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High-Resolution ^{1}H-NMR Spectroscopy of Free and Glycosidically Linked *O*-Acetylated Sialic Acids

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A number of naturally occurring and synthetic, partially *O*-acetylated derivatives of *N*-acetylneuraminic and *N*-glycoloylneuraminic acids have been investigated by 360-MHz ^{1}H-NMR spectroscopy. *O*-Acetylation causes strong downfield shifts for the resonances of neighbouring sugar-skeleton protons. The chemical shifts of these resonances, together with their characteristic multiplet shapes, can be used for localisation of the position of *O*-acetyl substituents in the molecule. The number of such substituents in the molecule can be inferred from the number of acetyl-methyl singlets, which also have characteristic resonance positions.

This method for determination of the number and position of *O*-acetyl substituents in sialic acid residues is very powerful for structural analysis of underivatized carbohydrate chains derived from glycoconjugates. This is demonstrated for the oligosaccharide *N*-acetyl-4-*O*-acetylneuraminosyl-(α2→3)-lactose isolated from echidna milk.

Sialic acids (a collective noun for derivatives of neuraminic acid; 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid) occur in nature in a great variety of forms. Generally, they are present in carbohydrate chains of bacterial and animal oligosaccharides, polysaccharides, glycoproteins and glycolipids occupying reducing terminal, non-reducing terminal, or internal (intra-chain) positions [1–5], whereas their glycosidic linkage to other sugar residues always is of the α-type. From a biochemical point of view the group of *N*,*O*-acylated sialic acids, comprising derivatives of *N*-acetylneuraminic and *N*-glycoloylneuraminic acids with one or more of their hydroxyl groups acetylated or lactylated, is intriguing [2]. However, the biological importance of these sialic acid modifications is only partly understood. One function of the acyl substituents seems to be their influence on the action of sialidases. An *O*-acetyl group at C-4 renders the sialic acid resistant to the action of viral, bacterial and mammalian sialidases and an *O*-acetyl group at C-9 reduces the rate of cleavage by about 50% when compared to the non-*O*-acetylated compound [6]. Sialic acids with an *N*-glycoloyl residue are hydrolyzed at a much lower rate by sialidases than those with an *N*-acetyl group [7]. The observation that *O*-acetylation of colominic acid from *Escherichia coli* K1 increases the antigenicity when compared with the non-*O*-acetylated polysaccharide points to a possible influence of sialic acid *O*-acyl residues on the immunological behaviour of complex carbohydrates [8]. Incorporation of ester groups into sialic acids may also influence the physico-chemical, e.g. amphiphilic, properties of cell-membrane glycoproteins.

To obtain insight into the distribution and biological functions of the different sialic acids much effort has been invested in elucidating their structures, i.e. linkage types and substitution patterns. A general strategy is the cleavage of the linkage between sialic acid and carbohydrate by acidic or enzymic hydrolysis, followed by chromatographic purification and subsequent structural analysis of the monomeric sialic acids [2]. A serious drawback of this approach, however, is that an appreciable loss of *O*-acyl substituents occurs during the hydrolysis and isolation. Furthermore, the possibility of migration of *O*-acyl groups cannot be excluded.

A breakthrough in the characterization of sialic acids was the development of gas chromatography/mass spectrometry for identification of microgram samples [9,10]. In this context, the vinylation technique [11,12] for blocking the free hydroxyl groups in a partially *O*-acetylated sialic acid residue followed by methylation analysis should also be mentioned. This procedure has the advantage that it can be applied to intact glycoconjugates.

Another method for analysis of *N*,*O*-acylneuraminic acid residues in carbohydrate chains is high-resolution ^{1}H nuclear magnetic resonance (NMR) spectroscopy. This method can give detailed structural information about native, underivatized oligomeric carbohydrate chains, viz. type and sequence of monosaccharide units and their substitution patterns, type and position of glycosidic linkages and also information about conformational aspects of the molecule. Furthermore, this approach eliminates the necessity for hydrolysis and purification procedures that may give rise to artificial modifications in the substitution pattern. The high-resolution ^{1}H-NMR spectrum of βNeu5Ac (compound 1) has been described in detail by Brown et al. [13] and the

