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Note

Identification of the internal acetal 5-acetamido-2,7-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranose

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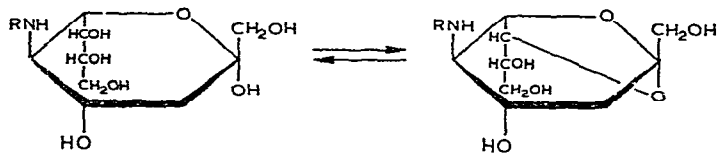
The reduction[1] of glycosidically bound *N*-acetylneuraminic acid methyl ester with sodium borohydride gives the glycoside of an *N*-acetylnonulosamine (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulose), and mild acid hydrolysis of the ketoside yields the free *N*-acetylnonulosamine **1**. During the course of our studies on the properties of ganglioside internal esters[2], we noted that reduction of ganglioside ester with sodium borohydride, followed by mild acid hydrolysis leads to the formation of nonulosamine **1** and a less polar product (**3**) (as observed by t l c) which gave the



1 R = Ac
2 R = $HOCH_2CH$

3 R = Ac
4 R = $HOCH_2CO$

same characteristic pink color with the resorcinol spray-reagent as gave **1**. Compound **3** was also formed by mild acid treatment of **1**. In this report, we present evidence that **3** is an internal acetal, namely 5-acetamido-2,7-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranose. The *N*-glycolylnonulosamine **2** was shown to form an analogous anhydride **4**. It is known that heptuloses in the presence of dilute aqueous acid are in equilibrium with their 2,7-anhydrides[3–5]. The data presented here indicate that nonulosamines behave similarly.

A mixture of *N*-acetylneuraminosyllactosylceramide and *N*-glycolylneuraminosyllactosylceramide (*N*-acetyl- and *N*-glycolyl-G_{M3}) was treated with glacial acetic acid to form the internal ester, reduced, and hydrolyzed. The dialyzable products formed were per(trimethylsilyl)ated and analyzed by g l c (bottom trace of Fig. 1). Standard nonulosamine **1**, acid-treated **1**, and acid-treated nonulosamine **2** were similarly chromatographed, as shown in Fig. 1. Peaks B and D were formed when

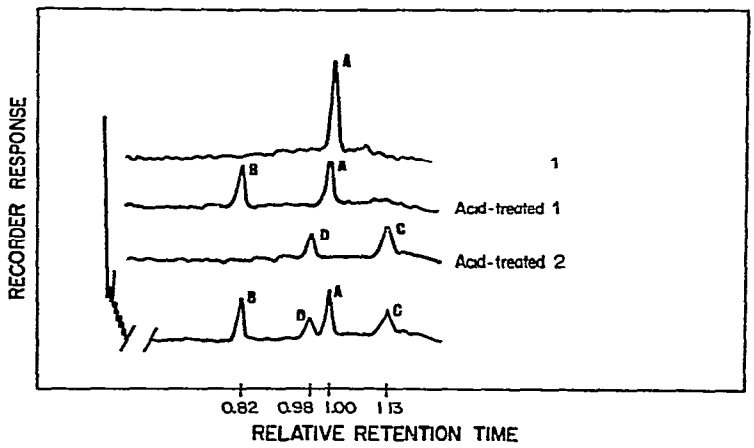


Fig 1 Gas–liquid chromatography of compound **1** and products formed by mild acid treatment of **1** and of **2** The per(trimethylsilyl)ated samples were injected on a 3% OV-1 column, and the temperature was programmed from 190° to 230° at 2° min^{-1} G_{M3} is sialosyllactosylceramide Untreated **2** gave only peak C The lower curve was given by reduced, acid-treated *N*-acetyl- and *N*-glycolyl-G_{M3} internal ester.

1 and **2**, respectively, were subjected to mild acid treatment Low-resolution mass-spectra of these compounds were obtained by combined g l c –m s The spectra of the per(trimethylsilyl)ated derivatives of **1** and **3** (peaks A and B, Fig 1) are shown in Figs 2 and 3 The trimethylsilyl derivative of **3** gave rise to a molecular ion having an *m/e* value of 565, which is 162 mass units below the mol wt of nonulosamine The molecular ion of **1** (*m/e* 727) was not observed, but a strong signal is visible at *m/e* 712 ($M^+ - 15$) These results suggested a difference of one atom of oxygen and two trimethylsilyl groups. Thus, it appears that **3** is an anhydro derivative of **1** The strong signal at *m/e* 360 ($M^+ - 205$) in the spectrum of **3** indicates the removal of the C-8–C-9 fragment with maintenance of the anhydride structure The fragmentation pattern of **3** is consistent with a 2,7-internal acetal form of an *N*-acetylnonulosamine High-resolution mass spectra were obtained by direct-probe analysis and some of the most important ions and their deduced compositions are given in Table I The exact masses of these ions provide confirmatory evidence of the relationship of the formulas of **1** and **3**, the anhydro fragments derived from **3** being 162 mass units below the corresponding fragments derived from **1** (Table I) Thus, the structural change of **1** to **3** involves the loss of one molecule of water with the formation of a 2,7-anhydro ring Studies of the *N*-glycolyl derivatives gave similar mass-spectral data, but the mass numbers of the corresponding ions that have an *N*-acyl group were increased by 88 mass units.

