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Synthesis of 9-O-Acetyl- and 4,9-Di-O-Acetyl Derivatives of the Methyl Ester of N-Acetyl-β-D-Neuraminic Acid Methylglycoside

Their Use as Models in Periodate Oxidation Studies

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Summary: Reaction of the methyl ester of *N*-acetyl-β-D-neuraminic acid methyl glycoside with *N*-acetylimidazole yielded the corresponding 9-*O*-acetyl- and 4,9-di-*O*-acetyl derivatives. The structures of these compounds were confirmed by mass spectrometry and both ^{1}H and ^{13}C NMR spectroscopy. The compounds served as model substances in a comparative study of the rate of periodate oxidation of unsubstituted and of 9-*O*-acetylated *N*-acetyl-neuraminic acids. This reaction was strongly hampered by the presence of the 9-*O*-acetyl group. The low molar absorbancy coefficient of *N*-acetyl-9-*O*-acetylneuraminic acid in the periodic acid/thiobarbituric acid assay can be explained by this retardation.

Synthese der 9-O-Acetyl- und 4,9-Di-O-acetylderivate des N-Acetyl-β-D-neuraminsäure-methylester-methylglykosids – Ihre Anwendung als Modelle in Oxidationsstudien mit Perjodat

Zusammenfassung: 9-*O*-Acetyl- und 4,9-Di-*O*-acetylderivate des Methylesters des *N*-Acetyl-β-D-neuraminsäuremethylglykosids wurden mit *N*-Acetylimidazol als Acetylierungsmittel synthetisiert. Ihre Strukturen wurden durch Massenspektrometrie und ^{1}H- bzw. ^{13}C-NMR-Spektroskopie bewiesen. Diese Substanzen dienten als Modelle in einer vergleichenden Studie über die Reaktionsgeschwindigkeit unsubstituierter bzw. 9-*O*-acetylierter *N*-Acetylneuraminsäuren. Die Oxidationsrate wurde durch die Gegenwart der 9-*O*-Acetylgruppe stark behindert. Der niedrige molare Absorptionskoeffizient der *N*-Acetyl-9-*O*-acetylneuraminsäure in der Perjodsäure/Thiobarbitursäure-Reaktion läßt sich durch diesen Befund leicht erklären.

O-Acetylated *N*-acylneuraminic acids are widespread in animal and human tissues[1,2]. The *O*-acetyl groups have mainly been found at C-4 of *N*-acetylneuraminic acid, as in equine submandibular gland glycoproteins[3-5], or in position 9, as in bovine submandibular gland glycoproteins[5]. In earlier experiments, the positions of the *O*-acetyl groups were assigned by measurement of the molar periodate uptake of the acylneuraminic acids and the rate of the oxidation reaction[3,4,6]. From the low consumption of periodate, it was concluded that the *O*-acetyl group of the main acylneuraminic acid from bovine submandibular gland should be present at C-8[4,6]. However, it

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has been proven by mass spectrometry that the ester group is localised at C-9[5].

For a more detailed study of the unexpected behaviour in the periodate oxidation of the 9-*O*-acetylated *N*-acetylneuraminic acid, the model substance *N*-acetyl-9-*O*-acetyl-β-D-neuraminic acid methyl ester methyl glycoside and also its 4,9-di-*O*-acetyl analogue were synthesised. We have already mentioned their mass spectral data, which are characteristic for the substitution pattern, in an earlier publication[5].

These compounds are the first chemically synthesised neuraminic acid derivatives having one or two *O*-acetyl groups in definite positions. *N*-Acetyl-9-*O*-acetyl-β-D-neuraminic acid methyl ester methyl glycoside was recently mentioned in a ^{13}C NMR study reported by Bhattacharjee[7], although a synthesis was not described.

Materials and Methods

Thin-layer chromatography

Thin-layer chromatography of the synthetic acetyl-β-D-neuraminic acid methyl ester methyl glycosides was performed on 0.5-mm plates of Silica Gel 60 (Fertigplatten, E. Merck, Darmstadt) using the solvent system chloroform/methanol, 85:15 (v/v). The compounds were stained with the orcinol/$Fe^{3\oplus}$/HCl spray reagent[8], or for preparative separations by ~ 15 sec exposure to iodine vapour at room temperature, on which only the main acetylneuraminic acid bands were coloured yellowish-brown. After evaporation of the iodine at room temperature, these zones were eluted with chloroform/methanol, 1:1 (v/v). Thin-layer chromatography of naturally occurring acylneuraminic acids was carried out on 0.2 mm cellulose MN 300 layers (Macherey, Nagel & Co., Düren) using the solvent system butanol/propanol/0.1N HCl, 1:2:1 (v/v/v)[9].

Gas-liquid chromatography

Gas-liquid chromatography of the trimethylsilyl ethers of the acetyl-β-D-neuraminic acid methyl ester methyl glycosides was carried out on a Varian Aerograph 2740-30-01 gas chromatograph equipped with flame ionisation detector and glass columns (2.00 m × 4.0 mm internal diameter) packed with 3.8% SE-30 on Chromosorb W-AW-DMCS, HP, 80 - 100 mesh. The column-oven temperature was 205 °C and the nitrogen flow rate 40 ml/min. The retention times (R_1) are given relative to the trimethylsilyl derivative of *N*-acetyl-β-D-neuraminic acid methyl ester methyl glycoside (1): Trimethylsilylation of the methyl ester methyl glycosides of acetylneuraminic acids was carried out with *N*-trimethylsilylimidazole[10] or with hexamethyldisilazane and trimethylchlorosilane in pyridine[5].

