Eur. J. Biochem. *152*, 459–463 (1985)

Structural parameters and natural occurrence of 2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid

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(Received February 26/July 10, 1985) – EJB 85 0191

1. 2-Deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid has been found to occur in porcine, bovine and equine submandibular glands as well as in the urine of pig, horse and rat. This novel, unsaturated sialic acid was isolated by gel filtration and ion-exchange chromatography. Final purification was achieved by column chromatography or by preparative thin-layer chromatography on cellulose. The structural analysis was performed by combined capillary gas-liquid chromatography/mass spectrometry. The various data were compared with those from synthetic 2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid. Besides of the unsaturated *N*-glycoloylated sialic acid, also the corresponding *N*-acetylated derivative was present in the materials analyzed.

2. The inhibitory effect of 2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid on *Vibrio cholerae* sialidase using *N*-acetylneuraminyl-(α2→3)-lactose as substrate is slightly higher (50% inhibition at 10 μM) when compared with 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (50% inhibition at 15 μM).

Among the various sialic acids occurring in nature [2, 3] the only unsaturated species found up to now is 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (5-acetamido-2,6-anhydro-2,3,5-trideoxy-D-glycero-D-talo-non-2-enonic acid, Neu2en5Ac). This sialic acid lacks the glycosidic hydroxyl group at C-2. Neu2en5Ac has been found in low concentrations in serum, saliva and urine of healthy individuals [4]. It occurs in much higher concentrations in the serum and urine of a sialuria patient [4, 5], wherein the substance was discovered. Beau et al. [6] demonstrated that Neu2en5Ac could be obtained from CMP-Neu5Ac at pH values above 7, probably in a non-enzymic elimination reaction. Correspondingly, other 2-deoxy-2,3-didehydro-sialic acids may also originate from their respective CMP-glycosides already described [7, 8]. Recently, it has been postulated for mammalian brain that Neu2en5Ac might be obtained enzymically from sialoglycoconjugates and not from CMP-Neu5Ac [9].

As CMP-Neu5Gc has been detected in porcine submandibular glands [7], and Neu5Gc is the predominant sialic acid in porcine tissues [2, 3, 10], the occurrence of Neu2en5Gc in this tissue was anticipated. Here we report on the identification of Neu2en5Gc in porcine submandibular gland and in other biological materials. Furthermore, its inhibitory effect on sialidase activity will be compared with that of Neu2en5Ac.

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Preliminary data were presented at the Joint Meeting of the Nordic Biochemical Societies and the *Gesellschaft für Biologische Chemie* in Damp 2000 near Kiel, FRG, September 27–29, 1982 [1].

Abbreviations. Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid; Neu2en5Ac, 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid; Neu2en5Gc, 2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid; GLC-MS, gas-liquid chromatography/mass spectrometry; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; Me_3Si, $SiMe_3$, trimethylsilyl.

Enzyme. Sialidase or acylneuraminyl hydrolase (EC 3.2.1.18).

MATERIALS AND METHODS

Neu5Ac was prepared from edible bird's nest substance [10, 11], while Neu2en5Ac was bought from Boehringer, Mannheim. Neu5Gc was isolated from porcine submandibular gland mucin [10] and *N*-acetylneuraminyl-(α2→3)-lactose was obtained from bovine colostrum [12]. *Vibrio cholerae* sialidase (1 U/ml) was bought from Behringwerke, Marburg; Sephadex G-25 from Pharmacia, Uppsala; Bio-Gel P-2 (minus 400 mesh), Dowex 50 WX4, H^+-form (either 20–50 mesh or 100–200 mesh), Dowex 2X8, Cl^--form (200–400 mesh), Aminex A-28 (particle size 9 μm), and Aminex A-29 (particle size 7 μm) from Bio-Rad, Munich; Cellulose MN 2100 ff from Macherey-Nagel & Co., Düren; silica gel (70–230 mesh) and thin-layer plates (0.2 mm cellulose or 0.5 mm silica gel) from E. Merck, Darmstadt. The following derivatization reagents were used in GLC analysis: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Macherey-Nagel & Co., Düren), hexamethyldisilazane and trimethylchlorosilane (E. Merck, Darmstadt). The glycoloylation reagent 1,3-dioxolane-2,4-dione was prepared as described [13, 14]. All other reagents were of analytical grade.

