite. The filtrate was purified by on a Dowex-1 (formate) column eluted with aqueous 0.5 M formic acid, and the product was freeze-dried. The residue was submitted to HPLC, and the chromatogram is shown in Fig. 2D. In Fig. 2D, the peaks at 7.0

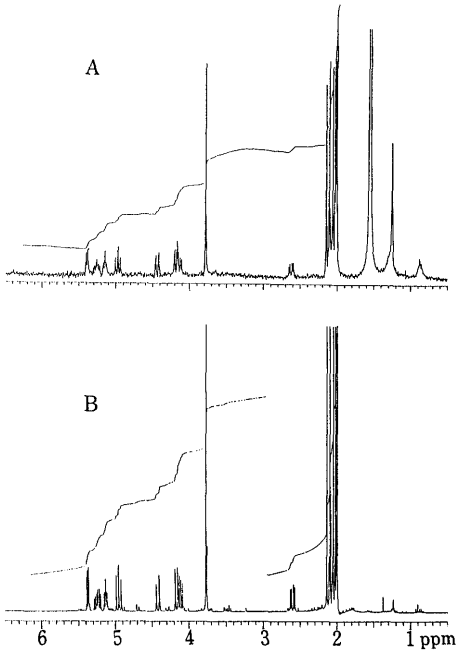
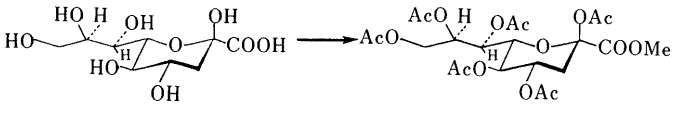


Fig. 3. ^{1}H-NMR Spectra of (A) Isolated **2** and (B) Authentic **2**

and 6.2 min were identical with those of authentic **1** and NeuAc, respectively.

Isolation of 1 from Chum Salmon The freeze-dried powder[7] (20 g) was suspended in water, stirred at room temperature, and filtered through Celite. The filtrate was washed with hexane, and the aqueous layer was evaporated to dryness. The residue was purified on a Dowex-1 (formate) column eluted with aqueous 2 M formic acid, and evaporation of the eluete gave a crude syrup. The syrup was esterified by treatment with Dowex-50 (H^+) in MeOH, and acetylated with acetic anhydride. The crude product was purified by silica gel column chromatography and fractions containing a component with *Rf* 0.67 (4:1, ether–hexane) were combined and concentrated to dryness. About 2 mg of the isolated component was submitted to 300 MHz proton nuclear magnetic resonance (^{1}H-NMR) measurement, and the spectrum is shown in Fig. 3A.

Synthesis of an Authentic Sample and Its Structural Elucidation The authentic sample, methyl 2,4,5,7,8,9-hexa-*O*-acetyl-3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (**2**), was synthesized by the following procedures. The carboxyl group of **1** was protected by esterification with Dowex-50 (M^+) in MeOH, and further protection of hydroxy groups were carried out by acetylation with acetic anhydride. The crude product was purified by silica gel column chromatography and recrystallized with ether–hexane to give **2** as colorless needles. The structure of **2** was elucidated by ^{1}H-NMR comparison with benzyl 2,4,5,7,8,9-hexa-*O*-acetyl-3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate.[5] The ^{1}H-NMR spectrum of **2** is shown in Fig. 3B.



1 2

Chart 1. Synthesis of **2**

Results and Discussion

Analysis and quantitation of **1** could be achieved by strongly basic anion-exchange HPLC, a method which is generally applied in sialic acid chemistry. Though the intensity of UV absorption of **1** is lower than that of NeuAc at 210 nm, this method can distinguish between **1** and NeuAc.

