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IDENTIFICATION BY GAS-LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY OF 4-*O*-ACETYL-9-*O*-LACTYL-*N*-ACETYL-NEURAMINIC ACID, A NEW SIALIC ACID FROM HORSE SUBMANDIBULAR GLAND

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Summary

The novel sialic acid 4-*O*-acetyl-9-*O*-lactyl-*N*-acetylneuraminic acid has been identified as a constituent of horse submandibular gland glycoproteins in addition to the already know equine sialic acids, *N*-acetylneuraminic acid, 4-*O*-acetyl-*N*-acetylneuraminic acid, 9-*O*-acetyl-*N*-acetylneuraminic acid, 4,9-di-*O*-acetyl-*N*-acetylneuraminic acid, *N*-glycolylneuraminic acid, 4-*O*-acetyl-*N*-glycolylneuraminic acid and 9-*O*-acetyl-*N*-glycolylneuraminic acid. The structure has been established by combined gas-liquid chromatography-mass spectrometry.

Previously, the occurrence of several sialic acids as constituents of glycoproteins from horse submandibular glands has been reported, viz. *N*-acetylneuraminic acid (Neu5Ac) [1], 4-*O*-acetyl-*N*-acetylneuraminic acid ($Neu4,5Ac_2$) [1, 2], 4,9-di-*O*-acetyl-*N*-acetylneuraminic acid ($Neu4,5,9Ac_3$) [2], *N*-glycolylneuraminic acid (Neu5Gl) [1] and 4-*O*-acetyl-*N*-glycolylneuraminic acid (Neu4Ac5Gl) [2]. Recently, 9-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,9Ac_2$) and 9-*O*-acetyl-*N*-glycolylneuraminic acid (Neu9Ac5Gl), widely distributed in nature [3], have been detected in equine submandibular glands also (un-

Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; $Neu4,5Ac_2$, 4-*O*-acetyl-*N*-acetylneuraminic acid; $Neu4,5,9Ac_3$, 4,9-di-*O*-acetyl-*N*-acetylneuraminic acid; Neu5Gl, *N*-glycolylneuraminic acid; Neu4Ac5Gl, 4-*O*-acetyl-*N*-glycolylneuraminic acid; $Neu5,9Ac_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid; Neu9Ac5Gl, 9-*O*-acetyl-*N*-glycolylneuraminic acid; $Neu4,5Ac_29Lac$, 4-*O*-acetyl-9-*O*-lactyl-*N*-acetylneuraminic acid; Neu5Ac9Lac, 9-*O*-L-lactyl-*N*-acetylneuraminic acid.

published results). In this paper, the characterization of an additional sialic acid, 4-*O*-acetyl-9-*O*-lactyl-*N*-acetylneuraminic acid (Neu4,5Ac$_2$9Lac) will be reported.

Submandibular glands from horse (400 g) were acquired immediately after death of the animals and were kept on ice. The following procedures were carried out at 2—4°C, unless otherwise stated. The tissue was homogenized with three volumes of distilled water and centrifuged for 10 min at 10 000 × *g*. The sediment was rehomogenized in the same volume of water and centrifuged again. The combined supernatants were adjusted to pH 3.5 with formic acid to precipitate the major amount of glycoproteins. After centrifugation (10 min; 10 000 × *g*), the sediment was suspended in water (100 mg wet sediment/ml). The suspension was brought to pH 2 with formic acid and heated for 60 min at 70°C. The hydrolysate was dialysed three times against the 10-fold amount of deionised water (5 h each). The sialic acids present in the combined dialysates (200 mg) were adsorbed on a column (20 × 3.5 cm) of Dowex 2 X8, $HCOO^-$ form, 100—200 mesh, and subsequently eluted with 2 l of a linear gradient of 0 to 2 N formic acid; 10 ml fractions were collected. Sialic acid-containing fractions, detected by the orcinol/Fe^{3+}/HCl reagent [3, 4], were pooled and lyophilised. This residue (190 mg) was dissolved in the system water/*n*-propanol/*n*-butanol (1:2:1, by vol.) (100 mg/ml) and fractionated on a column (100 × 3.5 cm) of cellulose MN 2100 ff (Macherey, Nagel & Co., Düren, F.R.G.) using the same solvent system. The fractions (5 ml) were analysed for sialic acids as mentioned above. Sialic acid-positive fractions were screened by thin-layer chromatography on 0.1 mm cellulose sheets (Merck AG, Darmstadt) with water/*n*-propanol/*n*-butanol (1:2:1, by vol.) as solvent system. The di-O-acetylated-*N*-acetylneuraminic acid-containing fractions were pooled and lyophilised (7 mg).

