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# Nature and biosynthesis of sialic acids in the starfish *Asterias rubens*. Identification of sialo-oligomers and detection of *S*-adenosyl-L-methionine: *N*-acylneuraminate 8-*O*-methyltransferase and CMP-*N*-acetylneuraminate monooxygenase activities

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**Summary** — Mass spectrometric and NMR spectroscopic analyses of bound sialic acids from the starfish *Asterias rubens* revealed the presence of *N*-acetylneuraminic acid (4 %), *N*-acetyl-8-*O*-methylneuraminic acid (12 %), *N*-acetyl-9-*O*-acetyl-8-*O*-methylneuraminic acid (< 1 %), *N*-glycoloylneuraminic acid (19 %), *N*-glycoloyl-8-*O*-methylneuraminic acid (47 %), and *N*-glycoloyl-9-*O*-acetyl-8-*O*-methylneuraminic acid (18 %). Analysis of sialo-oligomeric material, obtained after mild acid hydrolysis, demonstrated that *N*-glycoloyl-8-*O*-methylneuraminic acid can occur as di- and tri-oligomers, linked through the anomeric center and the *N*-glycoloyl moiety, Neu5Gc8Me-α(2→O5)-Neu5Gc8Me and Neu5Gc8Me-α(2→O5)-Neu5Gc8Me-α(2→O5)-Neu5Gc8Me. Studies on the biosynthesis of *N*-acyl-8-*O*-methylneuraminic acid in *A rubens*, using the tracer *S*-adenosyl-L-[methyl-$^{14}$C]methionine, showed that *N*-acylneuraminate 8-*O*-methyltransferase activity was present predominantly in the membrane fraction. CMP-*N*-acetylneuraminic acid monooxygenase activity was found in the soluble protein fraction, in agreement with investigations on the corresponding vertebrate enzyme.

**CMP-*N*-acetylneuraminic acid monooxygenase / *N*-acylneuraminic acid 8-*O*-methyltransferase / *N*,*O*-acylneuraminic acids / *O*-methylated sialic acids / starfish *Asterias rubens***

## Introduction

The echinodermata are thought to be the most primitive animals containing sialic acids [1]. In addition to the common sialic acids *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycoloylneuraminic acid (Neu5Gc), they possess rather unusual sialic acids. *N*-Acetyl-8-*O*-methylneuraminic acid (Neu5Ac8Me) was found to occur in the starfish *Distolasterias nipon* [2], whereas *N*-glycoloyl-8-*O*-methylneuraminic acid (Neu5Gc-8Me) was identified in *Asterias rubens* [3], *Asterias forbesi* [4], *Asterias amurensis* [5], *Aphelasterias japonica* [6], and *Asterina pectinifera* [7]. Recently, Neu5Gc, *N*-glycoloyl-9-*O*-acetyl-8-*O*-methylneuraminic acid (Neu9Ac5Gc8Me), and *N*-glycoloyl-7,9-di-*O*-acetyl-8-*O*-methylneuraminic acid (Neu7,9Ac$_2$-5Gc8Me) were detected in the starfish *Pisaster brevispinus* [8]. Preliminary reports have appeared concerning the occurrence of Neu9Ac5Gc8Me and Neu5,9Ac$_2$8Me in *A rubens*, besides Neu5Gc8Me [9, 10]. 8-*O*-Sulfated sialic acids have been isolated from sea urchin species [11–14], wheras *N*-glycoloyl-9-*O*-acetylneuraminic acid (Neu9Ac5Gc) was identified in the eggs of the sea urchin *Pseudocentrotus depressus* [15]. In echinoderms, sialic acids have been found in terminal and internal positions of the oligosaccharide chains [1, 6, 15, 16].

The biochemical reactions giving rise to such a variety of unusual sialic acids are poorly understood. Preliminary data have suggested that the 8-*O*-methylation of sialic acids in the starfish *A rubens* is cata-

*Abbreviations:* SAM, *S*-adenosyl-L-methionine; PSM, porcine submandibular gland mucin; BSM, bovine submandibular gland mucin; CM, collocalia mucin; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac8Me, *N*-acetyl-8-*O*-methylneuraminic acid; Neu5,9Ac$_2$8Me, *N*-acetyl-9-*O*-acetyl-8-*O*-methylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; Neu5Gc-8Me, *N*-glycoloyl-8-*O*-methylneuraminic acid; Neu9Ac5Gc-8Me, *N*-glycoloyl-9-*O*-acetyl-8-*O*-methylneuraminic acid; 2-D-HOHAHA, two-dimensional homonuclear Hartmann-Hahn; MLEV, Malcom Levitt; 2-D-ROESY, two-dimensional rotating frame Overhauser enhancement spectroscopy; GLC-EIMS, gas-liquid chromatography electron impact mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; TLC, thin layer chromatography.

lysed by an *S*-adenosylmethionine-dependent *O*-methyltransferase [17]. Although the biosynthesis of Neu5Gc has been shown to occur via the specific hydroxylation of CMP-Neu5Ac in vertebrates [18, 19], the presence of CMP-Neu5Ac hydroxylase in lower animals has not been demonstrated so far.

Here, a more detailed survey of the sialic acids present in conjugated form in the starfish *A rubens*, as determined by mass spectrometry and NMR spectroscopy, is reported, together with structural data on sialic acid oligomers. Further, insight into the properties of the enzyme *N*-acylneuraminate 8-*O*-methyltransferase and CMP-Neu5Ac monooxygenase is provided.

## Materials and methods

*Materials*

Starfish *Asterias rubens* were collected from the Baltic Sea. Porcine submandibular gland mucin (PSM) and bovine submandibular gland mucin (BSM) were isolated according to Tettamanti *et al* [20]. Collocalia mucin (CM) was purchased from Asitra (Germany). Neu5Ac and Neu5Gc were isolated from CM and PSM, respectively [21], and α(2→3)-and α(2→6)-sialyllactose from bovine colostrum [22]. *S*-Adenosyl-L-methionine (SAM) was purchased from Boehringer, and *Vibrio cholerae* sialidase from Behringwerke (Germany). Neu5Ac-α(2→8)-Neu5Ac and Neu5Ac-α(2→8)-Neu5Ac-α(2→8)-Neu5Ac, isolated from colominic acid of *E coli*, were kindly donated by Dr H Voshol (Department of Bio-Organic Chemistry, Utrecht University). *S*-Adenosyl-L-[methyl-$^{14}$C]methionine (58 mCi/mmol) ([$^{14}$C]SAM) and cytidine-5'-mono-phospo-*N*-acetyl-[4,5,6,7,8,9-$^{14}$C]neuraminic acid (316 mCi/mmol) were obtained from Amersham, supplied as solutions in dilute sulfuric acid (pH 2.5–3.5) and in 20 mM $NH_4HCO_3$ containing 2 % ethanol, respectively.

*Isolation and purification of sialic acids and/or sialic acid oligomers from the starfish Asterias rubens*