Mass spectrometry

The 70 eV mass spectra were recorded on an AEI MS-902 mass spectrometer at an ion-source temperature of 100 - 120 °C, a trap current of 500 μA and an accelerating voltage of 8 kV. High resolution mass measurements were carried out as described earlier[5].

Nuclear magnetic resonance spectroscopy

^1H NMR spectra (90 MHz) of 0.25 - 0.5M solutions of the neuraminic acid derivatives in [$^2H_4$]methanol were recorded at room temperature in 5-mm tubes on a Brucker WH-90 pulse spectrometer operating in the Fourier transform mode. The pulse width was 0.5 μsec, the pulse rate 4.5 sec (4 K real part after Fourier transformation). Proton-noise-decoupled Fourier-transform ^{13}C NMR spectra of the same samples of neuraminic acid derivatives were recorded at 22.63 MHz on the above-mentioned instrument (room temperature, specific width 6000 Hz, pulse width 5 μsec, pulse rate 0.68 sec, data 8192), or at 25.16 MHz on a Varian XL-100-15 Fourier-transform spectrometer (room temperature, specific width 5713 Hz, acquisition time 0.7 sec, delay 4.5 sec, pulse width 80 μsec, data 8191). ^1H and ^{13}C chemical shifts are given in ppm relative to internal tetramethylsilane on the δ scale.

Preparation of the methyl esters of N-acetyl-9-O-acetyl-β-D-neuraminic acid-methylglycoside (2) *and of N-acetyl-4,9-di-O-acetyl-β-D-neuraminic acid-methylglycoside* (3)

N-Acetyl-β-D-neuraminic acid methyl ester methyl glycoside (1) was prepared by the procedure of Yu and Ledeen[11]. [^1H NMR data in [$^2H_4$]methanol*: 2.00 (s, *N*-acetyl-methyl), 3.26 (s, 2-*O*-methyl), 3.79 (s, ester-methyl), 1.62 (q, $J_{3,3'}$ = 12.8 Hz; $J_{3',4}$ = 10.9 Hz, H-3′ *ax*), 2.32 (q, $J_{3,3'}$ = 12.8 Hz; $J_{3,4}$ = 4.5 Hz, H-3 *eq*), other protons 3.4 - 4.1].

Partial acetylation of this compound was carried out as follows: 40 mg of *N*-acetylimidazole (E. Merck, Darmstadt) in 1 ml of dry pyridine was added to a solution of 100 mg of 1 in 1.5 ml of pyridine. The solution was concentrated in a rotatory evaporator (water bath 40 °C) to about 0.4 ml and kept at room temperature. The progress of the acetylation reaction was then followed by thin-layer chromatography on silica gel [R_F-values: 1, 0.13; 2, 0.41; and 3, 0.69]. After 24 h the mixture contains mainly 2. The relative amount of 3 was increased in a second period of 24 h by a further addition of 20 mg *N*-acetylimidazole in 0.5 ml of pyridine and subsequent

* s = singlet, q = quartet, *ax* = axial, *eq* = equatorial

concentration of the solution. At the end of the reaction, pyridine was removed as far as possible by evaporating twice with methanol. To remove imidazole and residual pyridine from the remaining sirup, the latter was applied to a column of 20 ml of Dowex 50, $H^{\oplus}$-form, at 4 °C. Subsequently, the neuraminic acid derivatives were eluted with 80 ml of water/methanol, 1 : 1 (v/v). [Test reagent, orcinol/$Fe^{3\oplus}$/HCl[8].] The components were separated by preparative thin-layer chromatography. In this way 45 mg of 2 and 42 mg of 3 were isolated as clear sirups, corresponding together to 74% of theory. The purity of 2 and 3 was tested by gas-liquid chromatography: R_1 of 2, 1.20; R_1 of 3, 1.46.

Reference compounds

N-Acetyl-β-D-neuraminic acid methyl ester (4) and *N*-acetyl-α-D-neuraminic acid methyl ester methyl glycoside (5) were synthesised according to methods described by Yu and Ledeen[11]. Similarly, the following substances specifically labelled with trideuteriomethyl groups were synthesised by reaction of the appropriate neuraminic acid derivatives with trideuteriomethanol in the presence of Dowex 50 ($H^{\oplus}$): *N*-acetyl-β-D-neuraminic acid trideuteriomethyl ester trideuteriomethyl glycoside, *N*-acetyl-β-D-neuraminic acid trideuteriomethyl ester methyl glycoside, *N*-acetyl-β-D-neuraminic acid trideuteriomethyl ester, and *N*-acetyl-α-D-neuraminic acid trideuteriomethyl ester trideuteriomethyl glycoside. *N*-Acetyl-4-*O*-methylneuraminic acid and its ethyl ester were synthesised by *J.-M. Beau* and *P. Sinaÿ* (Carbohydrate Research, publication in preparation).