For structural studies part of the lyophilised material (100 μg) was esterified with diazomethane and then trimethylsilylated as described earlier [2]. Gas-liquid chromatographic analysis on 3.8% SE-30 at 215°C [5, 6] shows the presence of four peaks which could be attributed to sialic acids (Fig. 1). The peaks with R_{Neu5Ac} values of 1.00, 1.18 and 1.33 (relative to the pertrimethylsilyl derivative of the methyl ester of Neu5Ac) correspond with the pertrimethylsilyl derivatives of the methyl esters of Neu5Ac (peak 1), Neu4,5Ac$_2$ (peak 2) and Neu4,5,9Ac$_3$ (peak 4), respectively, as was proven by combined gas-liquid chromatography-mass spectrometry [2, 5]. Table I gives a survey of the characteristic fragment ions present in the mass spectra of these sialic acid derivatives [2, 5].

The mass spectrum obtained from the gas chromatographic peak 5 with R_{Neu5Ac} 3.01 (Fig. 2) was interpreted on guidance of the specific fragment ions A—G (see Table I). The latter fragments contain the information about the number, type and position of substituents in sialic acids [2, 5]. The peaks in the high-mass range at *m/z* 710 and 666 were assigned to the fragments A ($M^{+\cdot} - {}^{\cdot}CH_3$) and B ($M^{+\cdot} - {}^{\cdot}COOCH_3$), respectively, pointing to a molecular weight of 725. The peaks at *m/z* 448 and 298 are also present in the mass spectra of the Neu4,5Ac$_2$ and Neu4,5,9Ac$_3$ derivatives and correspond with the fragments C ($M^{+\cdot} - {}^{\cdot}CHOR_8\text{-}CH_2OR_9$) and D ($M^{+\cdot} - {}^{\cdot}CHOR_8\text{-}CH_2OR_9 - R_2OH - R_4OH$), respectively. These data indicate the presence of an OMe_3Si

group at C-7 (fragments C and D are hardly observed if C-7 bears an O-acyl group), an NHAc group at C-5 and an OAc group at C-4. The absence of peaks at *m/z* 173 (fragment G, being $R_5CONH{=}CH\text{-}\dot{C}HOR_4$, has shifted to *m/z* 143) and 317 (fragment E, being $M^{+\cdot} - {}^{\cdot}CHOR_7\text{-}CHOR_8\text{-}CH_2OR_9 - R_5CONH_2$, is detected only if C-4 bears an ether group) is in accordance with these assignments. On the basis of the peaks at *m/z* 710, 666 and 448, it could be concluded that the C-8/C-9 part of the molecule contains a substituent which causes an increment of +72 a.m.u. with regard to the $Neu4,5Ac_2$ derivative. Therefore, the peak at *m/z* 277 can be assigned to fragment F ($CH_2OR_9\text{-}CH{=}\overset{+}{O}R_8$). The latter peak in combination with the main peak at *m/z* 117 ($CH_3\text{-}CH{=}\overset{+}{O}Me_3Si$) gives evidence for the presence of a trimethylsilylated O-lactyl group at C-9 and an OMe_3Si group at C-8. It should be noted that fragment F can hardly be observed if C-8 bears an O-acyl group. The peaks at *m/z* 277 and 117 are also present in the mass spectrum of the 9-*O*-L-lactyl-*N*-acetylneuraminic acid (Neu5Ac9Lac) derivative, described previously [6, 7]. The observation of a peak at *m/z* 217 ($CH_2{=}C[OMe_3Si]\text{-}CH{=}\overset{+}{O}Me_3Si$) formed by elimination of $CH_3\text{-}CHOMe_3Si\text{-}COOH$ from the C-7/C-8/C-9 part of the molecule, in combination with the absence of *m/z* 289 (elimination of Me_3SiOH from the C-7/C-8/C-9 part of the molecule) is