*Procedure A*

Starfish (650 g) were freed from intestine and homogenized with an Ultraturrax in 700 ml water to give 1300 ml homogenate, which was adjusted to pH 1.5 with 50 % HCl, and hydrolyzed for 1 h at 70°C. During hydrolysis, the pH was readjusted several times. The hydrolysate was filtered and the filtrate (1080 ml) dialyzed against water at 4°C (five times 1 l for 16 h). The dialysate was combined with the residue from the filtration, adjusted to pH 1.0 with 50 % HCl, and hydrolyzed for 1.5 h at 80°C, with several readjustments of the pH, followed by filtration and dialysis as described above. The combined diffusates were concentrated to 120 ml by rotary evaporation, and fractionated on a column (10 x 66 cm) of Sephadex G-25 (Pharmacia), using water as eluent at a flow rate of 4 ml/min. The 5-ml fractions were tested for sialic acid by the quantitative orcinol/$Fe^{3+}$/HCl assay [21] to yield, after lyophilization, three pools containing 60 mg (pool I), 78 mg (pool II), and 53 mg (pool III) sialic acid, respectively. To identify the various sialic acids present, pools I–III were analyzed by GLC-EIMS. Pools I and II were further subfractionated on a column (2.3 x 81 cm) of cellulose MN2100ff (Macherey-Nagel), using 1-propanol/1-butanol/water (2:1:1, vol) as eluent. Fractions of 5 ml were collected and sialic-acid-containing fractions (orcinol/$Fe^{3+}$/HCl test) were analyzed by thin layer chromatography (TLC) on silica gel and on cellulose as described below. Fractions containing sialic acid material with a lower migration rate than the known monomeric sialic acids were combined, concentrated by rotary evaporation, and lyophilized. The residue was dissolved in 1 ml water and aliquots were fractionated on a Mono Q HR 5/5 anion-exchange column (Pharmacia FPLC system) with detection at 214 nm. After washing with 2 ml water, the column was eluted with a linear concentration gradient from 0 to 50 mM NaCl in 8 ml water, followed by a steeper gradient from 50 to 500 mM NaCl in 8 ml water at a flow rate of 120 ml/h. The fraction eluting at the position of reference Neu5Ac-α(2→8)-Neu5Ac was collected, and desalted on a column (1 x 28 cm) of Bio-Gel P-2 (200-400 mesh, Bio-Rad) using water as eluent and monitoring at 206 nm. Further subfractionation was carried out on a Kratos SF 400 HPLC system (ABI analytical, Kratos Division), equipped with a 10-μm Partisil 10 SAX column (4.6 x 250 mm, Whatman). Elutions were performed with 30 mM $KH_2PO_4$, pH 4.7/acetonitrile (25:75, vol) at a flow rate of 120 ml/h and detection at 205 nm. The major fraction was desalted on Bio-Gel P-2 and analyzed by mass spectrometry and $^1$H-NMR spectroscopy.

*Procedure B*

In a second working-up procedure the combined diffusates were concentrated by rotary evaporation and lyophilization, and the residue, dissolved in 375 ml water, was fractionated on a column (10 x 78 cm) of Sephadex G-25 (Pharmacia), using water as eluent at a flow rate of 4 ml/min. The 10-ml fractions were tested for sialic acid using the quantitative orcinol/$Fe^{3+}$/HCl assay [21]. The void volume pool was passed through a column (2.5 x 5 cm) of Dowex 50WX8 ($H^+$) resin (20–50 mesh, Fluka), and the resin was washed with 125 ml water. After lyophilization, the residue was fractionated on Mono Q as described above. The retarded sialic-acid-containing pool was lyophilized, redissolved in 10 ml water, and fractionated on a column (3 x 47.5 cm) of Bio-Gel P-4 (200–400 mesh, Bio-Rad) using water as eluent at a flow rate of 35 ml/h. The 10-ml fractions were tested for sialic acid using the orcinol/$Fe^{3+}$/HCl assay. A sialic-acid-containing pool eluting before the column volume on Bio-Gel P-4 was lyophilized and fractionated on Mono Q as described above. In each case of Mono Q fractionation, the fractions, co-eluting with oligomers of colominic acid, were collected and desalted on a column (1 x 28 cm) of Bio-Gel P-2 using water as eluent at a flow rate of 15 ml/h. Sialic-acid-positive fractions were further purified by HPLC on Partisil 10 SAX as described above.

*Procedure C*

The bulk of the digestive system was removed from 400 g starfish, and the remaining material was homogenized in water to 0.5 g wet tissue/ml using a Waring blender, followed by an Ultraturrax step. The homogenate was centrifuged at 1080 *g* for 20 min to precipitate residual tissue. The supernatant was adjusted to pH 2.0 with formic acid and heated for 1 h at 70°C. After dialysis against 0.5 l water, with intermediate change of solvent, for 48 h at 4°C, the diffusate, containing released sialic acids, was collected. The dialysate was adjusted to pH 1.0 with concentrated HCl, and heated for 1 h at 80°C, followed by dialysis against 0.5 l water, with intermediate change of solvent, for 48 h at 4°C. The combined diffusates were applied to a column (2.6 x 15 cm) of Dowex 1X8 ($HCOO^-$) resin (200–400 mesh), and after washing with 100 ml water, the adsorbed

sialic acids were eluted with 0.4 M formic acid, and lyophilized. The total non-retarded fraction was processed for a second time to collect sialic acids, which were not bound in the first run due to overloading. The combined sialic acid fraction was dissolved in 2 ml water, applied to a SEP-Pak C-18 cartridge (Millipore; prewashed with 5 ml methanol, 5 ml chloroform/methanol (1:1, vol), 5 ml chloroform/methanol (2:1, vol), 5 ml methanol, and 4 ml water), which was eluted with 4 ml water, and the eluate was lyophilized. This sialic acid sample (0.5 mg sialic acid/g wet tissue), characterized by GLC-EIMS analysis, was used as a radio-TLC sialic acid standard series.

*Preparation of particulate and supernatant fractions from starfish*

Starfish were washed with water and blotted dry. The following operations were carried out at 0–4°C. The animals were cut into pieces and homogenized using an Ultraturrax (two times for 3 min). After each centrifugation step, the upper layer of fatty material was removed and discarded. Protein concentrations were determined with the Bio-Rad protein assay reagent, using bovine serum albumin as a standard.

*Preparation of fractions for testing O-methyltransferase activity*

The procedure followed is essentially based on that reported in [23]. Starfish were homogenized in 0.1 M Tris/HCl buffer, pH 8.9, in a concentration of 1 g wet weight tissue/ml. The homogenate was centrifuged at 700 *g* for 20 min to sediment large particles, and the obtained supernatant was centrifuged at 10 800 *g* for 20 min, yielding the heavy membrane fraction P1. The supernatant was carefully removed and centrifuged at 120 000 *g* for 1 h, giving a membrane pellet P2 and a supernatant S. The membrane fractions P1 and P2 were washed by homogenization in 60 ml 0.1 M Tris/HCl buffer using a Potter-Elvehjem homogenizer, and then recentrifuged at 10 800 *g* for 20 min and at 120 000 *g* for 1 h, respectively. The P1 and P2 sediments were resuspended in 30 ml 0.1 M Tris/HCl buffer by homogenization, to yield 7.5 mg and 23 mg protein/ml, respectively. The soluble protein fraction S was subjected to a further centrifugation at 120 000 *g* for 1 h, to remove remaining suspended material, and finally contained 22 mg protein/ml.

*Preparation of fractions for testing monooxygenase activity*

Starfish were homogenized in 0.1 M HEPES/NaOH buffer, pH 6.7, in a concentration of 1 g wet weight tissue/ml. Large particles were sedimented by centrifugation at 700 *g* for 20 min, and the membrane and soluble protein fractions were collected by centrifugation at 120 000 *g* for 1 h. The sediment was resuspended in 30 ml HEPES buffer and centrifuged at 120 000 *g* for 1 h. The combined supernatants were recentrifuged at 120 000 *g*, to remove remaining particulate material, to give a clear solution containing 25 mg protein/ml (fraction S'). The washed sediment was resuspended in 30 ml HEPES buffer using a Potter-Elvehjem homogenizer to give a membrane suspension containing 24 mg protein/ml (fraction P').

*Incubation of soluble substrates with S-adenosyl-L-methionine: N-acyl-D-neuraminate 8-O-methyltransferase preparations P1, P2 and S*

*Glycoconjugate-bound sialic acid substrates*

PSM (300 µg sialic acid) in 100 µl water, CM (330 µg Neu5Ac [1]) in 60 µl water, BSM (300 µg sialic acid) in 100 µl water, 150 µl mouse serum (300 µg sialic acid), α(2→3)-sialyllactose (300 µg Neu5Ac) in 50 µl water, and α(2→6)-sialyllactose (300 µg Neu5Ac) in 100 µl water, were each incubated with 2 ml of fraction P1, P2, and S containing each 0.8 µM [$^{14}$C]SAM (0.1 µCi) for 3 h at 37°C. Before use, stock [$^{14}$C]SAM was diluted ten times with Tris/HCl buffer. After incubation, the assay mixtures except those containing BSM, were adjusted to pH 1.0 with HCl and heated for 1 h at 80°C; in the case of BSM, the solution was adjusted to pH 2.0 with formic acid and heated for 1.5 h at 70°C. The various hydrolysates were dialyzed twice against 0.25 l water, and each of the diffusates was applied to a column (1.6 x 6 cm) of Dowex 1X8 ($HCOO^-$) resin (200–400 mesh). After washing with 100 ml water, sialic acids were eluted with 50 ml 0.4 M formic acid. For each sample, the sialic-acid-containing fraction was lyophilized, resuspended in 50 µl water, and analyzed by radio-TLC. Reference non-radioactive Neu5Ac and a mixture of sialic acids from the starfish containing predominantly Neu5Gc and Neu5Gc8Me (see working-up procedure C) were co-chromatographed on the same lane with each sample. Control incubations were performed in the absence of exogenous sialic-acid-containing acceptor, using 2 ml heat-denatured (95°C, 5 min) fractions P1, P2, and S, under the standard conditions. Using PSM as exogenous sialic-acid-containing acceptor, fraction P2 containing 0.8 µM [$^{14}$C]SAM (0.1 µCi) was also incubated in the presence of 1 mM EDTA, 10 mM EDTA or 4 mM *S*-adenosyl-L-homocysteine. Liquid scintillation counting was performed in Beckman Readisolv MP scintillation fluid using a Beckman LS 9000D liquid scintillation counter with appropriate correction for quenching.