Isolation of N-acetyl-9-O-acetylneuraminic acid

This compound was prepared from bovine submandibular gland glycoproteins[4,6]. The purity was tested by thin-layer[4] and gas-liquid chromatography[10].

Periodate oxidation

Periodate oxidation experiments were carried out in the dark at room temperature. 1 ml of a 5mM solution of a neuraminic acid derivative in dioxane/water, 1 : 1 (v/v) was mixed with an equal volume of 0.02M periodic acid or sodium metaperiodate in water. The periodate consumption was followed spectrophotometrically at 220 nm. 20-μl samples of the reaction mixture were withdrawn at appropriate time intervals, diluted with 1 ml of water and measured. In an experiment in which the oxidation by periodic acid (pH 2.5) of the compounds 1, 2 and 3 was followed over a period of 48 h, the ester content of the samples was determined quantitatively with the alkaline/hydroxylamine/$Fe^{3\oplus}$ reagent according to Hestrin[12]. 200-μl samples of the reaction mixture were taken at different times and treated with 100 μl 0.1M ethylene glycol. Since iodine formed on addition of the perchlorate reagents would interfere in the colorimetric test, it was extracted with chloroform. This procedure was calibrated with ethylacetate in 0.01M iodate solution. The hydrolysis of the 9-*O*-acetyl group of the natural *N*-acetyl-9-*O*-acetylneuraminic acid in aqueous acetic acid, pH 2.5, in the absence of periodic acid was also followed over this period of time.

Results and Discussion

Acetylation reaction

The methyl ester of *N*-acetyl-9-*O*-acetyl-β-D-neuraminic acid methyl glycoside and its 4,9-di-*O*-acetyl analogue could be synthesised in reasonable yields using *N*-acetylimidazole. As anticipated, this mild acetylating reagent reacts preferably with the primary hydroxyl group (at C-9) of compound 1. However, the reaction is not quite selective; at high molar ratios of *N*-acetylimidazole to substrate, the hydroxyl group at C-4 is also acetylated to a considerable extent. The ratio of mono- to di-*O*-acetyl derivatives in the reaction mixture can be regulated by the molar excess of acetylating reagent used. For instance, with 1.2 mol *N*-acetylimidazole to 1 mol neuraminic acid, about 15% product 3 was formed in 24 h. The use of 2.6 mol of *N*-acetylimidazole leads after 48 h mainly to 3 as end product. In addition, under the latter conditions, a small band with an R_F-value of 0.8 was observed on thin-layer chromatography. This compound was identified by mass spectrometry as *N*-acetyl-4,8,9-tri-*O*-acetyl-β-D-neuraminic acid methyl ester methyl glycoside, based on the presence of the following fragment ions described in ref.[5]: A, *m/e* 520; B, *m/e* 476; C, *m/e* 390; D, *m/e* 298 and G, *m/e* 143. Fragments E and F are absent.

Spectral analysis of the reaction products 2 *and* 3

1) Mass spectrometry

The mass spectra of the trimethylsilyl derivatives of 2 and 3 are presented in Figs. 1 and 2. Tables 1 and 2 summarise a number of important fragmentions. The brutoformulae were deduced from high-resolution mass measurements. In these figures and tables the fragments A - G, which are characteristic for the number, type and positions of the ester groups in acylneuraminic acids (see ref.[5,13]) are included. From the latter fragments, it was deduced that in compound 2 one *O*-acetyl group is present at C-9, whereas in 3 two *O*-acetyl groups occur, attached to C-4 and C-9, respectively.

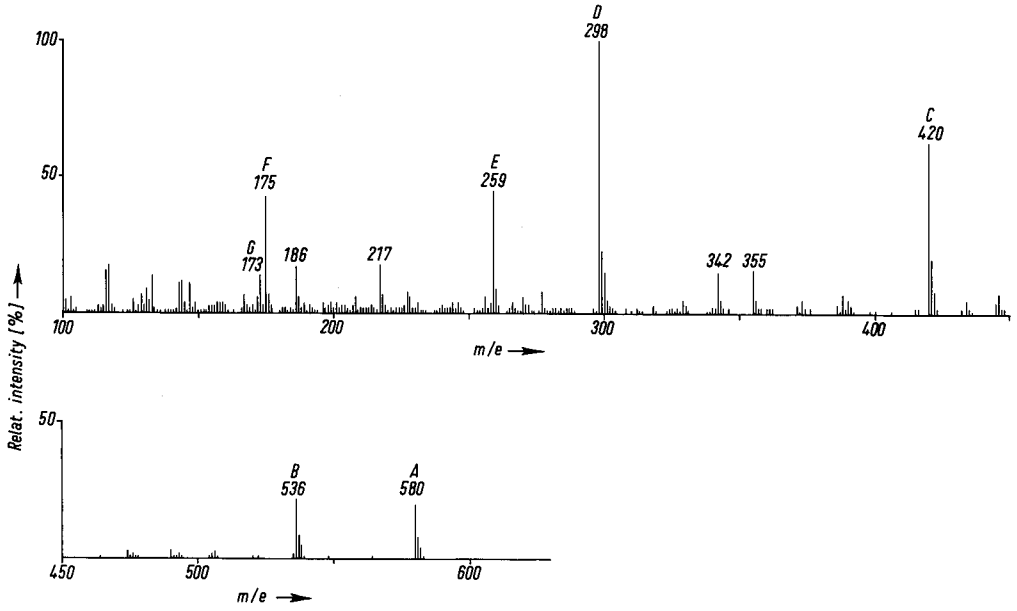

Fig. 1. Mass spectrum of the trimethylsilyl derivative of *N*-acetyl-9-*O*-acetyl-β-D-neuraminic acid methyl ester methylglycoside.

