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# FUCOPYRANOSYL-(1 → 4)-*N*-GLYCOLYLNEURAMINIC ACID, A CONSTITUENT OF GLYCOPROTEINS OF THE CUVIERIAN TUBULES OF THE SEA CUCUMBER *HOLOTHURIA FORSKALI* DELLA CHIAJE

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**Analysis of the sialic acid fraction obtained by mild acid hydrolysis of the Cuvierian tubules of the sea cucumber *Holothuria forskali* Della Chiaje revealed the presence of *N*-glycolylneuraminic acid and fucopyranosyl-(1 → 4)-*N*-glycolylneuraminic acid. Furthermore, methylation analysis of a pronase-digest of the tubules demonstrated that all *N*-glycolylneuraminic acid residues are substituted at C-4, explaining the earlier reported resistance of sialic acid in tubules to cleavage by neuraminidase.**

In a previous study on the carbohydrate moieties of the Cuvierian tubules of the sea cucumber *Holothuria forskali* Della Chiaje, Isemura et al. [1] were confronted with an unknown sialic acid. The so-called Hf-neuraminic acid was found to occur in a high molecular weight sulfated glycopeptide fraction, prepared by pronase digestion of the mentioned tubules. The sialic acid proved to be resistant to enzymic cleavage by neuraminidase of *Clostridium perfringens*. After treatment with mild alkali (to remove hypothetical *O*-acyl groups), the behaviour to the enzyme did not change. It was possible to isolate the Hf-neuraminic acid after mild acid hydrolysis of the glycopeptide fraction, but a final structure could not be proposed.

In the present paper, the occurrence of fucopyranosyl-(1 → 4)-*N*-glycolylneuraminic acid in the carbohydrate chains of glycoproteins of the tubules will be reported, and its relation with 'Hf-neuraminic acid' will by discussed.

Isolation of a low molecular weight sialic acid-positive fraction by partial hydrolysis of tubules was done as follows: minced tubules of the sea cucumber [1] (0.6 g) were treated with 50 ml formic acid, pH 2, at 70°C for 2h [2]. After dialysis against 400 ml distilled water at 4°C for 6 h, the suspension of the tubules was lyophilized. The residue was treated with 50 ml 0.1 M HCl at 80°C for 1 h, and dialyzed again. The combined diffusates (800 ml) were concentrated to 5 ml by rotary evaporation in vacuo at 30°C and subjected to ion-exchange chromatography at 4°C. The solution was passed through a column of Dowex 50W-X8, $H^+$ form, 50–100 mesh (10 × 1 cm). The resin was washed with 175 ml distilled water and the total eluate was concentrated to 4 ml by rotary evaporation. Then, the anionic material was adsorbed on a column of Dowex-2X8, $HCOO^-$ form, 50–100 mesh (10 × 1 cm). The column was washed

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Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Fuc, fucose; *p*, pyranose.

with 75 ml distilled water, and anionic compounds were eluted with 1 M formic acid. The eluate was monitored for sialic acid with the orcinol/$Fe^{3+}$/HCl reagent [3]. The sialic acid-positive fractions were pooled and lyophilized.

Permethylation of reduced sialic acid-positive fraction was done as follows: An aliquot of the fraction (corresponding to 0.3 g tubules) was dissolved in 3 ml distilled water and reduced with 30 mg $NaBH_4$ at room temperature for 2 h. After neutralisation with Dowex 50W-X8, $H^+$ form, the resulting solution was evaporated in vacuo. Boric acid was removed by three consecutive co-evaporations with 5 ml methanol. The residue was dried over $P_2O_5$ in a desiccator, dissolved in 1 ml methylsulfoxide and permethylated with 1 ml 2 M sodium methylsulfinylmethanide in methylsulfoxide/1 ml methyl iodide [4,5]. The permethylated preparation obtained by chloroform extraction was directly subjected to gas-liquid chromatography-mass spectrometry without additional purifications.