*Free sialic acid*

Neu5Ac and Neu5Gc (100 µg/10 µl water) were each incubated with 250 µl of fraction P1, P2 and S containing 1.6 µM [$^{14}$C]SAM (0.025 µCi) under the conditions described in the previous section. In each case the incubation was stopped by addition of 30 µl 2 M HCl, followed by cooling on ice and centrifugation at 12000 *g* for 12 min to remove precipitated protein. A 10-µl aliquot of each supernatant was subjected to radio-TLC analysis as described in the previous section. Control incubations in the presence of 1.6 µM [$^{14}$C]SAM were performed either in the absence of free sialic acid or with 250-µl heat denatured fractions P1, P2, and S for 3 h at 37°C.

*Incubation of immobilized PSM with a S-adenosyl-L-methionine: N-acyl-D-neuraminate 8-O-methyltransferase preparation*

A crude preparation of 8-*O*-methyltransferase was prepared by homogenization of starfish, without stomach, in 0.1 M Tris/glycine buffer, pH 8.8, containing 5 mM 2-mercaptoethanol, in a concentration of 1 g wet weight tissue/2 ml buffer. The homogenate was centrifuged at 1080 *g* for 15 min, and the supernatant was recentrifuged at 48 000 *g* for 1 h. The pellet was solubilized in Tris/glycine buffer containing 0.1 % Triton X-100 using a Potter-Elvehjem homogenizer, subjected to two 10-s bursts of sonication (40 W). The sonicated material was centrifuged at 120 000 *g* for 2 h, to afford a supernatant containing 1.2 mg protein/ml. PSM was immobilized on Sepharose 4B (Pharmacia) by the cyanogen bromide method [24, 25].

Incubations were carried out in 1 ml 0.1 M Tris/glycine buffer, pH 8.8, containing 5 mM 2-mercaptoethanol at 37°C, using various incubation times between 10 and 240 min, and variable final concentrations of substrate and protein. For routine assays typically 5 mM [$^{14}$C]SAM (0.05 µCi), 0.5 mM Neu5Gc (PSM immobilized on 200 µl Sepharose 4B) and crude enzyme preparation (0.3 mg protein) were used. Assays were stopped by placing the reaction vials on ice, and the

Sepharose beads were washed with 10 ml Tris/glycine buffer, pH 8.8, 10 ml 0.1 M NaCl, and 10 ml water. Radioactivity incorporated into Sepharose-bound Neu5Gc was quantified by liquid scintillation counting (see above). Sialic acids were identified by radio-TLC after mild acid hydrolysis with HCl at pH 1.0, as described above.

*Determination of CMP-N-acetyl-D-neuraminate monooxygenase activty*

Supernatant S' (6 mg protein) and pellet P' (6 mg protein) in HEPES buffer, pH 6.7, were incubated for 3 h at 37°C in the presence of 1 mM NADH, 0.5 mM $FeSO_4$ and 0.53 μM CMP-[$^{14}$C]Neu5Ac (0.05 μCi) in a total volume of 300 μl at pH 6.2, 6.7, 7.2, and 7.7 for S' and at pH 6.6, 7.0, 7.7, and 8.2 for P'. The incubations were stopped by addition of 30 μl 2 M HCl and the released [$^{14}$C]sialic acids were analyzed by radio-TLC. Reference non-radioactive Neu5Ac and Neu5Gc were co-chromatographed on the same lane. Control incubations were performed using heat denatured (95°C, 5 min) protein fractions.

*Sialidase treatment of A rubens sialic acid disaccharide*

Disaccharide (10 μg), dissolved in 100 μl 20 mM sodium phosphate buffer, pH 5.6, containing 1 mM $CaCl_2$ was incubated with 5 μU *Vibrio cholerae* sialidase. The progress of the digestion was followed with time by monitoring the amount of free sialic acid on an Aminex A-29 anion-exchange column (4.6 x 40 mm, Bio-Rad; Kratos SF 400 HPLC system), using 0.75 mM $Na_2SO_4$ as eluent at a flow rate of 0.5 ml/min at ambient temperature, and detection at 200 nm [26].

*Thin layer chromatography*

TLC separations were performed using the following solvent systems: i) glass plates (10 x 10 cm) coated with 0.2 mm HPTLC-silica gel (Merck), developed in ethanol/1-butanol/pyridine/water/acetic acid (100:10:10:30:3, vol); ii) glass plates as in system i), but developed in 2-propanol/water (7:3, vol); iii) glass plates (10 x 20 cm) coated with 0.1 mm HPTLC-cellulose (Merck), developed in 2-propanol/1-butanol/0.1 M HCl (2:1:1, vol). Before use, cellulose plates were run in 2-propanol/1-butanol/0.1 M HCl (2:1:1, vol). Sialic acid standards (3–10 μg) were visualized with the orcinol/$Fe^{3+}$/HCl reagent [21]. For radio-TLC, 5 to 10-μl aliquots of samples were applied to the plates in a 2-cm line. Appropriate amounts of radioactive compounds were co-chromatographed with non-radioactive reference substances. Zones of radioactivity were detected using a Berthold Tracemaster 40 LB 2821 (Berthold) linear analyser, interfaced with a computer.

*Combined gas-liquid chromatography electron impact mass spectrometry and fast atom bombardment mass spectrometry*

Dry sialic acids (30 μg) were converted into the corresponding per-*O*-trimethylsilyl derivatives by treatment with 50 μl pyridine/hexamethyldisilazane/chlorotrimethylsilane (5:1:1, vol) for 2 h at room temperature [27, 28], and analyzed on a capillary CP-Sil 5 fused silica column (0.32 cm x 25 m, Chrompack) using a Varian 3700 gas chromatograph. The injector temperature was 250°C, the oven temperature was kept at 140°C for 5 min and then raised to 250°C at 2°C/min. The carrier gas velocity was 1 ml/min. The gas chromatograph was coupled to a Varian MAT 44S mass spectrometer and a Varian spectrospin 200 data system. The 70 eV electron impact (EI) mass spectra were obtained at an ionization current of 500 μA and an ion-source temperature of 220°C.

Positive- and negative-ion FAB mass spectra of oligosaccharides (0.1 mg dispersed in glycerol) were recorded on a Jeol 505W mass spectrometer. The primary beam was composed of Xe atoms with a maximum energy of 6 keV. The sputtered ions were extracted and accelerated with a potential of 3 kV. A VG Analytical ZAB-2F mass spectrometer was used to obtain a linked mass scan for *m/z* 661. Ions were generated from the glycerol matrix by FAB with Xe atoms with a maximum energy of 7 keV, and the precursor ion at *m/z* 661 was extracted, selected, accelerated at 8 kV, and a spectrum of the He gas collision induced ions was recorded.