Values > *m/e* 100 and intensities ≥ 1% are given.

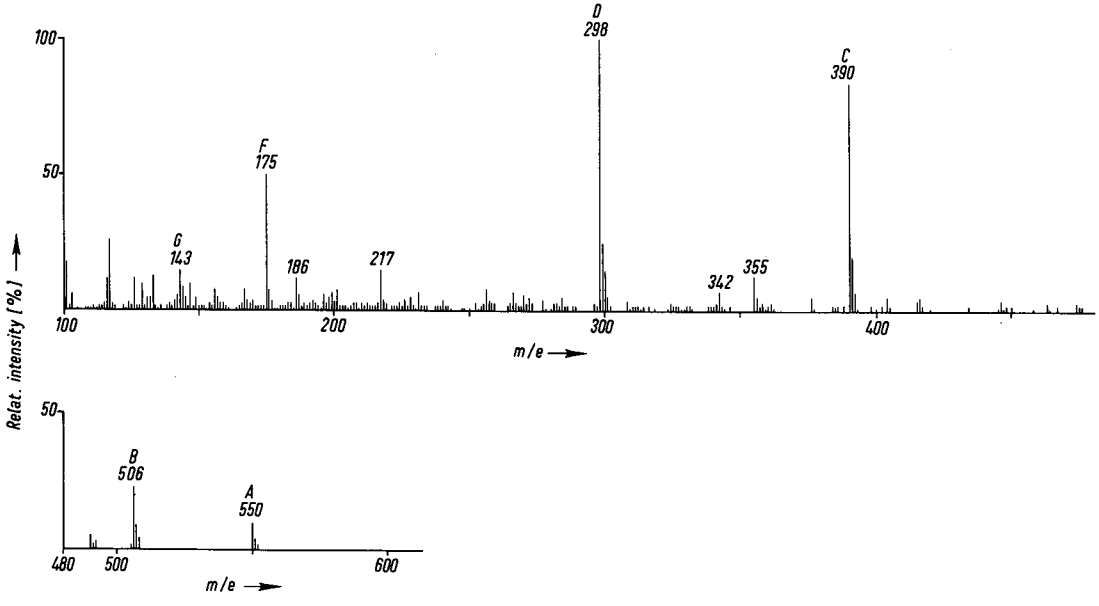

Fig. 2. Mass spectrum of the trimethylsilyl derivative of *N*-acetyl-4,9-di-*O*-acetyl-β-D-neuraminic acid methyl ester methylglycoside.

Values > *m/e* 100 and intensities ≥ 1% are given.

Table 1. Interpretation of some important fragment ions present in the mass spectrum of the trimethylsilyl derivative of *N*-acetyl-9-*O*-acetyl-β-D-neuraminic acid methyl ester methylglycoside (2).