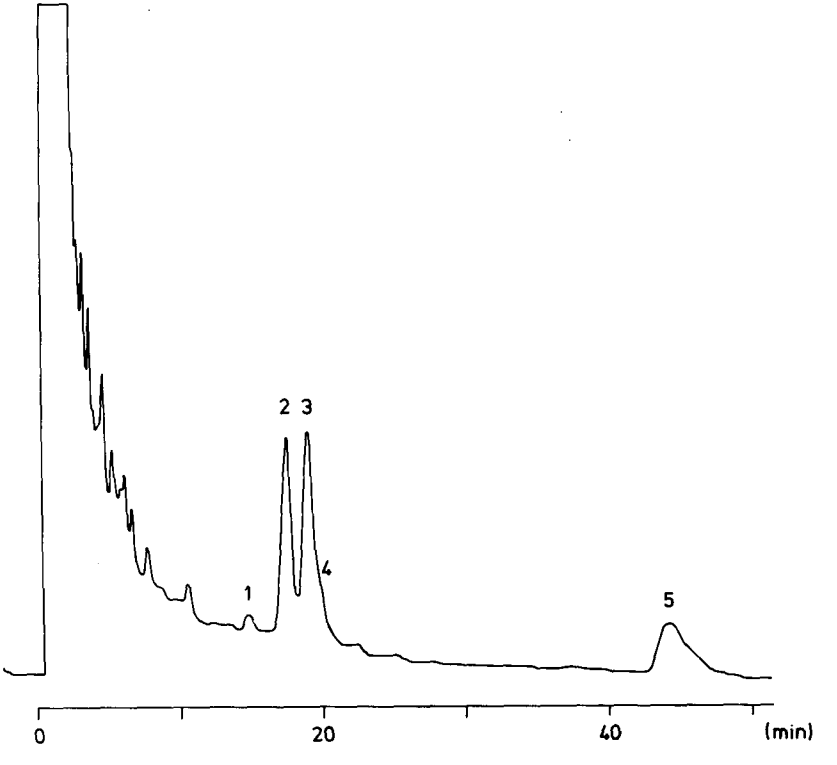


Fig. 1. Gas-liquid chromatogram on 3.8% SE-30 at 215°C of the di-O-acetylated *N*-acetylneuraminic acid-containing fraction. Peaks are identified by combined gas-liquid chromatography-mass spectrometry. Peak 1, Neu5Ac; peak 2, $Neu4,5Ac_2$; peak 3, phthalic acid ester; peak 4, $Neu4,5,9Ac_3$; peak 5, $Neu4,5Ac_2$9Lac.

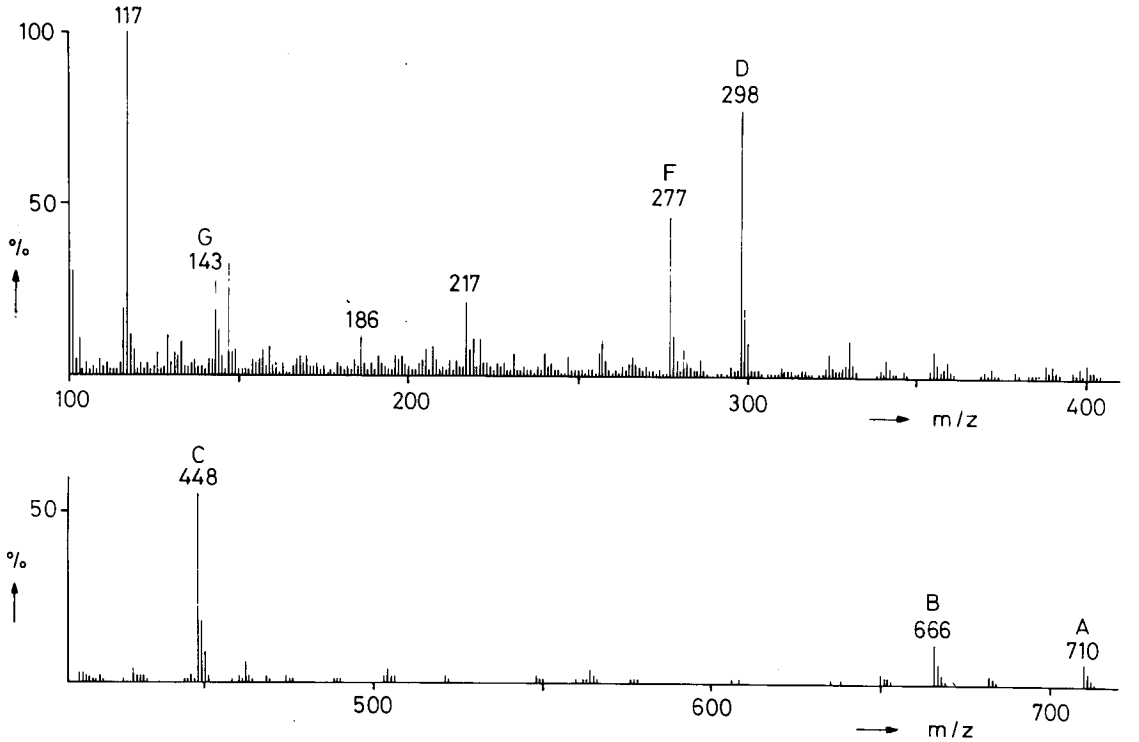
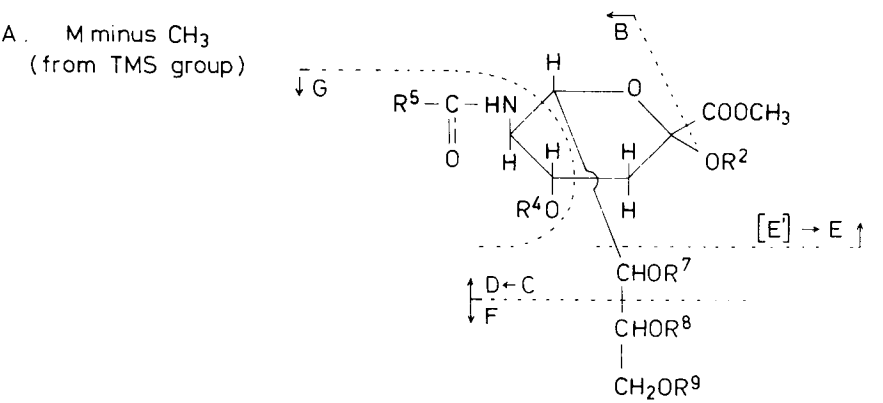