*NMR spectroscopy*

Prior to $^1$H-NMR spectroscopic analysis, samples were repeatedly exchanged in $^2H_2O$ (99.96 % $^2$H, MSD isotopes) at p$^2$H 7 with intermediate lyophilization [29, 30]. 500-MHz 1-D and 2-D $^1$H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR-spectroscopy, Utrecht University) at a probe temperature of 300 K. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 2.225) [29]. The 2-D Homonuclear Hartmann-Hahn (HOHAHA) spectra were recorded using the pulse sequence 90°-$t_1$-SL-Acq, wherein SL stands for a multiple of the MLEV-17 sequence [31, 32]. The spin-lock field-strength corresponded to a 90° pulse-width of 28.6 μs (dimer) or 27.2 μs (trimer) and a total spin-lock mixing time of 120 ms. A 406 x 2048 (dimer) or a 455 x 2048 (trimer) data point set was recorded at 300 K, and the spectral width was 1600 Hz (dimer) or 3165 Hz (trimer) in each dimension. The time domain data were multiplied with a phase-shifted sine-bell, and a phase-sensitive Fourier transformation was performed after zero-filling to a 1024 x 2048 data matrix size. The 2-D rotating-frame nuclear Overhauser enhancement (ROESY) spectrum [33] was recorded at 500 MHz, and was obtained using the pulse sequence $90°_{\Phi}$-$t_1$-$(\text{spinlock})_{\Phi+\pi/2}$-Acq($t_2$), with a spin-lock mixing pulse of 200 ms at a field-strength corresponding to a 90° pulse-width of 115 μs. The carrier-frequency was placed at the left side of the spectrum at 5.7 ppm in order to minimize HOHAHA type magnetization transfer. A 512 x 2048 data point set was recorded at 300 K, and the spectral width was 3200 Hz in each dimension. Phase-sensitive handling of the data in the $\omega_1$ dimension was carried out by the time-proportional phase increment method [34]. The time domain data were multiplied with a phase-shifted sine-bell, and a phase-sensitive Fourier transformation was performed after zero-filling to a 1024 x 4096 data matrix size.

600-MHz 2-D ROESY $^1$H-NMR spectra were recorded on a Bruker AM-600 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) at a probe temperature of 280 K, under the conditions described above, except that the carrier frequency was placed on water at 5.0 ppm. The sialyl-disaccharide was dissolved in 500 μl 20 mM potassium phosphate, containing 92.5 % $^1H_2O$, 7.5 % $^2H_2O$, and 0.02 % sodium azide [35], and the pH was adjusted to 5.6 with 1 M HCl. The water signal was suppressed by presaturation. A 512 x 4096 data point set was recorded, and the spectral width was 4200 Hz in each dimension. Data processing was carried out on a VAX station 3100 using the TRITON NMR software package (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University). The time domain data were multiplied with a phase shifted sine-

bell. After Fourier transformation, the resulting data set of 1024 x 4096 points was baseline-corrected in the $\omega_2$-direction by a fourth-order polynomal fit.

75-MHz proton-decoupled $^{13}$C-NMR spectra were recorded in $^2H_2O$ on a Bruker AC-300 spectrometer (Department of Organic Chemistry, Utrecht University) operating in the Fourier transform mode at 300 K. Chemical shifts (δ) are expressed in ppm relative to internal acetone (δ 31.55).

## Results

### *Analysis of sialic acids and sialic acid oligomers*

Following different procedures, bound sialic acids were released and isolated from starfish *A rubens*. Using GLC-EIMS in case of working-up procedure A, pool I showed the presence of Neu5Gc8Me (major) and Neu5Ac8Me, pool II Neu5Gc8Me (major), Neu5Ac, Neu5Ac8Me, and Neu9Ac5Gc8Me, and pool III Neu9Ac5Gc8Me (major), Neu5Ac8Me, Neu5,9-$Ac_2$8Me, and Neu5Gc8Me. The fragment ions A-H, which characterize the type, number and location of the substituents in pertrimethylsilylated neuraminic acid [27, 28], of each of the pertrimethylsilylated sialic acids are compiled in table I, together with the GLC retention times. As a typical example, in figure 1 the El mass spectrum of pertrimethylsilylated Neu5Gc8Me is depicted. Applying working-up procedure C, TLC of the sialic acid mixture in solvent systems i-iii and GLC-EIMS of the pertrimethylsilylated derivatives revealed the presence of six different sialic acids, namely, Neu5Ac (4 %), Neu5Ac8Me (12 %), Neu5,9$Ac_2$8Me (<1 %), Neu5Gc (19 %), Neu5Gc8Me (47 %), and Neu9Ac5Gc8Me (18 %).

In view of the oligomeric structures discussed below, the NMR parameters of the major sialic acid Neu5Gc8Me were established, and the $^1$H- and $^{13}$C-NMR data are presented in tables II and III. By comparison of the intensities of the H-3a and H-3e signals of the β- and α-anomers in the $^1$H-NMR spectrum (fig 2A), it can be deduced that the molar ratio of β-Neu5Gc8Me and α-Neu5Gc8Me amounts to 95:5, similar to that found for Neu5Gc [36]. Comparison of the $^1$H-NMR data of β-Neu5Gc [36] and β-Neu5Gc8Me shows that the introduction of a methyl group (δ 3.425) at O-8 not only gives rise to an upfield shift for H-8 (Δδ –0.345) and downfield shifts for the neighbouring H-7 (Δδ +0.010), H-9 (Δδ + 0.111), and H-9' (Δδ +0.039), but also to upfield shifts for H-3e (Δδ –0.024), H-4 (Δδ –0.017), H-5 (Δδ–0.019), H-6 (Δδ –0.089), and the *N*-glycoloyl methylene (Δδ –0.010), and a downfield shift for H-3a (Δδ + 0.023). Comparison of the $^{13}$C-NMR data of β-Neu5Gc [36] and β-Neu5Gc8Me demonstrates that the *O*-methylation (OMe, δ 58.98) causes a strong downfield shift of C-8 (Δδ +9.54) and upfield shifts for the neighbouring C-7 (Δδ –1.46) and C-9 (Δδ –3.61) atoms.

The compound having the same retention time as Neu5Ac-α(2→8)-Neu5Ac on Mono Q (see working-up procedure A), which was further purified on Partisil 10 SAX, revealed after enzymatic hydrolysis with *Vibrio cholerae* sialidase Neu5Gc8Me as the only constituent. The FAB mass spectrum recorded in the positive mode of the ammonium form, showed a protonated molecular ion $(M + H)^+$ at *m/z* 661 and a cationized ion $(M + NH_4)^+$ at *m/z* 678, whereas in the negative FAB mass spectrum a $(M–H)^-$ ion at *m/z*

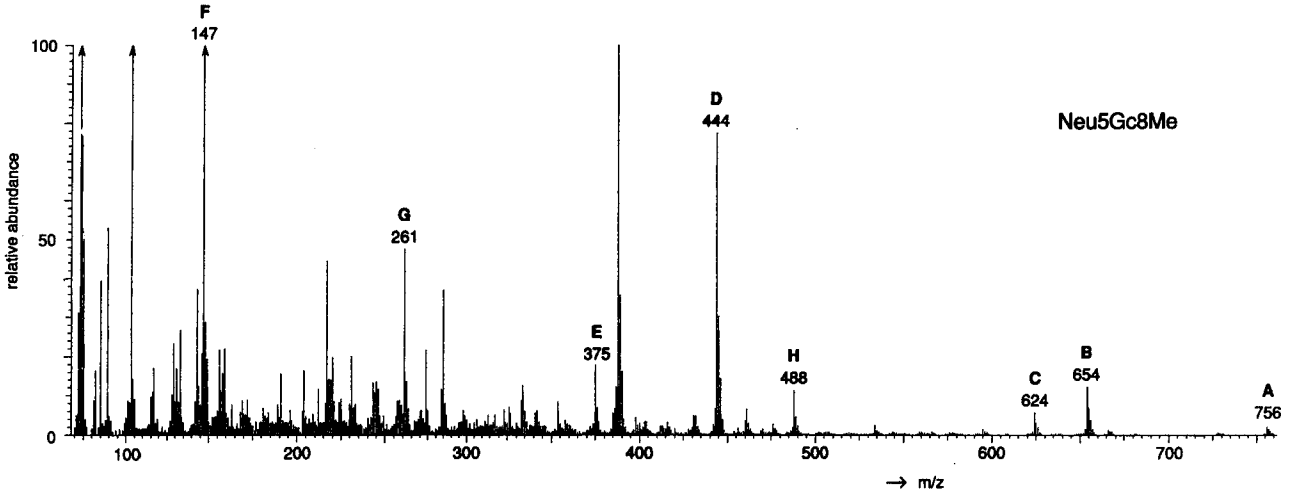


**Fig 1.** 70 eV El mass spectrum of pertrimethylsilylated Neu5Gc8Me.

**Table I.** Gas-liquid chromatographic retention times of pertrimethylsilylated sialic acids of *Asterias rubens* relative to pertrimethylsilylated β-Neu5Gc on CP-Sil 5 and *m/z* values (EIMS) of characteristic fragment ions A-H [27, 28].