m/e	Formula	Fragment
580	$C_{23}H_{46}NO_{10}Si_3$	$M^{\oplus\cdot} - {}^{\cdot}CH_3$
564	$C_{23}H_{46}NO_9Si_3$	$M^{\oplus\cdot} - {}^{\cdot}OCH_3$
548	$C_{22}H_{42}NO_9Si_3$	$M^{\oplus\cdot} - {}^{\cdot}CH_3 - CH_3OH$
536	$C_{22}H_{46}NO_8Si_3$	$M^{\oplus\cdot} - {}^{\cdot}COOCH_3$
506	$C_{21}H_{40}NO_9Si_2$	$M^{\oplus\cdot} - {}^{\cdot}OSi(CH_3)_3$
490	$C_{20}H_{36}NO_9Si_2$	$M^{\oplus\cdot} - (CH_3)_3SiOH - {}^{\cdot}CH_3$
474	$C_{20}H_{36}NO_8Si_2$	$M^{\oplus\cdot} - {}^{\cdot}OCH_3 - (CH_3)_3SiOH$
446	$C_{19}H_{34}O_8Si_2$	$M^{\oplus\cdot} - (CH_3)_3SiOH - NH_2Ac$
434	$C_{18}H_{40}NO_5Si_3$	$M^{\oplus\cdot} - {}^{\cdot}COOCH_3 - CH_2CO - CH_3COOH$
420	$C_{17}H_{34}NO_7Si_2$	$M^{\oplus\cdot} - CH_2OAc\text{-}{}^{\cdot}CHOSi(CH_3)_3$
388	$C_{16}H_{30}NO_6Si_2$	$M^{\oplus\cdot} - CH_2OAc\text{-}{}^{\cdot}CHOSi(CH_3)_3 - CH_3OH$
373	$C_{16}H_{29}O_6Si_2$	$M^{\oplus\cdot} - {}^{\cdot}CH_2OAc - (CH_3)_3SiOH - NH_2Ac$
355	$C_{16}H_{25}NO_6Si$	$M^{\oplus\cdot} - 2 \times (CH_3)_3SiOH - CH_3COOH$
342	$C_{15}H_{24}NO_6Si$	$M^{\oplus\cdot} - {}^{\cdot}CH_2OAc - 2 \times (CH_3)_3SiOH$
298	$C_{13}H_{20}NO_5Si$	$M^{\oplus\cdot} - CH_2OAc\text{-}{}^{\cdot}CHOSi(CH_3)_3 - CH_3OH - (CH_3)_3SiOH$
277	$C_{11}H_{25}O_4Si_2$	$CH_2OAc\text{-}CH[OSi(CH_3)_3]\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
259	$C_{11}H_{19}O_5Si$	$M^{\oplus\cdot} - CH_2OAc\text{-}CH[OSi(CH_3)_3]\text{-}{}^{\cdot}CHOSi(CH_3)_3 - NH_2Ac$
227	$C_{10}H_{15}O_4Si$	$M^{\oplus\cdot} - CH_2OAc\text{-}CH[OSi(CH_3)_3]\text{-}{}^{\cdot}CHOSi(CH_3)_3 - NH_2Ac - CH_3OH$
217	$C_9H_{21}O_2Si_2$	$CH_2{=}C[OSi(CH_3)_3]\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
186	$C_8H_{16}NO_2Si$	$AcNH\text{-}CH{=}CH\text{-}CH{=}O^{\oplus}Si(CH_3)_3$, $AcN^{\oplus}H{=}CH\text{-}C[OSi(CH_3)_3]{=}CH_2$
175	$C_7H_{15}O_3Si$	$CH_2OAc\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
173	$C_7H_{15}NO_2Si$	$AcN^{\oplus}H{=}CH\text{-}{}^{\cdot}CHOSi(CH_3)_3$
143	$C_6H_{11}O_2Si$	No reasonable structure can be proposed.

Table 2. Interpretation of some important fragment ions, present in the mass spectrum of the trimethylsilyl derivative of *N*-acetyl-4,9-di-*O*-acetyl-β-D-neuraminic acid-methyl ester methylglycoside (3).

m/e	Formula	Fragment
550	$C_{22}H_{40}NO_{11}Si_2$	$M^{\oplus\cdot} - {}^{\cdot}CH_3$
506	$C_{21}H_{40}NO_9Si_2$	$M^{\oplus\cdot} - {}^{\cdot}COOCH_3$
492	$C_{20}H_{38}NO_9Si_2$	$M^{\oplus\cdot} - {}^{\cdot}CH_2OAc$
490	$C_{20}H_{36}NO_9Si_2$	$M^{\oplus\cdot} - {}^{\cdot}CH_3 - CH_3COOH$
474	$C_{20}H_{36}NO_8Si_2$	$M^{\oplus\cdot} - {}^{\cdot}OCH_3 - CH_3COOH$
463	$C_{19}H_{37}NO_8Si_2$	$M^{\oplus\cdot} - CH_3COOH - CH_2CO$
446	$C_{19}H_{34}O_8Si_2$	$M^{\oplus\cdot} - CH_3COOH - NH_2Ac$
415	$C_{18}H_{29}NO_8Si$	$M^{\oplus\cdot} - (CH_3)_3SiOH - CH_3COOH$
404	$C_{17}H_{34}NO_6Si_2$	$M^{\oplus\cdot} - {}^{\cdot}COOCH_3 - CH_3COOH - CH_2CO$
390	$C_{16}H_{28}NO_8Si$	$M^{\oplus\cdot} - CH_2OAc\text{-}{}^{\cdot}CHOSi(CH_3)_3$
355	$C_{16}H_{25}NO_6Si$	$M^{\oplus\cdot} - 2 \times CH_3COOH - (CH_3)_3SiOH$
342	$C_{15}H_{24}NO_6Si$	$M^{\oplus\cdot} - {}^{\cdot}CH_2OAc - CH_3COOH - (CH_3)_3SiOH$
298	$C_{13}H_{20}NO_5Si$	$M^{\oplus\cdot} - CH_2OAc\text{-}{}^{\cdot}CHOSi(CH_3)_3 - CH_3OH - CH_3COOH$
277	$C_{11}H_{25}O_4Si_2$	$CH_2OAc\text{-}CH[OSi(CH_3)_3]\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
217	$C_9H_{21}O_2Si_2$	$CH_2{=}C[OSi(CH_3)_3]\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
186	$C_8H_{16}NO_2Si$	$AcNH\text{-}CH{=}CH\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
175	$C_7H_{15}O_3Si$	$CH_2OAc\text{-}CH{=}O^{\oplus}Si(CH_3)_3$
156	$C_7H_{10}NO_3$	$AcN^{\oplus}H{=}CH\text{-}C(OAc){=}CH_2$
143	$C_6H_9NO_3$	$AcN^{\oplus}H{=}CH\text{-}{}^{\cdot}CHOAc$