Isolation of Neu2en5Gc from natural material

The isolation procedures are slight modifications of those described in [10, 15]. For the colorimetric determination of sialic acid micro-adaptations [10] of the periodic acid/thiobarbituric acid and orcinol/HCl/Fe^{3+} assays were used.

Isolation from tissues. Porcine or bovine submandibular glands (200 g, each) or equine submandibular glands (50 g) obtained from the local slaughter house were homogenized and then dialyzed four times for 8 h each against a 10-fold

volume of water. The permeates containing the free sialic acids were rinsed through a column with 50 ml Dowex 50 WX4, H^+-form, 100–200 mesh. Sialic acids in the effluent were adsorbed on a column with 10 ml Dowex 2X8, 200–400 mesh, $HCOO^-$-form. After washing with water, sialic acids were eluted from the anion-exchange resin with 80 ml 0.2 M pyridinium-acetate buffer pH 5.4. The eluate was concentrated to 1 ml by rotary evaporation, applied to a column (1.5 × 20 cm) with Bio-Gel P-2, minus 400 mesh, and eluted with 50 ml water. The fractions (2 ml) being positive for sialic acids in the orcinol/Fe^{3+}/HCl assay [10] were pooled and concentrated by lyophilization. Further separation of the sialic acids was achieved on a cellulose column (1 × 40 cm) using butan-1-ol/propan-1-ol/water (1:2:1, by vol.) [11]. A screening of the sialic acid-containing fractions (1 ml) was carried out by HPLC and capillary GLC(-MS).

Isolation from urines. Urine samples of 1 l each from pig, horse or rat were concentrated to 100 ml by rotary evaporation and applied to a column of Sephadex G-25 (10 × 70 cm), which was eluted with water [15]. Fractions (20 ml) containing sialic acids were rinsed through a column with 500 ml Dowex 50 WX4, 20–50 mesh, H^+-form. The sialic acids of the eluate were adsorbed on 200 ml Dowex 2X8, 200–400 mesh, $HCOO^-$-form, washed with water, eluted with 1.6 l 0.2 M pyridinium-acetate buffer pH 5.4 and concentrated. They were further purified by filtration on Bio-Gel P-2 as described above, followed by a second chromatography on Dowex 2X8, 200–400 mesh, $HCOO^-$-form (10 ml) using a gradient of 0–0.2 M pyridinium-acetate buffer pH 5.4 for elution. A screening of the sialic acids present in the freeze-dried fractions was carried out by HPLC and TLC. To facilitate GLC-MS analysis, fractions were further purified by preparative cellulose thin-layer chromatography at 4 °C using butan-1-ol/propan-1-ol/0.1 M HCl (1:2:1, by vol.) as solvent.

*Preparation of 2-deoxy-2,3-didehydro-*N*-glycoloylneuraminic acid*

Neu2en5Gc was prepared in analogy to the synthesis of Neu2en5Ac as reported by Nöhle et al. [16]. To this end Neu5Gc was converted into the penta-*O*-*p*-nitrobenzoyl derivative of its methyl ester. After transformation to the 2-bromo derivative and subsequent elimination of HBr by treatment with triethylamine, the protecting groups were removed with aqueous sodium hydroxide. The resulting Neu2en5Gc was purified on Dowex 2X8, $HCOO^-$-form.

Other procedures for the preparation of Neu2en5Ac have been applied successfully for obtaining Neu2en5Gc: CMP-Neu5Gc as precursor [6, 17], 2-deoxy-2,3-didehydroneuraminic acid as synthon [14, 16], and incubation of Neu5Gc methyl ester with acetic anhydride/conc. sulfuric acid [18]. In the latter procedure the C-4 epimer of Neu2en5Gc is formed as a side-product.

All four synthetic routes produced Neu2en5Gc in similar overall yields of about 30%.

High performance liquid chromatography

Sialic acids were analyzed on Aminex A-28 or A-29 using 0.75 mM Na_2SO_4 at a flow-rate of 0.5 ml/min [19].