Methylation analysis of a pronase digest of tubules: Tubules of the sea cucumber were digested with pronase essentially as described earlier [1]. The lyophilized pronase digest (0.1 g) was then dissolved in 10 ml methylsulfoxide and permethylated with 10 ml 2 M sodium methylsulfinylmethanide in methylsulfoxide/10 ml methyl iodide [4,5]. Triphenylmethane was used as an indicator for the presence of excess of base [6]. The permethylated pronase digest, obtained by chloroform extraction, was purified by gel filtration on a column (40 × 2 cm) of Sephadex LH-20 using chloroform/ethanol (1 : 1, v/v) as solvent. The eluate was monitored for carbohydrate employing the orcinol/$H_2SO_4$ procedure. Carbohydrate-positive fractions were pooled and evaporated in vacuo. The residue was dried over $P_2O_5$ in a desiccator and treated with 1.5 ml 0.5 M methanolic-HCl at 85°C for 24 h. The reaction mixture was then neutralized with $Ag_2CO_3$, treated with acetic anhydride and trimethylsilylation reagents as described earlier [7,8]. The final preparation was analysed for methylated sialic acid methyl ester methyl glycosides using gas-liquid chromatography-mass spectrometry.

Chromatography and mass spectrometry: Sialic acids were analysed by thin-layer chromatography on 0.1 mm cellulose plates (DC-Fertigplatten; E. Merck, Darmstadt, F.R.G.) using the solvent system *n*-butanol/*n*-propanol/0.1 M HCl (1 : 2 : 1, v/v) [9].

Sugar analyses after methanolysis, *N*-(re)acetylation, and trimethylsilylation [7] were carried out by capillary gas-liquid chromatography on SE-30 (column: 25 m × 0.35 mm inner diameter). The oven temperature was programmed from 130 to 220°C at 2°C/min.

Gas-liquid chromatography of trimethylsilylated sialic acid methyl esters on 3.8% SE-30 was performed as described previously [10–12]. Combined gas-liquid chromatography-mass spectrometry of trimethylsilylated sialic acid methyl esters [10–12], trimethylsilylated partially methylated sialic acid methyl ester methyl glycosides [12–14], and permethylated (oligosaccharide-)alditols were carried out on a Carlo Erba GC/Kratos MS 80/Kratos DS55 system (electron energy, 70 eV; accelerating voltage, 2.7 kV; ionising current, 100 μA; ion-source temperature, 225°C; stationary phase, 3% OV-1 on Gaschrom Q, 80–100 mesh). The oven temperature was selected according to the type of compounds analysed.

To determine the monosaccharide composition of the Cuvierian tubules of *Holothuria forskali* Della Chiaje, the tubules are methanolysed, *N*-(re)acetylated and trimethylsilylated [7]. Gas chromatographic analysis of the obtained trimethylsilylated methyl glycosides demonstrated the presence of fucose, mannose, galactose, glucose, *N*-acetylgalactosamine, *N*-acetylglucosamine, glucuronic acid, and *N*-acetylneuraminic acid (Neu5Ac) in a molar ratio of 1.00 : 0.22 : 0.37 : 0.56 : 0.23 : 0.12 : 0.19 : 0.15. The identification of the uronic acid as glucuronic acid has not been reported earlier [1].

The method employed for the sugar analysis does not reveal original *N*-/*O*-acyl and *O*-sulfate substituents of the monosaccharide residues. With respect to sialic acid, this means that the native sialic acid of the tubules may be different from Neu5Ac. Therefore, the tubules were also subjected to mild acid hydrolysis, known to release sialic acids without cleaving the *N*-acyl group, but to split partially the *O*-acyl groups [2]. The released sialic acid-positive fraction subjected to thin-layer chromatography gave rise to a single

distinct band with the same $R_F$ value as the reference *N*-glycolylneuraminic acid (Neu5Gc). An aliquot of the fraction was further analysed by gas-liquid chromatography-mass spectrometry [10–12] after esterification with diazomethane and trimethylsilylation, and revealed only one sialic acid derivative with the same gas chromatographic retention time ($R_{Neu5Ac}$ 1.81; internal standard, trimethylsilylated Neu5Ac methyl ester) and the same mass spectrum [9] as the trimethylsilylated methyl ester of reference Neu5Gc. In conclusion, the fraction contains free Neu5Gc.