Larger amounts of **1** and **3** were obtained from colominic acid [poly(*N*-acetylneuraminic acid)] by treatment with glacial acetic acid to form the polylactone, then reduction with sodium borohydride, and subsequent mild acid hydrolysis Compound **1** was separated from the anhydro derivative **3** by silica gel column chromatography

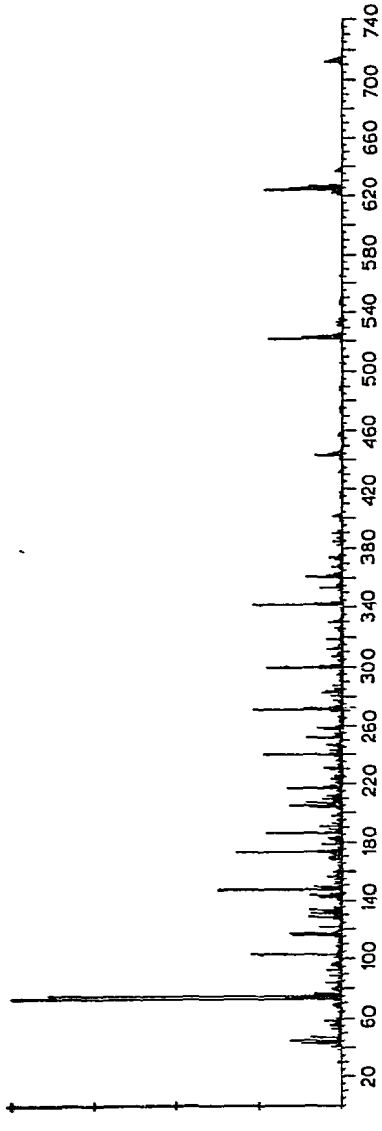


Fig. 2. Low-resolution mass spectra of the per(trimethylsilyl)ated derivative of 1.

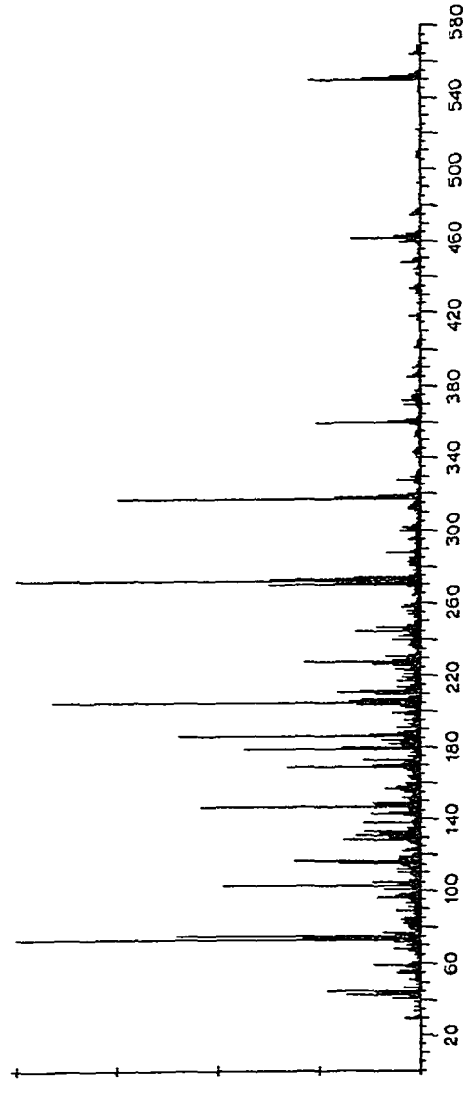


Fig. 3. Low-resolution mass spectra of the per(trimethylsilyl)ated derivative of 3

TABLE I

PRECISE MASSES OF FRAGMENTATION IONS FROM PER(TRIMETHYLSILYL)ATED COMPOUNDS 1 AND 3 OBTAINED BY HIGH-RESOLUTION MASS SPECTROMETRY