Capillary gas-liquid chromatography

Isolated and synthetic sialic acids (100 µg samples) were trimethylsilylated by dissolving in 30 µl dry pyridine, addition of 50 µl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide, thorough mixing and keeping at room temperature for 30–60 min [20]. The resulting per-*O*-$SiMe_3$, (*N*-$SiMe_3$) derivatives were analyzed on a Packard Becker gas chromatograph (model 428), using OV-101 and OV-17 fused-silica capillary columns (25 m × 0.4 mm and 20 m × 0.4 mm, respectively) with flame-ionization detection. The oven temperature was programmed from 150 °C to 280 °C (OV-101) or from 150 °C to 240 °C (OV-17) at 2 °C/min. The methyl esters of Neu2en5Gc and its C-4 epimer obtained from the synthesis according to Kumar et al. [18] were trimethylsilylated with hexamethyldisilazane/trimethylchlorosilane/pyridine (1:1:5, by vol.). The Me_3Si ethers were analyzed on a CPsil 5 WCOT fused-silica capillary column using flame-ionization detection, under the conditions described for the GLC analysis of trimethylsilylated methyl glycosides [21].

Gas-liquid chromatography/mass spectrometry

GLC-MS of sialic acid derivatives was performed on a Varian GC 3700/Varian MAT 44S/Varian Spectro Spin SS 200 system, operating in the electron impact mode: electron energy, 70 eV; ionization current, 0.5 mA; ion-source temperature, 220 °C; OV-101 fused-silica capillary column (25 m × 0.4 mm) using the oven temperature program, 150 °C to 280 °C at 2 °C/min; OV-17 fused-silica capillary column (20 m × 0.4 mm) using the oven temperature program, 150 °C to 240 °C at 2 °C/min.

Inhibition of sialidase by Neu2en5Gc

A 5 mM solution (20 µl) of *N*-acetylneuraminyl-(α2→3)-lactose in water was added to 70 µl of a solution containing various amounts of Neu2en5Gc or Neu2en5Ac in 0.02 M phosphate buffer pH 5.4. The final concentrations of the unsaturated sialic acids ranged from 1 µM to 10 mM; controls without inhibitors were run in parallel. After the addition of 10 µl of *Vibrio cholerae* sialidase (10 mU) the mixtures were incubated for 30 min at 37 °C and analyzed for released Neu5Ac by the periodic acid/thiobarbituric acid assay [10] and by HPLC [19].

RESULTS AND DISCUSSION

Mass spectrometry of synthetic Neu2en5Gc derivatives

In Fig. 1 the mass spectrum of the Me_3Si ester, per-*O*-$SiMe_3$ ether of Neu2en5Gc (compound 1) is shown. The most characteristic *m/z* values of compound 1, the Me_3Si ester, per-*O*-$SiMe_3$ ether, *N*-$SiMe_3$ derivative of Neu2en5Gc (compound 2), and the methyl esters, per-*O*-$SiMe_3$ ethers of Neu2en5Gc (compound 3) and its C-4 epimer (compound 4) together with intensities and explanations are summarized in Table 1.

The earlier reported mass spectrometric characteristics which define the methyl ester, per-*O*-$SiMe_3$ ether derivative of Neu2en5Ac [4, 5, 21] were also detected in the mass spectra of compounds 1 and 3, and in the spectrum of the Me_3Si ester, per-*O*-$SiMe_3$ ether derivative of Neu2en5Ac [9]. The spectra are dominated by a fragment ion *a*, occurring at *m/z* 227 for methyl ester derivatives and at *m/z* 285 for Me_3Si ester derivatives.

Another intense fragment ion corresponds with the structure $R'\overset{+}{N}R{=}CH{-}CH{=}CHOSiMe_3$ (R′=CH_3CO, *m/z* 186 in *N*-acetyl derivatives; R′=Me_3SiOCH_2CO, *m/z* 274 in trimeth-