We applied this analysis method to search for **1** from salmoid fishes, and detected **1** in water used to rinse fertilized eggs of chum salmon. We first attempted to isolate **1** by using a column of Dowex-1 (formate) resin only, but this failed. Therefore, free **1** was protected and isolated as a protected derivative, although the yield was poor. The amount actually present in the freeze-dried powder should be at least 340 mg/20 g, as judged from the HPLC assay result.[8] In earlier studies on biological sialic acids, most have been detected in glycosidically linked form.[9] Free sialic acid is generally present in low concentrations in tissues, for example trout egg[10] and mammalian brain.[11,12] Outside of tissues, free sialic acid at micromolar concentration has been found in normal human serum and urine.[13]

There has been a study on the rates of hydrolysis of glycosyl derivatives of NeuAc under various conditions.[14] The rate of hydrolysis was proved to be more dependent on the reaction temperature than on the acid concentration. Therefore, if an unknown glycoproteins containing **1** is presented in the freeze-dried powder, it seems very unlikely that **1** would have been released by the treatment with formic acid at room temperature (below 30 °C).

Although the biological function of **1** is obscure, our results may provide a basis for speculating on a possible role of **1** in fertilization and early embryogenesis.

Experimental

Melting points were measured with Yamato melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-181 digital polarimeter. Thin layer chromatography (TLC) was performed on Silica gel GF254 (Merck) plates, and detection was achieved by UV irradiation and with 5% sulfuric acid solution. Infrared (IR) and UV spectra were measured with JASCO IR-A2 and Hitachi 340 instruments, respectively. ^{1}H-NMR spectra were measured with a Varian VXR-300 spectrometer. Tetramethylsilane (TMS) in $CDCl_3$ was used as an internal reference. Column chromatography was conducted on Silica gel 60 (Merck, 70—230 mesh).

HPLC KDN and NeuAc were analyzed by anion exclusion chromatography using a Hitachi GEL 3013-N strongly basic anion-exchange resin column (4.6 × 150 mm) at 50 °C. A mobile phase of 5 mM sodium sulfate was used at a flow rate of 0.9 ml/min. The column effluent was monitored with a UV detector at 210 nm (Nihon Seimitsu Kagaku, model NS-310).

Methyl 2,4,5,7,8,9-Hexa-*O*-acetyl-3-deoxy-β-D-*glycero*-D-*galacto*-2-nonulopyranosonate (2) Dowex-50 (H^+) (88 mg) was added to a solution of KDN (440 mg, 1.64 mmol) in dry MeOH. The mixture was stirred for 18 h at room temperature, the reaction mixture was filtered, and the filtrate was evaporated to dryness. The residual syrup was dissolved in pyridine (5 ml) and then acetic anhydride (5 ml) and 4-dimethylaminopyridine (4 mg) were added. The reaction mixture was stirred for 5 h at room temperature, then poured into water, and the product was extracted with ethyl acetate. The extract was washed with 1 N HCl, aqueous $NaHCO_3$, and brine. The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The residual syrup was purified on a column of silica gel and the product was recrystallized from ether–hexane to give **2** (245 mg, 28%) as colorless needles. mp 99—101 °C. $[\alpha]_D^{19}$ −20.4° (*c*=0.71, $CHCl_3$). Mass (EI) *m/z*: 534 (M^+). *Anal.* Calcd for $C_{22}H_{30}O_{15}$: C, 49.44; H, 5.66. Found: C, 49.22; H, 5.70. IR ν_{max}^{KBr} cm^{-1}: 2950, 1750, 1440, 1370, 1230. ^{1}H-NMR (300 MHz, $CDCl_3$): 1.99—2.14 (3H × 6, s × 6, $COCH_3$), 2.06 (1H, dd, *J*=13.8, 11.8 Hz, 3-Hax), 2.60 (1H, dd, *J*=13.8, 5.2 Hz, 3-Heq), 3.77 (3H, s, CH_3), 4.12 (1H, dd, *J*=12.5, 5.8 Hz, 9-H), 4.17 (1H, dd, *J*=10.0, 2.4 Hz, 6-H), 4.42 (1H, dd, *J*=12.5, 2.7 Hz, 9′-H), 4.95 (1H, t, *J*=9.7 Hz, 5-H), 5.13 (1H, m, 8-H), 5.24 (1H, m, 4-H), 5.37 (1H, dd, *J*=6.3, 2.4 Hz, 7-H).

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