2) ^{1}H NMR spectroscopy

The ^{1}H NMR spectra of compounds 2 and 3 in [$^2H_4$]methanol are rather complex. The following signals have been assigned. Compound 2: 2.00 (s, *N*-acetyl-methyl), 2.07 (s, 9-*O*-acetyl-methyl), 3.25 (s, 2-*O*-methyl), 3.80 (s, ester-methyl), 1.63 (q, $J_{3,3'}$ = 12.9 Hz; $J_{3',4}$ = 10.6 Hz, H-3′ *ax*), 2.32 (q, $J_{3,3'}$ = 12.9 Hz; $J_{3,4}$ = 4.5 Hz, H-3 *eq*). Compound 3: 1.95 (s, 4-*O*-acetyl-methyl), 2.01 (s, *N*-acetyl-methyl), 2.07 (s, 9-*O*-acetyl-methyl), 3.29 (s, 2-*O*-methyl), 3.82 (s, ester-methyl), 1.75 (q, $J_{3,3'}$ = 13.0 Hz, $J_{3',4}$ ~ 11.2 Hz, H-3′), 2.43 (q, $J_{3,3'}$ = 13.0 Hz; $J_{3,4}$ ~ 5.2 Hz, H-3). The number of *O*-acetyl groups is directly evident from the above data.

3) ^{13}C NMR spectroscopy

The positions of the substituents in 2 and 3 have been established by ^{13}C NMR spectroscopy. The spectra of these compounds and of the reference substances 1 and 4 in [$^2H_4$]methanol are schematically presented in Fig. 3. Table 3 contains a survey of the chemical shift values. The assignment of the signals of compound 1 (in [$^2H_4$]methanol) was based on the spectrum of this compound in D_2O published recently by Bhattacharjee[7] and on comparison with the spectrum of the corresponding α-D-anomer 5 (Table 3). The signals in the 51-54 ppm range (representing 2-*O*-methyl, the ester-methyl carbon and C-5) were analysed by means of specific labelling with CD_3 groups and correlation with the spectrum of 4. It must be noted that the sequence of these resonances is changed with regard to the sequence in the spectrum in D_2O. The assignment of the signal near 65 ppm to C-9 was checked by correlation with the chemical shifts of the primary carbon atoms in structurally related polyols such as *meso*-erythritol (δC-1 and δC-4: 64.0 ppm) and arabitol (δC-1 and δC-5: 64.6 and 64.8 ppm; spectra in [$^2H_4$]methanol, unpublished results). The identification of the C-4 resonance (67.7 ppm), was verified by consideration of the relative resonance positions in the spectra of *N*-acetylneuraminic acid, its 4-*O*-methyl derivative and of the ethyl ester of *N*-acetyl-4-*O*-methylneuraminic acid (spectra in D_2O, unpublished results). On methylation, the resonance of C-4 is shifted 9.0 ppm downfield, whereas δC-3 and δC-5 are shifted upfield by 3.5 ppm and 2.2 ppm, respectively.

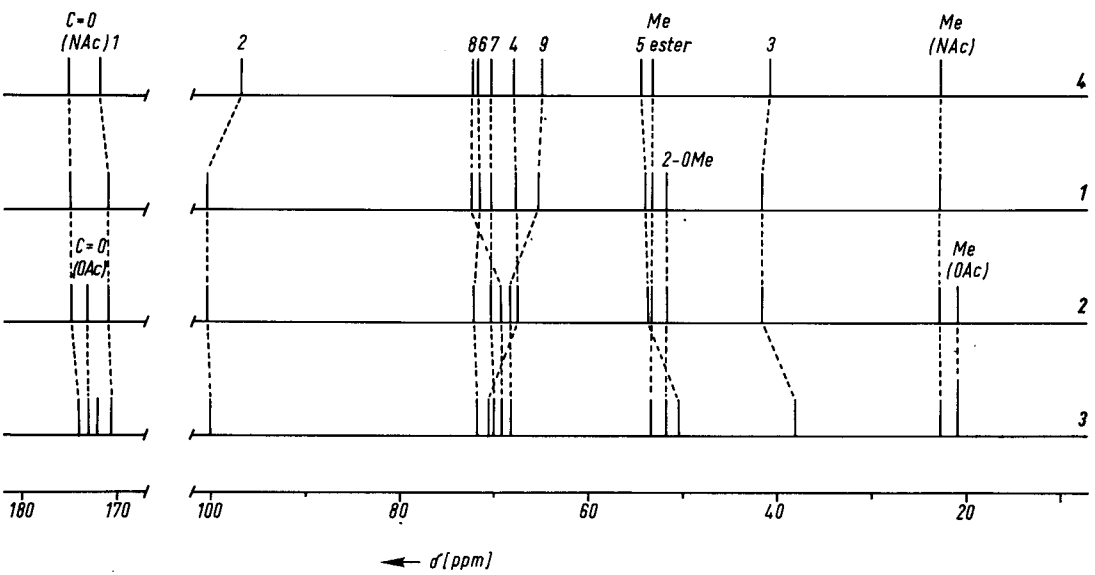

Fig. 3. Correlation of the ^{13}C NMR spectra of *N*-acetyl-β-D-neuraminic acid methyl ester methylglycoside (1), its 9-*O*-acetyl (2) and 4,9-di-*O*-acetyl (3) derivatives and of *N*-acetylneuraminic acid methyl ester (4).