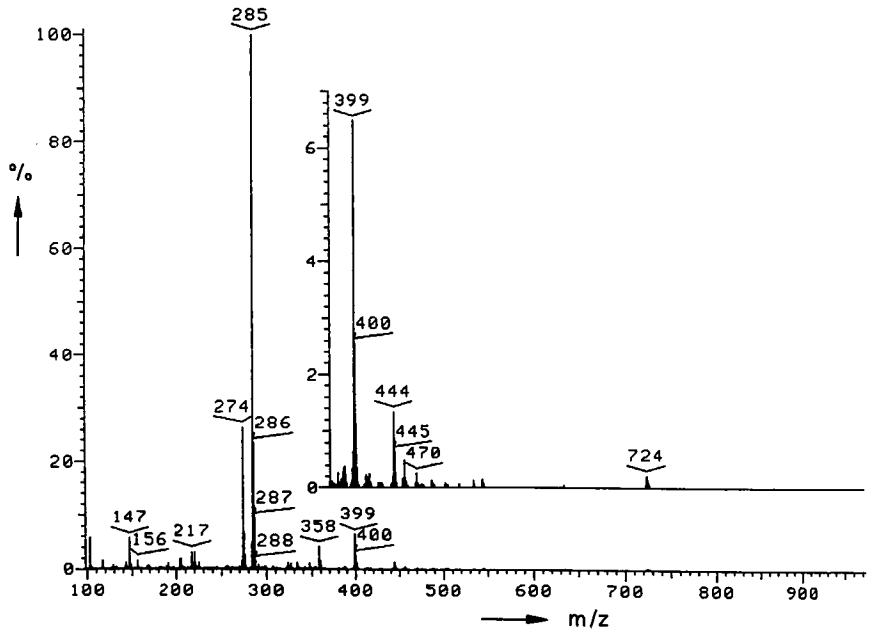

Fig. 1. *Mass spectrum (electron impact, 70 eV) of the trimethylsilyl ester, per-O-trimethylsilyl ether of Neu2en5Gc, compound 1*

Table 1. *Interpretation of a series of characteristic fragment ions of Neu2en5Gc, analyzed as Me_3Si ester, per-O-$SiMe_3$ ether (compound 1), Me_3Si ester, per-O-$SiMe_3$ ether, N-$SiMe_3$ derivative (compound 2), and methyl ester, per-O-$SiMe_3$ ether (compound 3), and of 4-epi-Neu2en5Gc, analyzed as methyl ester, per-O-$SiMe_3$ ether (compound 4)*

The figures in parentheses are the intensities of the ions relative to the base peak in the mass range higher than *m/z* 100. n.d., not detected; R, H or Me_3Si

Fragment	*m/z* values for compound			
	1	2	3	4
$M^{+\cdot}$ minus $^{\cdot}CH_3$	724 (0.3)	796 (0.6)	666 (3)	666 (5)
$M^{+\cdot}$ minus $^{\cdot}CHOSiMe_3$-CH_2OSiMe_3	534 (0.2)	n.d.	476 (0.5)	476 (21)
$M^{+\cdot}$ minus $^{\cdot}CH_2OSiMe_3$ minus Me_3SiOH minus Me_3SiOH	456 (0.5)	n.d.	398 (3)	398 (10)
$M^{+\cdot}$ minus $^{\cdot}CHOSiMe_3$-CH_2OSiMe_3 minus Me_3SiOH	444 (1)	516 (0.5)	386 (7)	386 (29)
$M^{+\cdot}$ minus $^{\cdot}CH_2OSiMe_3$ minus Me_3SiOH minus $RNHCOCH_2OSiMe_3$	399 (7)	399 (5)	341 (15)	341 (10)
Me_3SiOCH_2CO-$\dot{N}R$=CH-CH=$CHOSiMe_3$	274 (26)	346 (100)	274 (37)	274 (100)
$M^{+\cdot}$ minus $^{\cdot}CHOSiMe_3$-$CHOSiMe_3$-CH_2OSiMe_3 minus $RNHCOCH_2OSiMe_3$ (fragment *a*)	285 (100)	285 (26)	227 (100)	227 (33)
CH_2OSiMe_3-CH=$\dot{O}SiMe_3$	205 (2)	205 (5)	205 (7)	205 (10)

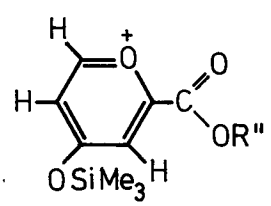

Fig. 2. *Structure of the fragment ion* a. The ion occurs at *m/z* 227 for methyl ester derivatives (R″=CH_3) and *m/z* 285 for Me_3Si derivatives (R″=Me_3Si)

ylsilylated *N*-glycoloyl derivatives; R=H). As is evident from a comparison of the mass spectral data of compounds 3 and 4 (Table 1), a reversed stereochemical orientation of the H and $OSiMe_3$ substituents at C-4, as is the case in compound 4, gives rise to a specific alteration in intensities of the fragments *a* and $R\dot{N}R$=CH-CH=$CHOSiMe_3$ (R=H). A similar alteration is observed when an additional Me_3Si group is introduced at the amido group, as in compound 2 and in the Me_3Si ester, per-*O*-$SiMe_3$ ether, *N*-$SiMe_3$ derivative of Neu2en5Ac (not shown).