| | *GLC* | *characteristic fragment ions (m/z)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *Sialic acid* | $R_{Neu5Gc}$ | *A* | *B* | *C* | *D* | *E* | *F* | *G* | *H* |
| Neu5Ac | 0.84 | 726 | 624 | 536 | 356 | 375 | 205 | 173 | 458 |
| Neu5Gc | 1.00 | 814 | 712 | 624 | 444 | 375 | 205 | 261 | 546 |
| Neu5Gc8Me | 0.96 | 756 | 654 | 624 | 444 | 375 | 147 | 261 | 488 |
| Neu9Ac5Gc8Me | 0.98 | 726 | 624 | 624 | 444 | 375 | 117 | 261 | 488 |
| Neu5Ac8Me | 0.82 | 668 | 566 | 536 | 356 | 375 | 147 | 173 | 400 |
| Neu5,9Ac$_2$8Me | 0.84 | 638 | 536 | 536 | 356 | 375 | 117 | 173 | 400 |

**Table II.** $^1$H-NMR data for Neu5Gc [30], Neu5Gc8Me, Neu5Gc8Me-α(2→O5)-Neu5Gc8Me, and Neu5Gc8Me-α(2→O5)-Neu5Gc8Me-α(2→O5)-Neu5Gc8Me. Chemical shifts are given in ppm relative to internal acetone (δ 2.225) in $^2H_2O$ at p$^2$H 7 and 300 K.

| *Compound* | *$^1$H-chemical shift (in ppm)* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *H-3a* | *H-3e* | *H-4* | *H-5* | *H-6* | *H-7* | *H-8* | *H-9* | *H-9'* | *5Gc/5Gc'*[a] | *8Me* | *NH* |
| α-Neu5Gc | 1.644 | 2.749 | nd[b] | nd | nd | nd | nd | nd | nd | 4.12 | – | nd |
| β-Neu5Gc | 1.840 | 2.243 | 4.127 | 4.002 | 4.106 | 3.549 | 3.777 | 3.821 | 3.613 | 4.143 | – | nd |
| α-Neu5Gc8Me | 1.643 | 2.551 | nd | nd | nd | nd | nd | nd | nd | 4.125 | nd | nd |
| β-Neu5Gc8Me | 1.863 | 2.219 | 4.110 | 3.983 | 4.017 | 3.559 | 3.432 | 3.932 | 3.652 | 4.133 | 3.425 | nd |
| Neu5Gc8Me-α(2→O5)-Neu5Gc8Me | | | | | | | | | | | | |
| α-Neu5Gc8Me | 1.767 | 2.669 | 3.846 | 3.959 | 4.064 | 3.558 | 3.496 | 3.979 | 3.670 | 4.124 | 3.479 | 8 31[c] |
| β Neu5Gc8Me (reducing unit) | 1.853 | 2.212 | 4.134 | 3.999 | 4.033 | 3.589 | 3.433 | 3.927 | 3.647 | 4.320/4.118 | 3.432 | 8.26[c] |
| Neu5Gc8Me-α(2→O5)-Neu5Gc8Me-α(2→O5)-Neu5Gc8Me | | | | | | | | | | | | |
| α-Neu5Gc8Me (terminal)[d] | 1.758[e] | 2.665 | 3.868 | 3.961 | 4.088 | 3.543 | 3.494 | 3.972 | 3.661[e] | 4.119 | 3.476 | nd |
| α-Neu5Gc8Me (internal)[d] | 1.756[e] | 2.655 | 3.831 | 3.950 | 4.067 | 3.584 | 3.504 | 3.984 | 3.664[e] | 4.309/4.099 | 3.488 | nd |
| β-Neu5Gc8Me (reducing unit) | 1.854 | 2.209 | 4.140 | 3.980 | 4.036 | 3.590 | 3.436 | 3.926 | 3.646 | 4.317/4.113 | 3.434 | nd |

[a] 5Gc/5Gc' represents the protons of the *N*-glycoloyl methylene group. In case of a singlet (2H) only one value is given, in case of two doublets (2 x 1H) two values are given.
[b] nd, not determined.
[c] Value obtained at 280 K, pH 5.6 and in $^1H_2O$, containing 20 mM sodium phosphate.
[d] The set of H-3a,3e,4,5,6 of the terminal and internal α-Neu5Gc8Me may be interchanged. The same holds for the set of H-7,8,9,9'. [e] Values may be interchanged.

**Table III.** $^{13}$C-NMR data for β-Neu5Gc [30] and β-Neu5Gc8Me. Chemical shifts are given in ppm relative to internal acetone (δ 31.55) in $^2H_2O$ at p$^2$H 7 and 300 K.

| *Compound* | *$^{13}$C-chemical shift (in ppm)* | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *C-1* | *C-2* | *C-3* | *C-4* | *C-5* | *C-6* | *C-7* | *C-8* | *C-9* | *5Gc ($CH_2$)* | *5Gc (C=O)* | *8Me* |
| β-Neu5Gc | 178.08 | 97.65 | 40.61 | 68.18 | 53.18 | 71.20 | 69.63 | 71.62 | 64.47 | 62.25 | 176.85 | – |
| β-Neu5Gc8Me | nd[a] | 96.75 | 40.58 | 67.81 | 53.23 | 71.64 | 68.17 | 81.16 | 60.86 | 62.31 | nd[a] | 58.98 |