Abbreviations. Neu, neuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac1Me, *N*-acetylneuraminic acid methyl ester; Neu5Ac2Me, *N*-acetylneuraminic acid methyl glycoside; Neu5Acl, $2Me_2$, *N*-acetylneuraminic acid methyl ester methyl glycoside; Neu5,9Ac_2, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu9Ac5Glc, *N*-glycoloyl-9-*O*-acetylneuraminic acid (other mono-*O*-acetyl derivatives are denoted similarly); Neu4,5,9$Ac_3$1,$2Me_2$, *N*-acetyl-4,9-di-*O*-acetylneuraminic acid methyl ester methyl glycoside, etc. These abbreviations for neuraminic acid derivatives conform to the new proposals for the nomenclature of natural sialic acids drawn up on the basis of a discussion during the 5th Int. Symp. on Glycoconjugates (Sept. 1979) in Kiel, FRG.

spectrum of βNeu5Gc (compound 2) by Jaques et al. [14]. Recently we mentioned the presence of characteristic signals for sialic acid moieties in the ^{1}H-NMR spectra of sialic acid glycosides, oligosaccharides and glycopeptides, viz. the H-3*ax.* and H-3*eq.* multiplets which are indicative of the type and configuration of the anomeric linkage of the sialic acid residue [15–18]. In this article we will focus on the effects of *O*-acetyl substitution on the NMR chemical shift data of the skeleton protons in a number of *N*,*O*-acylated neuraminic acids of natural origin, as well as on the application of the obtained NMR reference data for deduction of the position(s) of *O*-acetylation of sialic acid residues in a sialo-oligosaccharide.

MATERIALS AND METHODS

Sialic Acids

Neu4,5Ac$_2$ (compound 10) was isolated from equine submandibular gland glycoprotein after mild acid hydrolysis followed by ion-exchange and cellulose partition chromatography as described previously [2,19]. Neu5Ac (compounds 1, 17), Neu5Gc (compounds 2, 18), Neu5,9Ac$_2$ (compounds 7, 22), Neu9Ac5Gc (compounds 9, 23), Neu5,7Ac$_2$ (compounds 12, 24), Neu5,7,9Ac$_3$ (compounds 13, 25) and Neu5,8,9Ac$_3$ (compound 14) were isolated from bovine submandibular gland glycoprotein according to this procedure, modified in the following way. Sialic acid elution from Dowex 2X8 was carried out by a linear formic-acid gradient (0–0.5 M) of the 10-fold column volume, followed by another 5-fold volume of 0.5 M formic acid. The purity and nature of the sialic acids was determined by colorimetric methods [2], thin-layer chromatography [2,19], gas-liquid chromatography [2,10] and gas-liquid chromatography/mass spectrometry [9,10].

Neu5Ac1Me (compounds 3, 19) and the Neu5Ac1,2Me$_2$ anomers, compounds 5, 6 and 21, were synthesized according to the procedures described by Yu and Ledeen [20]. The Neu5Ac2Me glycosides, compounds 4 and 20, were prepared by saponification of the compounds 5 and 21 in aqueous triethylamine at p^{2}H 11, 40 °C, following the reaction by ^{1}H-NMR spectroscopy. The *O*-acetylated derivatives of βNeu5Ac1,2Me$_2$ (compound 5), i.e. compounds 8, 11, 15 and 27, were prepared by partial acetylation of compound 5 with *N*-acetylimidazole in pyridine [21] followed by thin-layer chromatographic separation of the various products. βNeu2,4,5,7,8,9Ac$_6$ (compound 16) was prepared following the procedure of Meindl and Tuppy [22]. αNeu4,5,7,8,9Ac$_5$-2Me (compound 26) was prepared by acetylation of compound 20 with acetic anhydride/pyridine at 95 °C for 1 h, followed by treatment with Dowex 50, H^+ in water at 0 °C and lyophilisation. The sialyllactoses, compounds 28 and 30 were gifts from Dr Strecker (Lab. of Biol. Chem., Univ. of Lille, France) and compound 29 was prepared by ozonolysis from ganglioside [Neu5Gc(α2→3)Gal(β1→4)Glcβ1→ceramide] isolated from equine erythrocyte membranes [23]. Compound 31 was isolated from echidna (*Tachyglossus aculeatus*) milk by Dr Messer (Dept of Biochem., Univ. of Sydney, Australia) [24].