Neu2en5Gc in biological materials

Using the mass spectrometric features of the model compound shown in Table 1 and discussed above, and the chromatographic data summarized in Table 2, Neu2en5Gc could be detected in various biological samples. Its occurrence was established in porcine, bovine and equine submandibular glands as well as in the urine of pig, horse and rat. The relative amount of the new, unsaturated sialic acid species is 1–

Table 2. *TLC and HPLC retention values of sialic acids investigated and GLC data of corresponding derivatives*
For details, see Materials and Methods

	TLC	HPLC	GLC		
			OV-101	OV-17	CPsil5
Neu2en5Gc	0.99/0.27[a]	2.1			
Me ester, per-*O*-$SiMe_3$-					1.01[b]
Me_3Si ester, per-*O*-$SiMe_3$-			1.16	1.45	
Me_3Si ester, per-*O*-$SiMe_3$-, *N*-$SiMe_3$			1.10	1.26	
Neu2en5Gc, 4-epi	0.48[a]				
Me ester, per-*O*-$SiMe_3$-					1.00[b]
Neu2en5Ac	1.20	1.7			
Me_3Si ester, per-*O*-$SiMe_3$-			0.93	1.09	
Me_3Si ester, per-*O*-$SiMe_3$, *N*-$SiMe_3$-			0.88	0.99	
Neu5Ac	1.00	1.0			
Me_3Si ester, per-*O*-$SiMe_3$-			0.91	1.04	
Me_3Si ester, per-*O*-$SiMe_3$-, *N*-$SiMe_3$-			1.00	1.00	
Neu5Gc	0.84	1.3			
Me_3Si ester, per-*O*-$SiMe_3$-			1.16	1.29	
Me_3Si ester, per-*O*-$SiMe_3$-, *N*-$SiMe_3$-			1.23	1.25	

[a] Chloroform/methanol (7:3).
[b] These retention times are relative to Me ester, per-*O*-$SiMe_3$ Neu5Gc.

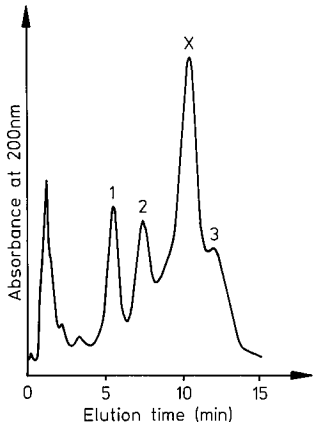

Fig. 3. *High performance liquid chromatography of free sialic acids from porcine submandibular gland on Aminex A-29 using 0.75 mM Na_2SO_4 as solvent system.* Flow rate, 0.5 ml/min; pressure, 15 bar; detection, 200 nm [19]. Peak identification: (1) Neu5Ac; (2) Neu5Gc; (3) Neu2en5Gc; (×) unidentified

3% of free sialic acids in all materials investigated. Besides Neu2en5Gc, Neu2en5Ac could also be identified in all materials.

Typical examples of the use of HPLC and GLC-MS in the various structural studies are presented in Figs3 and 4, respectively. In Fig.3 the HPLC profile of free sialic acids from porcine submandibular glands is demonstrated. Fig.4 presents the GLC-MS analysis of a sialic acid-containing fraction (trimethylsilylated with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide), obtained during the isolation of Neu2en5Gc from porcine urine. The upper trace represents the total ion current of the masses between *m/z* 100 and *m/z* 950. For the detection of Neu2en5Gc derivatives selected ion monitoring was carried out using *m/z* 285, *m/z* 274 and *m/z* 444 (for an explanation of these ions, see Table 1) [20]. It has to be noted that *m/z* 285 can also be used for the detection of Neu2en5Ac derivatives. The peaks at *m/z* 274 and *m/z* 444 are also observable in the Me_3Si ester, per-*O*-$SiMe_3$ ether of Neu5Gc.

Sialidase inhibition by Neu2en5Gc

The activity of *Vibrio cholerae* sialidase using 1 mM *N*-acetylneuraminyl-($\alpha 2 \rightarrow 3$)-lactose as substrate was inhibited by 50% at concentrations of 1×10^{-5} M Neu2en5Gc and 1.5×10^{-5} M Neu2en5Ac. The slightly higher inhibitory effect of Neu2en5Gc may be explained by the higher electronegativity of the *N*-glycoloyl chain when compared with the *N*-acetyl residue. This assumption is based on observations of Palese and Schulman [22], demonstrating that the inhibitory potency on viral sialidase preparations of 2-deoxy-2,3-didehydro-*N*-acylneuraminic acids increased with increasing electronegativity of the *N*-substituent, e.g. 2-deoxy-2,3-didehydro-*N*-trifluoroacetylneuraminic acid exhibiting the strongest influence.