	Compound 1		*Compound* 3	
Fragmentation ions	*Measured mass* (m/e)	*Deduced formula*	*Measured mass* (m/e)	*Deduced formula*
$M^{\ddagger}$	727 3639	$C_{29}H_{69}NO_8Si_6$	565 2743	$C_{23}H_{51}NO_7Si_4$
$M^{\ddagger}-15$	712 3404	$C_{28}H_{66}NO_8Si_6$	550 2508	$C_{22}H_{48}NO_7Si_4$
$M^{\ddagger}-89(90)$	638 3216	$C_{26}H_{60}NO_7Si_5$	475 2242	$C_{20}H_{41}NO_6Si_3$
$M^{+}-103$	624 3060	$C_{25}H_{58}NO_7Si_5$	462 2066	$C_{19}H_{40}NO_6Si_3$
$M^{\ddagger}-205$	522 2560	$C_{21}H_{48}NO_6Si_4$	360 1663	$C_{15}H_{29}NO_5Si_2$
$M^{+}-294$			271 1186	$C_{12}H_{23}O_3Si_2$
$M^{+}-456$	271 1186	$C_{12}H_{23}O_3Si_2$		

The visible absorption spectra of the resorcinol chromogens formed from **1** and **3** were identical (λ_{max} 560 nm) and were distinct from that obtained with *N*-acetylneuraminic acid The resorcinol reaction was shown to be applicable for the quantitative determination of both **1** and **3** Treatment of **3** with mild acid gave **1** Although optimal conditions for the formation of **3** from **1** and the reverse reaction were not sought, the interconversion under acid conditions was demonstrated and the equilibrium ratio under the mild acid conditions used is probably close to one The reducing power (as measured by the ferricyanide procedure) of **1** and **2**, relative to *N*-acetylneuraminic acid, was found to be 0 5 and 0 15, respectively Compound **1** was readily reduced within 15 min at room temperature with sodium borohydride, whereas **3** remained unchanged under these conditions When **1** was treated with sodium periodate, 3 equiv of this reagent were consumed per mole of the sugar Under similar conditions, **3** consumed only 1 equiv. of periodate These data are consistent with the structure proposed Examination of models indicates that only the α anomer is capable of forming an internal anhydride with the hydroxyl group at C-7 Experimental evidence for this configuration was obtained from optical rotation measurements Brossmer and Holmquist[1] showed that the rotations of the α glycosides of **1** are less negative than that of **1** We observed a large positive change, from $[\alpha]_D^{25}$ $-31°$ for **1** to $[\alpha]_D^{25}$ $+74°$ for **3** This difference suggests that the glycosyl linkage of **3** has the α configuration We, therefore, propose that **3** be assigned the structure 5-acetamido-2,7-anhydro-3,5-dideoxy-D-*glycero*-α-D-*galacto*-nonulopyranose

EXPERIMENTAL

General methods — The sialic acid content of gangliosides and of their internal esters was determined by the resorcinol method[6], which was also used for the quantitative determination of **1** and **3** by measurement of absorbance at 560 nm. The reducing power was determined by the ferricyanide method of Park and Johnson[7]

Preparative t l c. was performed on Silica Gel G (250 μm) plates (Analtech) with 60 35 8 (v/v) chloroform–methanol–water as the developing solvent Detection was achieved with the resorcinol reagent[8] G l c was performed on a 2760A Hewlett–Packard gas chromatograph equipped with a flame-ionization detector and a 1 8 m glass-column packed with 3% OV-1 on Chromosorb W Per(trimethylsilyl)ation was performed with hexamethyldisilazane–chlorotrimethylsilane–*N*,*N*-bis(trimethylsilyl)-trifluoroacetamide–pyridine (2 1·1 4) at room temperature for 30 min and aliquots were directly analyzed by g l c. or g l c–m s Low-resolution mass spectra were obtained with a Perkin–Elmer–Hitachi RMU-6L instrument, interfaced with a Perkin–Elmer 990 gas chromatograph Gas-chromatographic conditions were as just described, with interface valve and molecular separation held constant at 250°. The ionization voltage was 70 eV. High-resolution mass spectra were obtained with a DuPont CEC-110 photoplate instrument with acceleration voltage held at 6 eV Samples were introduced through the solid-probe inlet All data were gathered. processed, and presented by an IBM 1800 computer and related peripherals as presented elsewhere[9] Optical rotations were determined with a Zeiss polarimeter

Chemicals — *N*-acetylneuraminosyllactosylceramide (*N*-acetyl-G_{M3}) and *N*-glycolyl neuraminosyllactosylceramide (*N*-glycolyl-G_{M3}) were isolated from bovine adrenal glands[10] 5-Acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulose was a gift from Dr R Brossmer (Institut fur Biochemie der Universitat Heidelberg) Colominic acid was obtained from Koch–Light Laboratories, Colnbrook, England, and the Silica Gel 60 prepacked column was purchased from E Merck, Darmstadt, Germany