Fig. 2. Mass spectrum at 70 eV of the pertrimethylsilyl derivative of the methyl ester of Neu4,5Ac$_2$9Lac. Only *m/z* values over 100 are given. The specific fragment ions are indicated by A—G.

TABLE I

Specific fragment ions A—G in the mass spectra of the pertrimethylsilyl derivatives of the methyl esters of the sialic acids with R_{Neu5Ac} 1.00, 1.18, 1.33 and 3.01 (relative to the TMS derivative of the methyl ester of Neu5Ac).

Sialic acids	R_{Neu5Ac}	A	B	C	D	E	F	G
Neu5Ac	1.00	668	624	478	298	317	205	173
Neu4,5Ac$_2$	1.18	638	594	448	298	—	205	143
Neu4,5,9Ac$_3$	1.33	608	564	448	298	—	175	143
Neu4,5Ac$_2$9Lac	3.01	710	666	448	298	—	277	143



in agreement with an O-acyl group at C-9 [2, 6]. Finally, the occurrence of a peak at m/z 186 is an additional indication for an NHAc group at C-5 [2, 6]. Summarising the mass spectrometric data, it can be concluded that the gas chromatographic peak with R_{Neu5Ac} 3.01 belongs to the pertrimethylsilylated derivative of the methyl ester of Neu4,5$Ac_2$9Lac. The absolute configuration of the lactyl group was not determined.

This is the first report describing the presence of Neu4,5$Ac_2$9Lac in nature. Up to now the occurrence of *N*-acetylneuraminic acid having an O-lactyl group at C-9 has not been reported for equine tissues but only for human blood and saliva and for glycoproteins isolated from cow submandibular glands [6, 7].

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References

1 Schauer, R. and Faillard, H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 961—968
2 Kamerling, J.P., Vliegenthart, J.F.G., Versluis, C. and Schauer, R. (1975) Carbohydr. Res. 41, 7—17
3 Schauer, R. (1978) Methods Enzymol. 50, 64—89
4 Böhm, P., Dauber, S. and Baumeister, L. (1954) Klin. Wochenschr. 32, 289—292
5 Kamerling, J.P., Haverkamp, J., Vliegenthart, J.F.G., Versluis, C. and Schauer, R. (1978) in Recent Developments in Mass Spectrometry in Biochemistry and Medicine (Frigerio, A., ed), Vol. 1, pp. 503—520, Plenum Publishing Corporation, New York
6 Schauer, R., Haverkamp, J., Wember, M., Vliegenthart, J.F.G. and Kamerling, J.P. (1976) Eur. J. Biochem. 62, 237—242
7 Haverkamp, J., Schauer, R., Wember, M., Farriaux, J.-P., Kamerling, J.P., Versluis, C. and Vliegenthart, J.F.G. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1699—1705