[a]nd, not determined

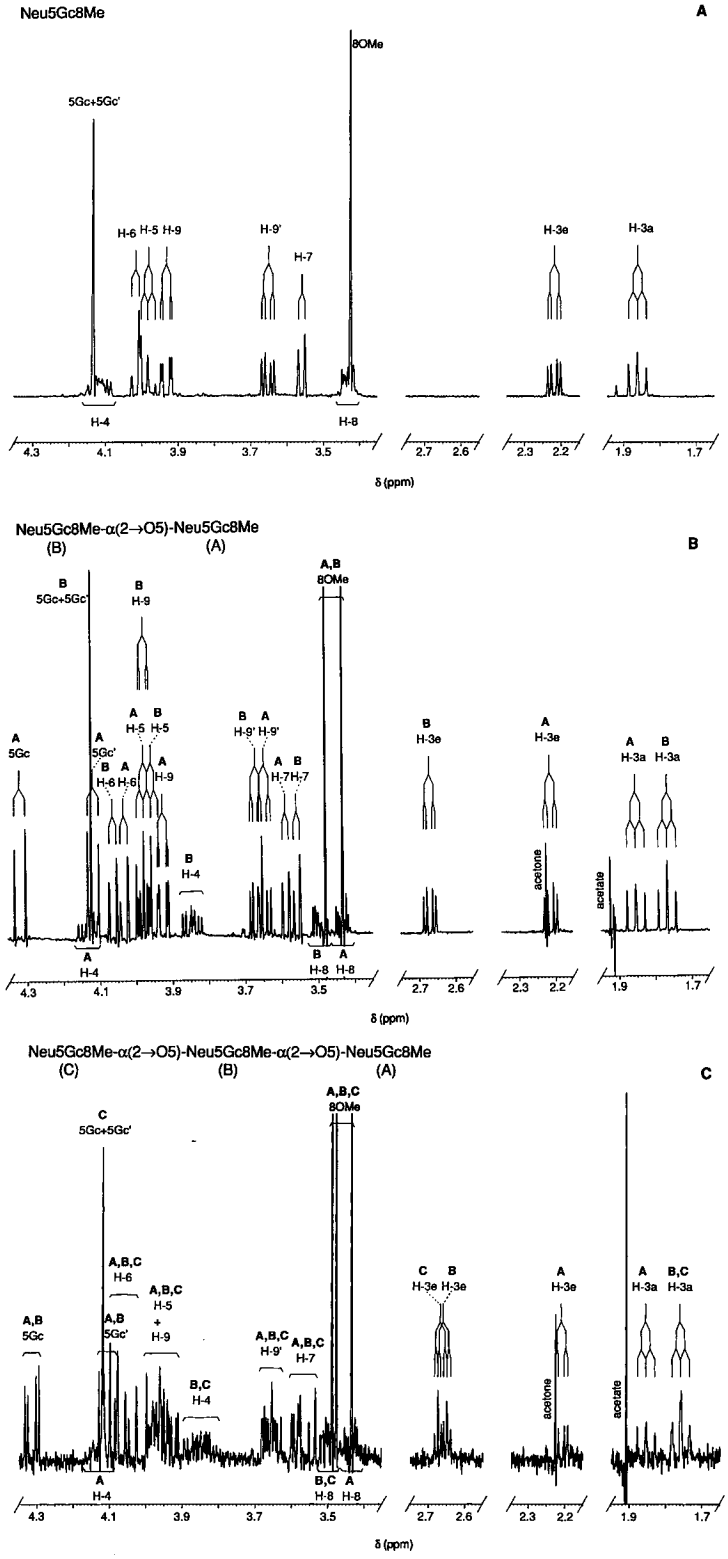


**Fig 2.** 500-MHz 1-D $^1$-HNMR spectra recorded in $^2H_2O$ at p$^2$H 7 and 300 K of (**A**) Neu5Gc-8Me, (**B**) Neu5Gc8Me-α(2→O5)-Neu5Gc8Me, and (**C**) Neu5Gc8Me-α(2→O5)-Neu5Gc8Me-α(2→O5)-Neu5Gc8Me, predominantly occurring in the β-anomeric form.

659 was observed, indicating a molecular mass of 660 Da, in accordance with a Neu5Gc8Me dimer with $H^+$ as counter ion. A collisional activation spectrum of the $(M + H)^+$ ion at *m/z* 661 contained peaks at *m/z* 643 $[(M + H\text{-}H_2O)^+]$, *m/z* 625 $[(M + H - 2H_2O)^+]$, *m/z* 340 $[(Neu5Gc8Me + H)^+$, cleavage of the glycosidic bond], *m/z* 322 $[(Neu5Gc8Me + H - H_2O)^+]$, and *m/z* 304 $[(Neu5Gc8Me + H - 2H_2O)^+]$.

The $^1$H-NMR spectrum of the Neu5Gc8Me dimer in $^2H_2O$ (fig 2B) showed two sets of signals in an intensity ratio of 1:1. On basis of the $^1$H-NMR data of Neu5Gc8Me, the set of signals of H-3a at δ 1.853, H-3e at δ 2.212, and H-4 at δ 4.134 indicates the presence of a β-Neu5Gc8Me unit, whereas the set of signals of H-3a at δ 1.767, H-3e at δ 2.669, and H-4 at δ 3.846 can be attributed to an α-Neu5Gc8Me residue. Since the dimer can be cleaved by sialidase (see above), its sequence has to be α-Neu5Gc8Me →β-Neu5Gc8Me. Each set of H-3a,3e,4 signals could be correlated with the corresponding H-5,6,7,8,9,9' resonances using 2-D-HOHAHA and 2-D-ROESY spectroscopy. Via cross-peaks on the H-3 tracks of both Neu5Gc8Me units in the 2-D-HOHAHA spectrum (fig 3), two sets of H-3e,3a,4,5,6 atoms could be deduced, whereas also two sets of H-7,8,9,9' atoms could be identified via cross-peaks. Since $^3J_{6,7}$ is small (0.9 Hz), no cross-peaks were observed correlating H-6 and H-7, and therefore it was not possible to relate a specific set of ring protons with a specific set of side-chain protons. However, the 2-D-ROESY spectrum of the Neu5Gc8Me dimer showed for each of the units a r0e between H-6 and H-7. The various

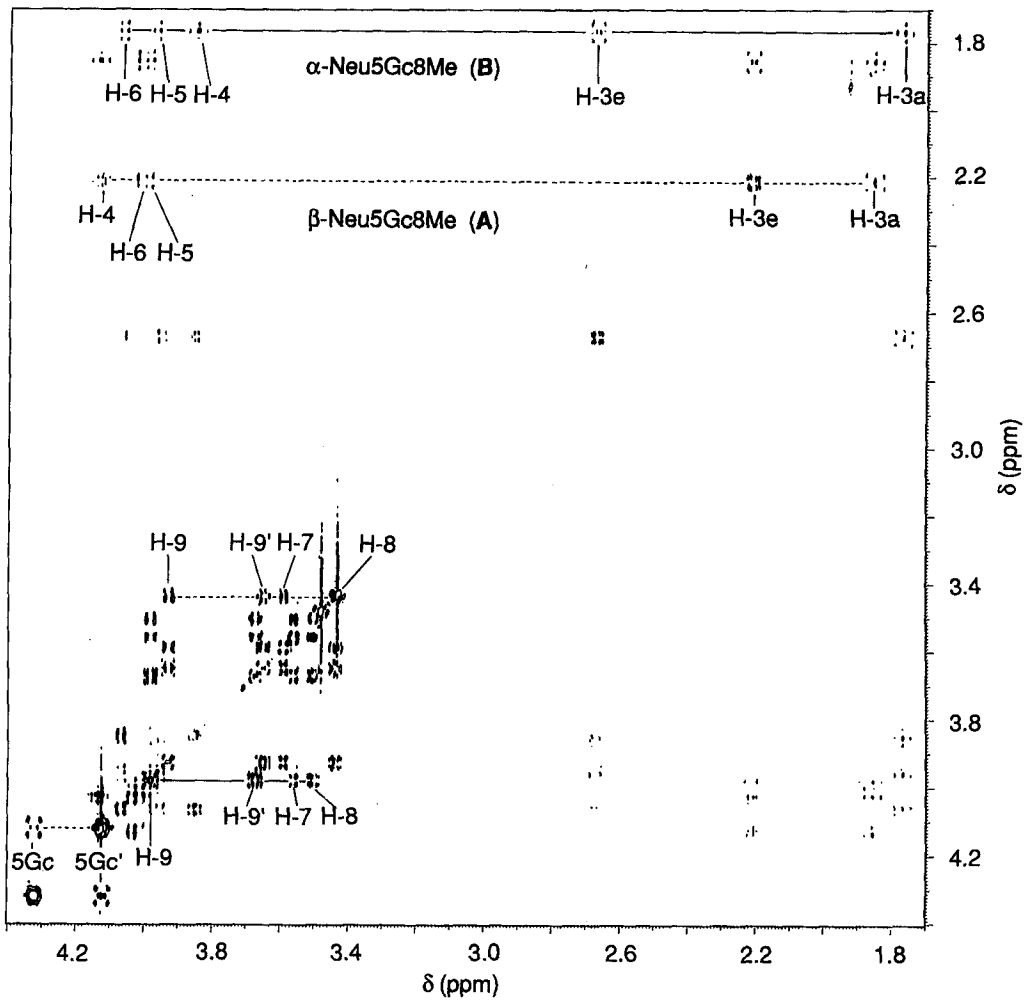


**Fig 3.** 2-D-HOHAHA spectrum of Neu5Gc8-Me-α(2→O5)-Neu5Gc-8Me recorded at 500 MHz in $^2H_2O$ at p$^2$H 7 and 300 K, with a mixing time of 120 ms. Tracks are drawn for H-3a and H-8 of the non-reducing α-Neu5Gc8Me residue (—) and for H-3a, H-8 and 5Gc' of the reducing β-Neu5Gc8Me residue (- - - -).

assignments are summarized in table II. The *O*-methyl groups of both residues resonate at different positions, namely, at δ 3.479 and δ 3.432. The *N*-glycoloyl methylene group of one Neu5Gc8Me residue appears as a singlet at δ 4.124 (2 H), whereas the *N*-glycoloyl methylene group of the other residue gives rise to two doublets at δ 4.320 (1 H) and δ 4.118 (1 H) with $^2J$ -15 Hz (figs 2B and 3), reflecting a distinct rotamer distribution.