Another portion of the fraction was analysed for additional carbohydrates. As expected, sugar analysis showed the main compound to be Neu5Gc (measured as Neu5Ac; see above). Also fucose was observed, although in much lower amounts; ratio Neu5Gc/Fuc, 1.0 : 0.2. Furthermore, relatively small amounts of xylose, glucose, galactose and *N*-acetylgalactosamine were detected ($\leqslant 0.07$). The data indicated that in addition to Neu5Gc, the fraction contains also small amounts of other anionic carbohydrate compounds, e.g., oligosaccharides containing Neu5Gc.

Gas-liquid chromatographic-mass spectrometric analysis of the remaining part of the fraction, after $NaBH_4$-reduction and permethylation, revealed the presence of three sialic acid derivatives. Fig. 1 shows the mass spectrum of the main compound, i.e. the permethylated alditol of Neu5Gc. The mass spectra of the other two compounds, in combination with the sugar analysis data of the fraction, led to the structures of the permethylated epimeric disaccharide-alditols of Fuc*p*-(1 → 4)-Neu5Gc (Fig. 2). The alditol with the highest retention time was only present in a very small amount. The pyranose ringform of fucose (Fuc) is indicated by the presence of the relatively intense peak at *m/z*

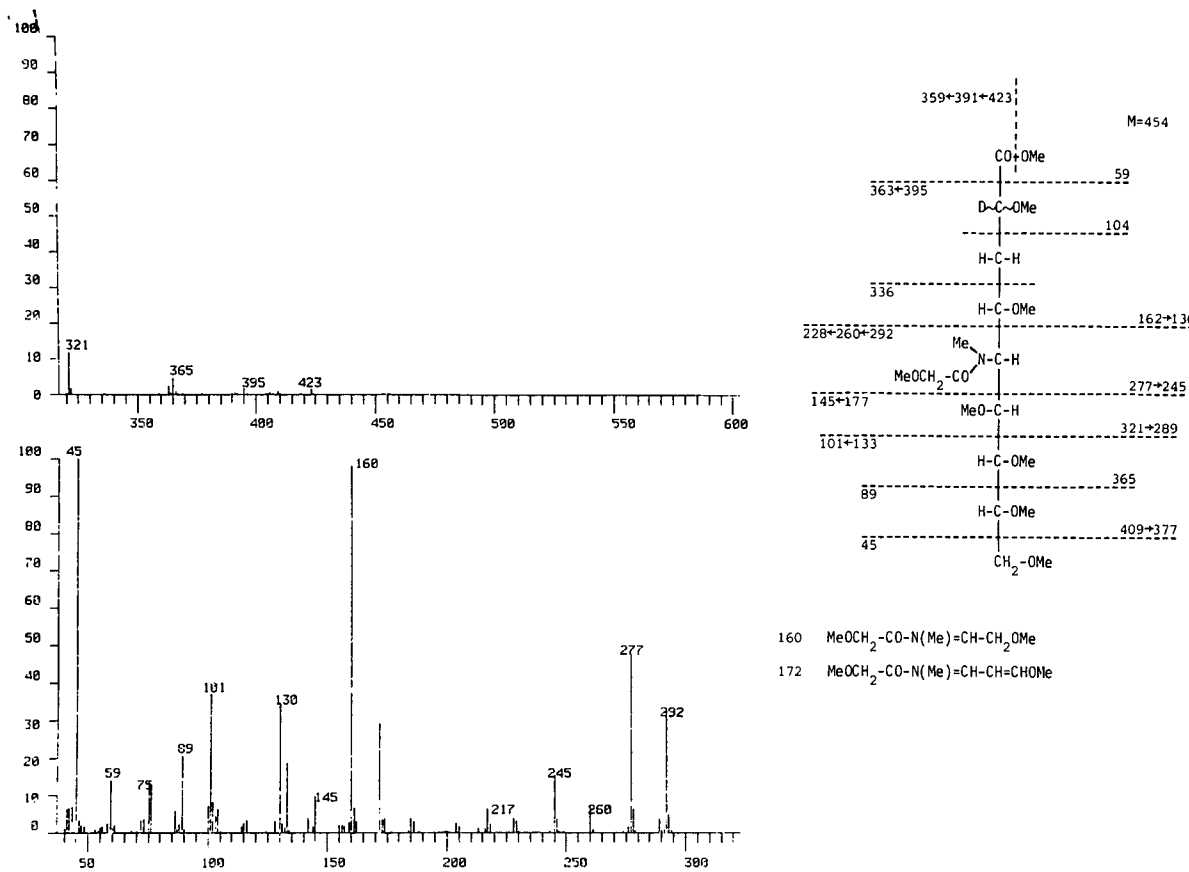



Fig. 1. Mass spectrum at 70 eV and structure with some peak assignments of the permethylated alditol of Neu5Gc.