Abbreviations, see Table 3. The numbers on top of the lines correspond with the C-atoms 1 - 9 of neuraminic acid.

Table 3. ^{13}C NMR chemical shifts (δ ppm) of *N*-acetylneuraminic acid derivatives.
Abbreviations: NeuNAc, *N*-acetylneuraminic acid; Me, methyl; OAc, *O*-acetyl; NAc, *N*-acetyl.
The assignments of the resonances marked with * may be interchanged.

	β-D-NeuNAc Me-ester-Me-glycoside (1)	β-D-NeuNAc-9-OAc Me-ester-Me-glycoside (2)	β-D-NeuNAc-4,9-di-OAc-Me-ester-Me-glycoside (3)	β-D-NeuNAc Me-ester (4)	α-D-NeuNAc-Me-ester-Me-glycoside (5)
C-1	170.9	170.9	170.5	171.8	170.9
C-2	100.4	100.4	100.0	96.7	100.4
C-3	41.6	41.5	38.3	40.8	41.4
C-4	67.7	67.5	70.6*	67.9	68.6
C-5	53.9	53.7	50.5	54.4	53.9
C-6	71.5	72.1	71.8	71.7	75.0
C-7	70.3	70.3	70.0*	70.3	70.4
C-8	72.4	69.3	69.2	72.2	72.5
C-9	65.3	68.3	68.2	64.9	64.8
CH_3 Me-ester	53.2[a,b]	53.3	53.4	53.2[c]	53.3[d]
CH_3 Me-glyc.	51.7[a]	51.7	51.8	–	52.0[d]
CH_3 NAc	22.8	22.8	22.7	22.7	22.8
CH_3 OAc	–	20.9	20.9	–	–
			20.9		
C=O NAc	174.9	174.8	174.0	175.1	175.4
C=O OAc	–	173.1	172.0	–	–
			173.0		

[a] Absent in the spectrum of β-D-NeuNAc-trideuteriomethyl ester trideuteriomethylglycoside
[b] Absent in the spectrum of β-D-NeuNAc-trideuteriomethyl ester methylglycoside
[c] Absent in the spectrum of β-D-NeuNAc-trideuteriomethyl ester
[d] Absent in the spectrum of α-D-NeuNAc-trideuteriomethyl ester trideuteriomethylglycoside

For compound 2, the signal of C-9 shows a 3.0 ppm downfield shift increment and the signal of C-8, a 3.1 ppm upfield shift increment compared with the resonance positions in the spectrum of 1. These shifts are in accordance with the presence of an *O*-acetyl group at C-9[7].

The spectrum of compound 3 shows, in addition to the shifts mentioned above for 2, a downfield shift increment of 2.9 ppm for C-4 and upfield shift increments of 3.3 and 3.4 ppm for C-3 and C-5, respectively. The carbonyl resonance of the *N*-acetyl group is shifted 0.9 ppm upfield. These shifts are in accordance with the presence of *O*-acetyl groups at C-4 and C-9.

Periodate oxidation studies

The periodate consumption in 1 h of *N*-acetylneuraminic acid, its 9-*O*-acetyl derivative and of the synthetic compounds 1, 2 and 3 is schematically represented in Fig. 4. *N*-acetylneuraminic acid and its methyl ester-β-D-methylglycoside (1) consume the theoretical amount of 2 mol periodate/mol within 10 min. However, *N*-acetyl-9-*O*-acetylneuraminic acid and compounds 2 and 3 consume only between 0.12 and 0.17 mol in 10 min, increasing to 0.2 - 0.3 mol in 1 h. This consumption was not significantly influenced by the use of sodium metaperiodate instead of periodic acid or by changing the reaction temperature from 20 °C to 0 °C. It corresponds to only 20 - 30% of the theoretical value. A fast consumption of 1 mol would be expected, because both the 9-*O*-acetylated and the 4,9-di-*O*-acetylated compounds have a vicinal diol function. The oxidation rate of *N*-acetyl-4-*O*-acetylneuraminic acid is identical with that of *N*-acetylneuraminic acid[6]. Therefore, the 4-*O*-acetyl residue does not affect the oxidation rate. An explanation for the low oxidation rate in the case of the acylneuraminic acids acetylated in position 9 is found in the conformation of the C-7–C-8 part of the molecule.