Ganglioside internal esters — A solution of ganglioside (1–5 mg) in glacial acetic acid (1 ml) was maintained for five days at room temperature The acetic acid was evaporated under a stream of nitrogen at 40° and the residue dissolved in 95% methanol Alternatively, the ganglioside sample was dissolved in aqueous salt solution and treated with 0 1M 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide at a pH of 5 1 for 0 5 h according to the method employed by Shrager and Profera[11] without added glycine methyl ester Isolation of the internal ester was accomplished by passing the reaction mixture through a mixed-bed column containing Dowex 50 (H^+) and DEAE-cellulose (AcO^-) The neutral internal ester was eluted from the column with aqueous methanol

5-Acetamido-3,5-dideoxy-D-glycero-D-galacto-*nonulose* (**1**) *and 5-glycolylamido analog* (**2**) *5-Acetamido-2,7-anhydro-3,5-dideoxy*-D-glycero-D-galacto-*nonulopyranose* (**3**) *and 5-glycolylamido analog* (**4**) — (*a*) *From the ganglioside internal ester.* A ganglioside internal ester preparation was dissolved in methanol (2 mg/ml) and the solution cooled in an ice-bath An equal volume of cold 2% sodium borohydride in methanol was added, and the reaction mixture was kept overnight at 4°. The solution was diluted with water (0 5 vol) and neutralized with Dowex 50 (H^+) After filtration, the solution was evaporated to dryness, and methanol was added and evaporated three times to remove methyl borate The residue was purified by preparative t l c A marker lane was sprayed with the resorcinol reagent, which produces a characteristic pink color with reduced gangliosides The reduced ganglioside was hydrolyzed with 12 5mM

sulfuric acid for 1 h at 80° The solution was neutralized with Dowex 2 (AcO^-), and the products of hydrolysis (**1**, **2**, **3**, and **4**) were isolated by preparative t l c When relatively large quantities of mixed gangliosides were processed, the hydrolysis mixture was dialyzed and the dialyzable products were collected and purified by t l c

(*b*) *From colominic acids* A suspension of colominic acid (1 g) in glacial acetic acid (50 ml) was kept for five days at room temperature The acetic acid was removed under reduced pressure, and the residue was suspended in water (50 ml) and cooled with an ice-bath Sodium borohydride (0 5 g), dissolved in cold water (10 ml), was added to the suspension The reaction mixture was kept overnight at 4° The mixture was neutralized with Dowex 50 and filtered The solution, which contained the reduced colominic acid, was evaporated to dryness under reduced pressure, and methanol was added and evaporated three times to remove methyl borate The reduced product was hydrolyzed either by treatment with 12 5mM sulfuric acid at 80°, as just indicated (this method yields a preponderance of nonulosamine), or it was boiled at reflux for 2 h in aqueous solution (50 ml) with Dowex 50 (5 g), and then filtered (this treatment shifts the equilibrium toward the anhydro derivative) The products were isolated after neutralization with Dowex 2 (AcO^-)

The nonulosamine was separated from the anhydro derivative by gradient elution from a Silica Gel 60, prepacked Merck column (size B) A 1-liter linear chloroform–methanol–water gradient was used to elute the column The solvent proportions by volume changed from 60 25 4 to 10 10 3 Fractions (5 ml) were collected, and aliquots tested with the resorcinol spray reagent The anhydro derivative appeared in fractions 78–94 and nonulosamine in fractions 135–145 These fractions were pooled and evaporated to dryness under reduced pressure The residue from each of the two pools was dissolved in 1 1 (v/v) methanol–water. The solution was partitioned with an equal volume of hexane The lower phase was evaporated to dryness and the products dried in an Abderhalden pistol in the presence of phosphorus pentaoxide The preparations were pure, as judged by t l c and detection with the resorcinol spray reagent and charring with 40% sulfuric acid, yield, 124 mg of nonulosamine and 154 mg of glycosan, $[\alpha]_D^{25}$ of **1**, −31° (*c* 2 8, methanol), $[\alpha]_D^{25}$ of **2**, +74° (*c* 2 5, methanol)

Periodate oxidation Absorbance of the sodium metaperiodate reagent was measured (light-path 1 0 mm) at its maximum wavelength (223 nm) with a Pye Unicam Model SP 1800 spectrophotometer A known equivalent of D-mannitol was added to 1 2mM sodium periodate and the optical density was continuously recorded until it stabilized With this technique, it was demonstrated that 1 mol of D-mannitol consumes 5 equiv. of periodate in less than 20 min **1** consumed 2 96 equiv of periodate per mol within 20 min, and **3** consumed only 0 955 equiv of periodate per mol in less than 20 min

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