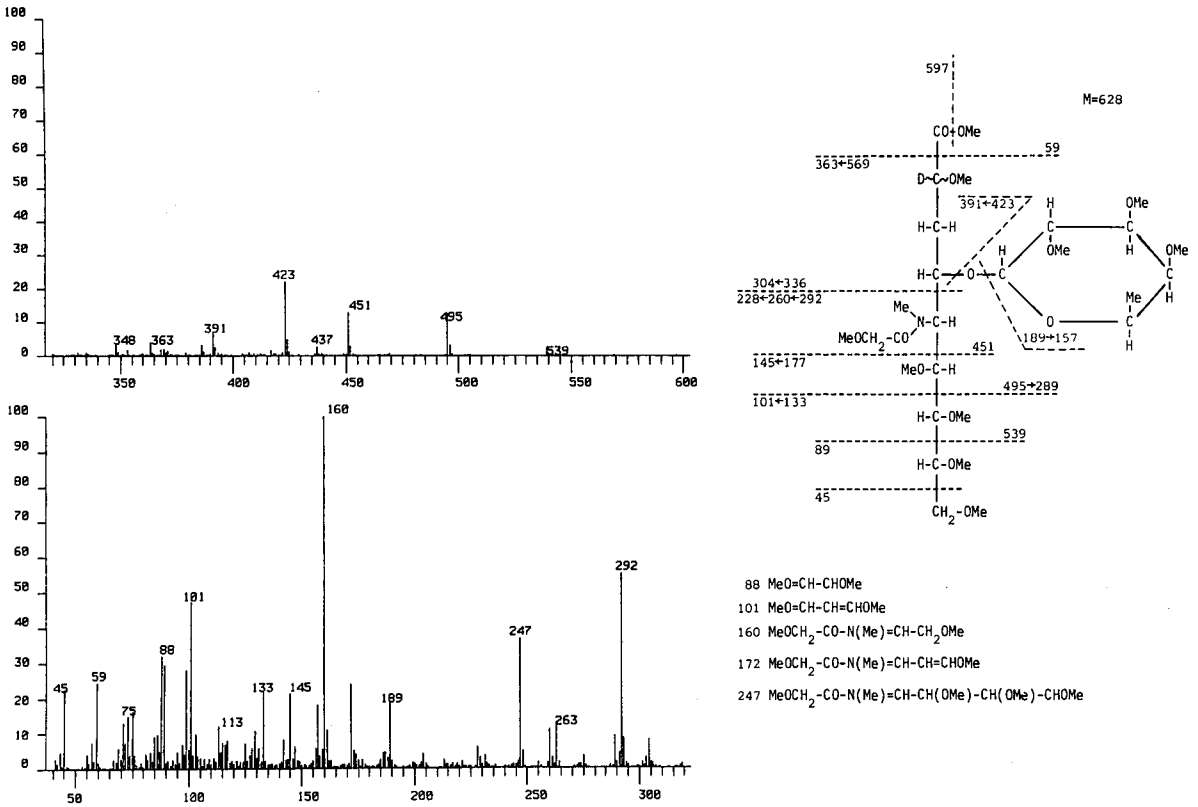
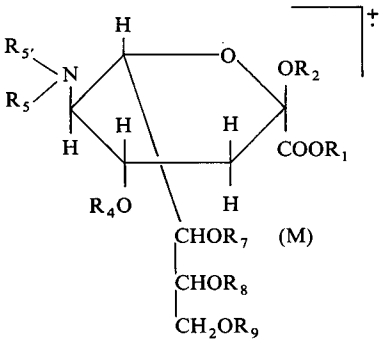



Fig. 2. Mass spectrum at 70 eV and structure with some peak assignments of the main permethylated alditol of Fuc*p*-(1 → 4)-Neu5Gc.