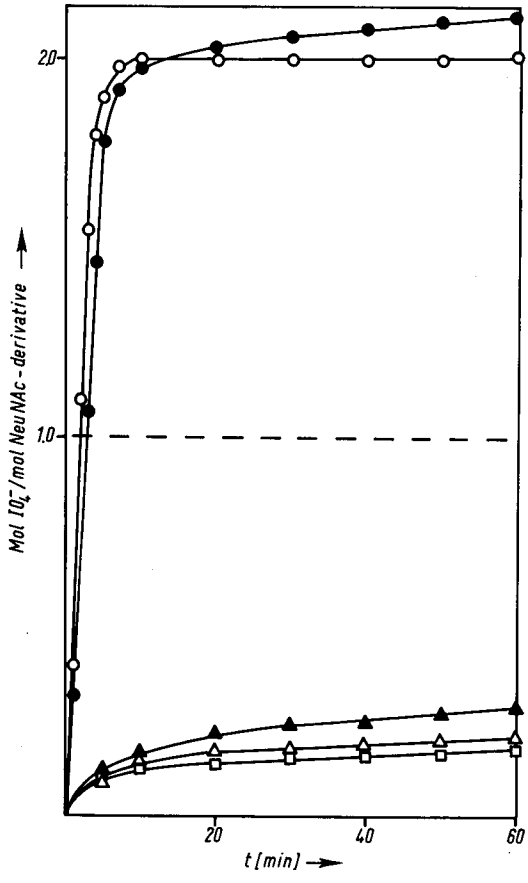

Fig. 4. Periodate consumption of *N*-acetylneuraminic acid (●——●); *N*-acetyl-β-D-neuraminic acid methyl ester methylglycoside (○——○); *N*-acetyl-9-*O*-acetylneuraminic acid (▲——▲); *N*-acetyl-9-*O*-acetyl-β-D-neuraminic acid methyl ester methylglycoside (△——△); and *N*-acetyl-4,9-di-*O*-acetyl-β-D-neuraminic acid methyl ester methyglycoside (□——□) at room temperature and pH 2.5.

For further experimental details see text.

The hydroxyl groups at these carbon atoms occupy preferentially a *trans* position to each other. This conformation, which can be deduced from the ^{1}H NMR data obtained for 4,7,8,9-tetra-*O*-acetyl-*N*-acetyl-α-D-neuraminic acid benzylglycoside methyl ester[14] and for free *N*-acetyl-β-D-neuraminic acid (L. Dorland, unpublished results), as well as from crystallographic data obtained for β-D-neuraminic acid methylglycoside trihydrate[15] and for *N*-acetyl-β-D-neuraminic acid dihydrate[16] causes a relative stability of this diol function against periodate[17]. Furthermore, it has to be noted that on treatment of *N*-acetylneuraminic acid with a small excess of periodate, the C-8–C-9 diol group is preferentially oxidised, as was shown by the high ratio of the aldehyde of the 8-carbon analogue of *N*-acetylneuraminic acid to the 7-carbon analogue obtained[18]. The 8-carbon analogue, being a 2-hydroxyaldehyde, is subject to further oxidation if the amount of periodate is sufficient. In conclusion, the oxidation of the C-7–C-8 diol group will be hindered if the formation of the mentioned aldehyde is blocked by the presence of an *O*-acetyl group at C-9. In addition, steric and/or electrostatic repulsion effects of the substituents at C-7 and C-8 can also hamper the formation of the diol-periodate complex.

It can be seen from Fig. 4 that free *N*-acetylneuraminic acid is further oxidised beyond its theoretical value, in contrast to its methyl ester-β-D-methylglycoside. This is due to oxidation of the C-6–C-7 vicinal diol function after opening of the pyranoside ring. Analogously, this might explain the differences in periodate oxidation rate of free *N*-acetyl-9-*O*-acetylneuraminic acid and the compounds 2 and 3.

In a period of 48 h, the compounds 2 and 3 were oxidised to 85% of their theoretical values of 1 mol periodic acid/mol. Exact kinetic data for the oxidation rates cannot be delineated, as the compounds were slowly de-*O*-acetylated, which was estimated by the hydroxamate test. However, the rate of oxidation was faster than that of hydrolysis of the 9-*O*-acetyl groups: as an example, no elimination of the ester group from *N*-acetyl-9-*O*-acetylneuraminic acid was observed after 10 min in aqueous acetic acid (pH 2.5, room temperature). The degree of de-*O*-acetylation of this compound was only 20% after 48 h.

In earlier experiments[6] the low oxidation rate of *N*-acetyl-9-*O*-acetylneuraminic acid from bovine submandibular gland glycoprotein led to the assumption that the *O*-acetyl residue of this acylneuraminic acid is localised at C-8. Comparison of this substance with the synthesised 9-*O*-acetylated neuraminic acid derivatives now offers a good explanation of the original assignment.

It is noteworthy that the low oxidation rate of *N*-acetyl-9-*O*-acetylneuraminic acid makes better understandable the molar extinction coefficient of about 38% of this acylneuraminic acid when compared with *N*-acetylneuraminic acid in the periodic acid/thiobarbituric acid assays of Aminoff[19] and Warren[20]. Under the conditions of the Aminoff assay (37 °C, 0.2N H_2SO_4, 30 min) the oxidation rate of *N*-acetyl-9-*O*-acetylneuraminic acid was 36% of the theoretical value, which corresponds well with its molar absorption coefficient. Concomitantly, 12% of the *O*-acetyl residues were hydrolysed within the reaction time of 30 min.

In analogy to these colorimetric methods, the presence of a 9-*O*-acetyl residue in an acylneuraminic acid may give rise to a low yield of chromophor in the periodate/resorcinol assay. Thus, the low values found recently with acylneuraminic acids from human and rat colon glycoproteins[21] do not necessarily mean that the hydroxyl groups of these acylneuraminic acids are acetylated at C-7 and/or C-8.

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