To get more information about the type of linkage, a 2-D-ROESY spectrum in $^1H_2O$ was recorded, showing amide protons for the two Neu5Gc8Me residues at δ 8.26 and δ 8.31. Each of the amide protons could be assigned through intra-residual connectivities with H-4,5,6,7 of a specific Neu5Gc8Me unit. The rOe's observed between an amide proton and the protons of a specific *N*-glycoloyl methylene group made the correlation with the individual Neu5Gc8Me residues possible (table II), establishing that the methylene singlet is derived from the *N*-glycoloyl group of the non-reducing unit and the methylene doublets from that of the reducing unit. Finally, the 2-D-ROESY spectrum showed a small inter-residual connectivity for α-Neu5Gc8Me H-3a at δ 1.767 with the proton at δ 4.320 of the *N*-glycoloyl methylene group of β-Neu5Gc8Me. This connectivity, together with the observed large downfield shift of the involved proton of the *N*-glycoloyl methylene group of β-Neu5Gc8Me (Δδ +0.187, as compared to the *N*-glycoloyl methylene group of β-Neu5Gc8Me at δ 4.133), indicate that the non-reducing α-Neu5Gc8Me residue is glycosidically linked to the hydroxyl group of the *N*-glycoloyl substituent of the reducing β-Neu5Gc8Me unit. As compared to free β-Neu5Gc8Me, relatively small downfield shifts are observed for H-4 (Δδ +0.024), H-5 (Δδ +0.016), H-6 (Δδ +0.016), and H-7 (Δδ +0.030) of the reducing β-Neu5Gc8Me residue. The somewhat larger increments for H-4 and H-7 can be explained on the basis of molecular models of Neu5Ac, in which HO-7 forms a hydrogen bond with HN and OH-4 a hydrogen bond with C=O of the *N*-acetyl group [30]. In conclusion, the structure of the Neu5Gc8Me dimer is proposed to be Neu5Gc8Me-α(2→O5)-Neu5Gc8Me, predominantly occurring as the β-anomer.

The 1-D $^1$H-NMR spectrum (fig 2C) of the compound, having the same retention time as Neu5Ac-α(2→8)-Neu5Ac-α(2→8)-Neu5Ac on Mono Q (see working-up procedure B), showed the presence of three Neu5Gc8Me residues, of which two occur in the α-configuration (H-3a, δ 1.758 and δ 1.756; H-3e, δ 2.665 and δ 2.655) and one in the β-configuration (H-3a, δ 1.854; H-3e, δ 2.209). In table II the assignments of the various protons of the Neu5Gc8Me trimer are presented, taking into account the $^1$H-NMR data of Neu5Gc8Me-α(2→O5)-Neu5Gc8Me (see above) and 2-D-HOHAHA measurements in $^2H_2O$. The three methyl groups give rise to three separated singlets at δ 3.476, δ 3.488, and δ 3.434. The three *N*-glycoloyl methylene groups are represented by a singlet at δ 4.119 (2 H), a set of two doublets at δ 4.309 (1 H) and δ 4.099 (1 H) with $^2J$ –15 Hz, and a set of two doublets δ 4.317 (1 H) and δ 4.113 (1 H) with $^2J$ –15 Hz, and were assigned to a terminal, internal, and reducing Neu5Gc8Me residue, respectively. Based on these data the structure of the Neu5Gc8Me trimer is proposed to be Neu5Gc8Me-α(2→O5)-Neu5Gc8Me-α(2→O5)-Neu5Gc8Me, predominantly occurring as the β-anomer.

*S-adenosyl-L-methionine: N-acyl-D-neuraminate 8-O-methyltransferase activity*

Incubation of the crude enzyme preparations P1, P2, and S with[$^{14}$C]SAM in the absence of an exogenous sialic-acid-containing acceptor gave rise to a higher incorporation of radioactivity per mg protein in the membrane fractions P1 and P2 than in the supernatant S. Incubations with the exogenous substrates PSM and CM, followed by cleavage and isolation of the sialic acids, resulted in a higher specific incorporation of radioactivity into the sialic acids from the samples containing the membrane fractions than those containing the supernatant. For PSM within 3 h these values are 0.51 ± 0.16 pmol, 0.59 ± 0.06 pmol, 0.10 ± 0.05 pmol per mg protein for P1, P2, and S, respectively (mean values of three experiments). Free Neu5Ac and Neu5Gc (both > 95 % β-form) are not substrates for *O*-methyltransferase, which can be explained as a specificity of the enzyme for α-glycosidically linked sialic acids. The amount of radioactivity incorporated into the same amount of sialic acids from PSM, BSM, CM, and mouse serum using fraction P2 were 28, 11, 18, and 34 pmol (3 h, 46 mg protein), respectively, whereas endogenous substrate gave rise to an incorporation of radioactivity of 5 pmol. No difference in incorporated radioactivity between the α(2→3) and α(2→6) isomers of sialyllactose was observed. Radio-TLC analysis of the isolated sialic acids from PSM, BSM, CM, mouse serum, α(2→3)-sialyllactose, and α(2→6)-sialyllactose revealed, in addition to the formation of [$^{14}$C]Neu5Gc8Me derived partially (PSM, BSM, mouse serum) or only (CM, sialyllactoses) from endogenous substrate (fig 4), also the formation of [$^{14}$C]Neu5Ac8Me (see fig 4 for PSM and CM). After incubation of immobilized PSM, [$^{14}$C]Neu-5Ac8Me, derived from exogenous substrate, and a small amount of [$^{14}$C]Neu5Ac8Me were detected.

With a crude membrane-bound enzyme preparation, solubilized by detergent and sonication, and PSM immobilized on Sepharose 4B as substrate, the pH optimum of the enzyme was determined to be 8.9. The

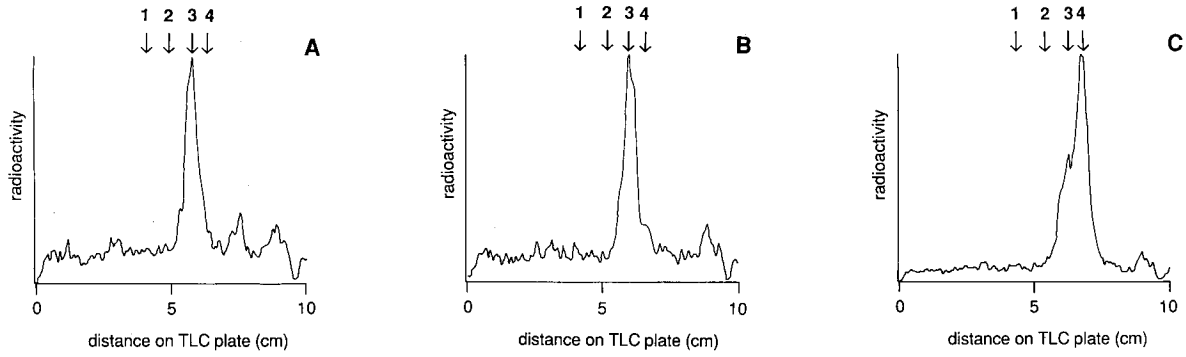


**Fig 4.** Radio-TLC analysis in solvent system i of *N*-acyl-8-*O*-[$^{14}$C]methylneuraminic acid, isolated after acid hydrolysis of sialic acids from endogenous substrate and from PSM and CM after incubation with crude 8-*O*-methyltransferase from fraction P2 and [$^{14}$C]SAM. Arrows 1, 2, 3, and 4 indicate the migration positions of Neu5Gc, Neu5Ac, Neu5Gc8Me, and Neu5Ac8Me, respectively.