88, originating mainly from the C-2/C-3 part of the 6-deoxyhexose [7,15,16] (compare Figs. 1 and 2). The finding of Fuc*p*-(1 → 4)-Neu5Gc demonstrated the presence of 4-substituted Neu5Gc residues in the carbohydrate chains of glycoproteins of the tubules.

To obtain more information on the substitution pattern of Neu5Gc, a pronase digest of tubules was directly subjected to methylation analysis. After permethylation, the methylated product was solvolysed with methanolic HCl. Because of the possible cleavage of methylated *N*-glycolyl groups [17], the neutralized methanolic solution was treated with acetic anhydride to *N*-acetylate $NH(CH_3)$ groups. After trimethylsilylation, the sample was analysed by gas-liquid chromatography-mass spectrometry. For the identification of methylated sialic acids, use was made of a series of characteristic fragment ions A—H (see Scheme 1), which indicate the molecular weight, the *N*-acyl substituent and the type, number and position of the *O*-protecting groups [12–14]. On the basis of the data obtained by mass spectrometry (mass fragmentography of characteristic fragment ions), two sialic acid derivatives could be identified. The main component was established to be 7,8.9-tri-*O*-methyl-4-*O*-trimethylsilyl-*N*,*N*-acetyl,methyl-neuraminic acid methyl ester methyl glycoside (A: *m/z* 450; B: *m/z* 406; C: *m/z* 376; D: *m/z* 254; E: *m/z* 259; F: *m/z* 89; G: *m/z* 187; H: *m/z* 298). Its mass spectrum was in agreement with that published earlier [14]. Furthermore, a small amount of the corresponding derivative with a methylated *N*-glycolyl group was suggested to be present. Because of the occurrence of Neu5Gc as the only sialic acid present in the tubules (see above), the structures of both sialic acid derivatives agree with that of 4-substituted Neu5Gc residues, present in the tubules.

In conclusion, the data presented in this paper



Scheme 1. Survey of the selected fragment ions A–H in sialic acid derivatives. $R_1$, $R_2$, $R_{5'}$, $R_7$, $R_8$, $R_9 = CH_3$; $R_5 = COCH_3$ or $COCH_2OCH_3$; $R_4 = Si(CH_3)_3$. A, M – $CH_3$; B, M – $COOR_1$; C, M – $CHOR_8CH_2OR_9$; D, M – $CHOR_8CH_2OR_9$ – $R_2OH$ – $R_4OH$; E, M – $CHOR_7CHOR_8CH_2OR_9$ – $NHR_5R_{5'}$; F, $CHOR_8CH_2OR_9$; G, $CHOR_4CHNR_5R_{5'}$; H, M – $CH_2OR_9$ – $R_4OH$ – $R_7OH$.

clearly demonstrate the presence of a Fuc*p*-(1 → 4)-Neu5Gc unit in the glycoprotein(s) of the Cuvierian tubules of the sea cucumber *Holothuria forskali* Della Chiaje. In view of this result, it is reasonably to propose that the structure of the unknown Hf-neuraminic acid [1], which was characterized by a $R_F$ value on paper chromatography that was lower than that of Neu5Gc, is identical to this disaccharide. Fuc*p*-(1 → 4)-Neu5Gc has been reported earlier for the egg jelly coat of the sea urchin *Pseudocentrotus depressus* (Misaki) [18].

The detection of only 4-substituted Neu5Gc residues in the methylation analysis of the pronase-digest of tubules explains the complete resistance of this sialic acid towards the action of sialidase, as observed earlier [1] (see also [19]). Because of the identification of Fuc*p*-(1 → 4)-Neu5Gc, it may be assumed that all Neu5Gc residues occur in internal positions. However, the glycoprotein material also contains sulfate groups [1], which have not been located so far. It is known that only marginal desulfation occurs when *O*-sulfated carbohydrates are subjected to alkaline conditions as applied during the permethylation of the pronase digest [20]. This means, that 4-substituted Neu5Gc as found in the methylation analysis of the pronase-digest, may also originate from terminal 4-*O*-sulfated Neu5Gc.

The various results indicate the occurrence of a new type of carbohydrate chain in glycoproteins, which is currently under investigation.

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