Concluding remarks

The discovery of Neu2en5Gc in different biological materials adds to the long list of natural neuraminic acid derivatives [2, 3]. Moreover, Neu2en5Ac does not only occur in man, but also in a variety of animals. Preliminary reports have appeared on the natural occurrence of *O*-acylated and *O*-methylated derivatives of 2-deoxy-2,3-didehydro-*N*-acylneuraminic acid [23]. It may be assumed that unsaturated sialic acids are of common occurrence in animals synthesizing sialic acids. It is not known, however, how these substances are formed *in vivo*. The chemical synthesis of Neu2en5Gc from CMP-Neu5Gc described here favours the assumption that this sugar nucleotide is a natural precursor of Neu2en5Gc.

Little is known about a biological function of unsaturated sialic acids, though Neu2en5Ac and Neu2en5Gc inhibit sialidase activity *in vitro*. It remains to be elucidated whether their local concentration inside the cell is high enough for the inhibition of sialidase action *in vivo* and in this way for participation in the regulation of sialic acid metabolism.

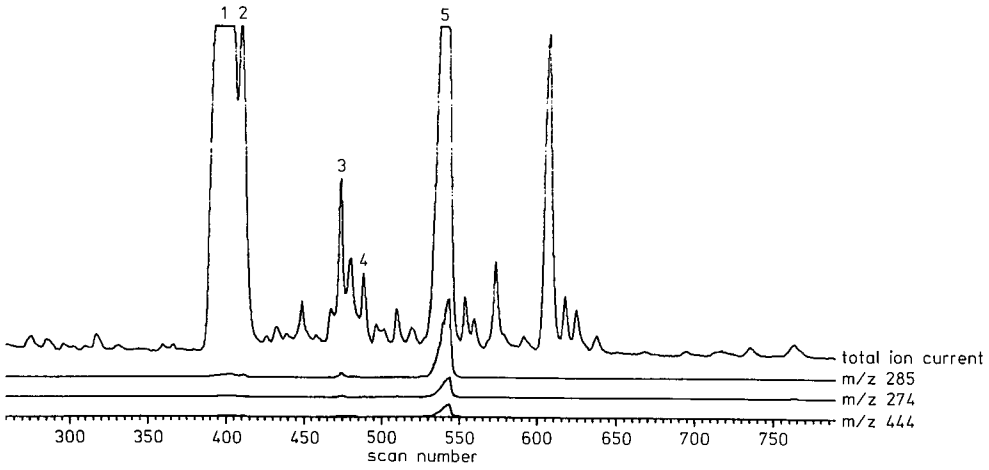

Fig. 4. *GLC-MS total ion current chromatogram (upper trace) and mass chromatograms (lower traces) on OV-17 of one sialic acid-containing fraction (trimethylsilylated with* N-*methyl*-N-*trimethylsilyltrifluoroacetamide) obtained during the isolation of Neu2en5Gc from porcine urine after anion-exchange chromatography and preparative thin-layer chromatography on cellulose (see Materials and Methods).* Peak identification: (1) Neu5Ac, Me_3Si ester per-*O*-$SiMe_3$ ether, *N*-$SiMe_3$ derivative; (2) Neu5Ac, Me_3Si ester per-*O*-$SiMe_3$ ether; (3) Neu2en5Gc, Me_3Si ester per-*O*-$SiMe_3$ ether, *N*-$SiMe_3$ derivative; (4) Neu5Gc, Me_3Si ester per-*O*-$SiMe_3$ ether; (5) Neu2en5Gc, Me_3Si ester per-*O*-$SiMe_3$ ether

The authors thank Jürgen Voss from the 'Victor-Hensen-House' of the University of Kiel for the collection of rat urine and Marzog El-Madani, Jutta Riedemann, Sabine Stoll, Claudia Szeiki, Silke Wiese and Henk Wagenaar for technical assistance. This work was supported by the *Deutsche Forschungsgemeinschaft* (grant Scha 202/10-4), the *Fonds der Chemischen Industrie* and the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organisation for the Advancement of Pure Research (ZWO).

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