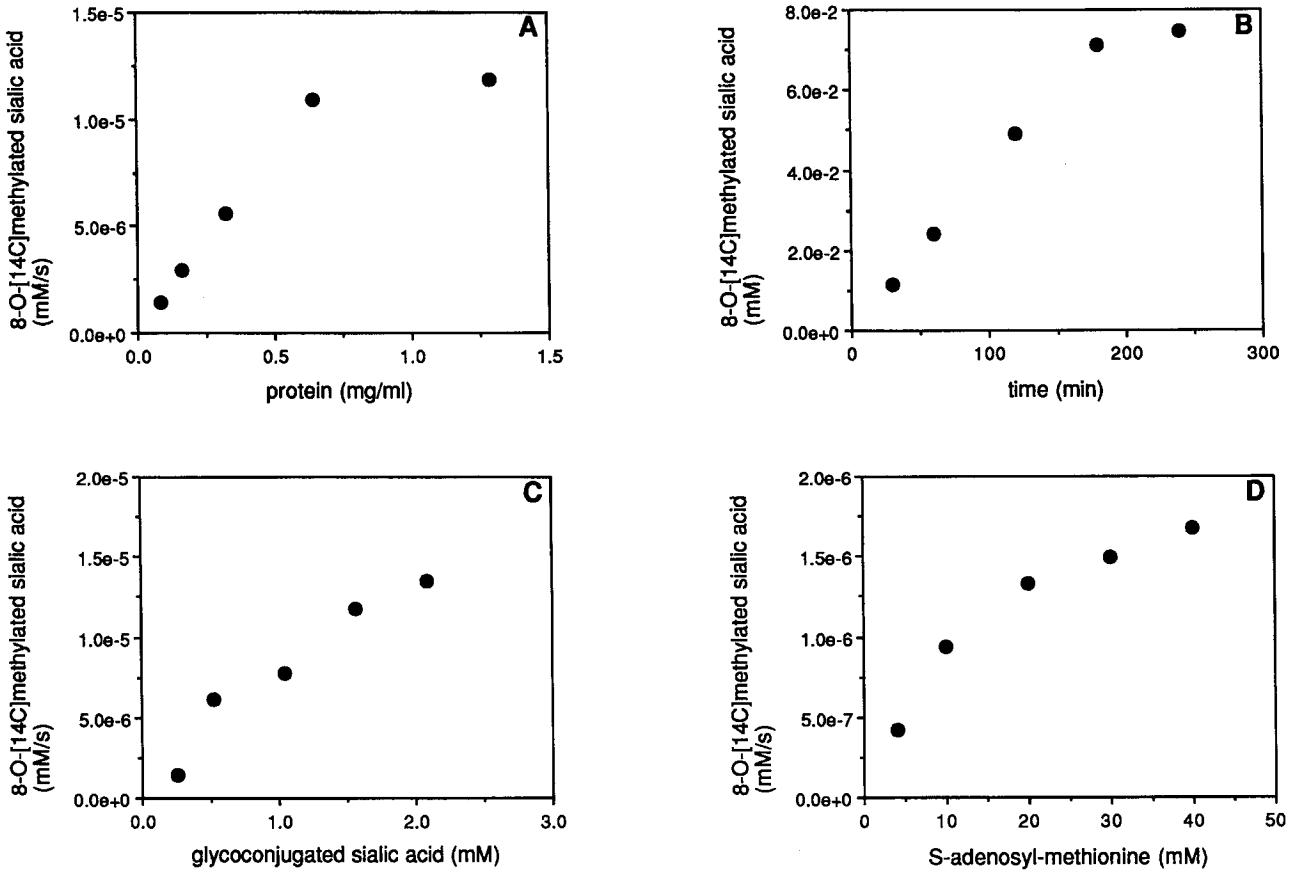


**Fig 5.** Protein (**A**), time (**B**), sialic acid (**C**), and *S*-adenosyl-L-methionine (**D**) dependency of *N*-acylneuraminate 8-*O*-methyltransferase by assaying the formation of *N*-acyl-8-*O*-[$^{14}$C]methylneuraminic acid in immobilized PSM. Further details are described in the text.

reaction with PSM (1.2 mM conjugated sialic acid) in the presence of 2.5 mM [$^{14}$C]SAM was linear with the enzyme preparation up to a protein concentration of 0.6 mg/ml (fig 5A), and up to 3 h of incubation (fig 5B). At 5 mM SAM, the sialic acid dependency (fig 5C) and at 1.3 mM conjugated sialic acid the SAM dependency of the enzyme preparation was assayed (fig 5D).

In additional experiments with particulate enzyme of fraction P2 and PSM as acceptor under the conditions described, it turned out that the *O*-methyltransferase activity can be inhibited by *S*-adenosyl-L-homocysteine and EDTA. *S*-Adenosyl-L-homocysteine at 4 mM reduced the enzyme activity by 80 %, whereas EDTA at 1 mM reduced the enzyme activity by 60 % and at 10 mM almost complete inhibition was found.

*CMP-N-acetyl-D-neuraminate monooxygenase activity*

CMP-Neu5Ac monooxygenase activity was detected in the supernatant fraction S'. It revealed an optimum pH at about 7, as deduced from the specific activities at pH 6.2, 6.7, 7.2, and 7.7 being 5, 29, 24, and 11 fmol/min per mg protein, respectively (with approximately 15 % error between duplicates). A small amount of activity was detectable in the pellet fraction P' at pH 7.0 (specific activity 3.7 fmol/min per mg protein). The presence of about 98 % of the total activity in the 120 000 *g* supernatant S' indicates that the monooxygenase is extractable as a soluble protein.

## Discussion

The sialic acids found so far in the starfish *A rubens* are Neu5Ac and Neu5Gc, and a series of 8-*O*-methylated *N*,*O*-acylneuraminic acids, namely, Neu5Ac 8Me, Neu5Gc8Me, Neu9Ac5Gc8Me, and Neu5,9Ac$_2$ 8Me. Neu5Gc8Me was shown to be a constituent of a main ganglioside isolated from the hepatopancreas tissue of *A rubens* [3]. In case of *A amurensis* a glycolipid was isolated with a terminal Neu5Gc8Me-α(2→3)-Neu5Gc8Me-α(2→6)-GalNAc sequence [5]. An unusual glycosidic linkage for a terminal dimer of Neu5Gc8Me was proposed for a disialoganglioside from the starfish *A japonica* [6, 37], namely, Neu5Gc8Me-α(2→O5)-Neu5Gc8Me. Recently, in a preliminary communication also longer sialo-oligomers of the latter type were suggested to occur in gangliosides from the starfish *A amurensis* [38]. In this study, we have identified after mild acid hydrolysis such di- and tri-sialo-oligomers from the starfish *A rubens* to be Neu5Gc8Me-α(2→O5)-Neu5Gc8Me and Neu5Gc8Me-α(2→O5)-Neu5Gc8Me-α(2→O5)-Neu5Gc8Me, using FAB-MS, and 1-D and 2-D NMR methods.

The metabolic studies presented in this report demonstrate that the biosynthesis of 8-*O*-methylated sialic acids occurs by an enzymatic transfer of a methyl group from *S*-adenosyl-L-methionine to O-8 of *N*-acylneuraminic acid. It is known that *O*-methyltransferases are SAM dependent and are inhibited by their transmethylation product, *S*-adenosyl-L-homocysteine [39-42]. These earlier findings hold also for the enzyme activity described in this study. Furthermore, the enzyme activity is inhibited in the presence of EDTA, which points to a metal-ion dependency of the enzyme. As is evident from the presented results, the *N*-acylneuraminate 8-*O*-methyltransferase is a membrane-bound enzyme. The presence of the activity especially in the membrane fraction obtained at 120 000 *g*, together with the sialic acid acceptor specificity, suggests that the transmethylation may occur in the Golgi-complex.

The presence of CMP-Neu5Ac monooxygenase activity in the soluble protein fraction conforms with previous observations in vertebrate tissues [18, 19] suggesting that the hydroxylation of CMP-Neu5Ac into CMP-Neu5Gc is conserved throughout evolution. The incorporation of an additional OH-group in CMP-Neu5Gc by CMP-Neu5Ac monooxygenase gives rise to a new potential glycosylation site in the growing carbohydrate chains.

Based on the various data, it can be suggested that the order of events in the biosynthesis of Neu5Gc8Me in glycoconjugates is as follows. It starts with the synthesis of Neu5Ac [43, 44], as is supported by the finding of Neu5Ac among the total sialic acids from *A rubens*. After activation of Neu5Ac into CMP-Neu5Ac, CMP-Neu5Ac is presumably for the greater part hydroxylated in the cytoplasm giving rise to CMP-Neu5Gc. After transport of the CMP-glycosides into the Golgi-apparatus, possibly by a transport system analogous to that in mouse liver [45], the sialic acids are transferred onto the growing glycan chains, where they may be *O*-methylated.

## Acknowledgments

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