^{1}*H-NMR Spectroscopy*

For ^{1}H-NMR spectroscopy the neuraminic-acid derivatives were exchanged four times in ^2H_2O (99.96% ^{2}H, Aldrich) with intermediate lyophilization. The solutions were neutral, except for the 7Ac and 7,9Ac$_2$ derivatives, which were kept at p^{2}H 2–4. The 360-MHz ^{1}H-NMR spectra of 3–10 mM solutions of the compounds in ^2H_2O were recorded on a Bruker HX-360 spectrometer operating in the Fourier-transform mode at a probe temperature of 25 °C. Resolution enhancement of the spectra was achieved by Lorentzian to Gaussian transformation, according to Ernst [25]. Chemical shifts are given relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in ^2H_2O: δ = 2.225 ppm).

RESULTS AND DISCUSSION

Spectral Data of Skeleton Protons of Monomeric Sialic Acid

Spectra were recorded of solutions in ^2H_2O of the series of *N*,*O*-acylated neuraminic acid derivatives, listed in Table 1. As typical spectra, those of the compounds Neu5,7Ac$_2$ (12, 24) and αNeu4,5,7,8,9Ac$_5$2Me (26) are given in Fig. 1 and 2, respectively. The positions of most of the sialic acid proton resonances are strongly dependent on the p^{2}H of the solution (e.g. compare the data for the pairs of compounds 1a-1b; 14a-14b and 17a-17b). For this reason the various substances were measured at p^{2}H ≈ 7, except for the *O*-acetylated neuraminic acid derivatives 12, 13, 24 and 25 which are unstable in neutral solutions. These compounds were analysed at p^{2}H ≈ 2. It should be noted that in aqueous solutions of free sialic acids the α and β anomeric forms occur in an equilibrium ratio of 7:93, as became evident from the ^{13}C-NMR investigations of Jaques et al. [26] and from the ^{1}H-NMR work of Haverkamp et al. [16] and Friebolin et al. [27]. In the spectrum of a free sialic acid the major part of the spectrum of the α anomer is hidden under the relatively intense resonance lines of the corresponding β anomer. Owing to a pronounced influence of the configuration of the anomeric centre on the shielding of H-3*ax.*, H-3*eq.* and the 5Ac methyl group their resonances in the α-anomeric form can clearly be observed. The singlet peak of the 5Gc methylene protons of the α anomer, however, is also hidden in the bulk region of the spectrum [28]. The listed chemical shift values for H-3*ax.*, H-3*eq.* and 5Ac of the α anomeric forms of the various *N*,*O*-acylated neuraminic acids are in principle suitable for the identification of bound sialic acid residues in natural glycoconjugates as these generally have α configuration.

Comparison of the chemical shift values of free Neu5Ac (compound 1a) and of βNeu5Ac2Me (compound 4) shows that glycoside formation has a significant influence on number of protons of the carbohydrate skeleton (viz. H-3*ax.*, H-3*eq.*, H-6, H-8 and H-9′). In the α anomers, shifts of resonance positions on methyl glycoside formation are observable just for H-3*ax.* and H-3*eq.*, but probably also other protons will be affected. Esterification of βNeu5Ac at C-1 also influences a number of proton resonance positions, viz. H-3*ax.*, H-3*eq.*, H-4, H-6 and H-7 (compare compounds 1a and 3). These effects of glycoside formation and esterification seem to be reasonably additive as can be concluded from comparison of the compounds 1a, 3, 4, 5 and 6; of compounds 7 and 8, and of compounds 10 and 11. Esterification of αNeu5Ac2Me (compound 20) leads to changes in the chemical shifts of all ring protons, whereas the protons of the glycerol side-chain are only slightly influenced (compare compounds 20, 21 and 26, 27).

Effects of O-Acetylation

The shifts for skeleton proton resonances, observed on *O*-acetylation of one of the hydroxyl functions, are generally

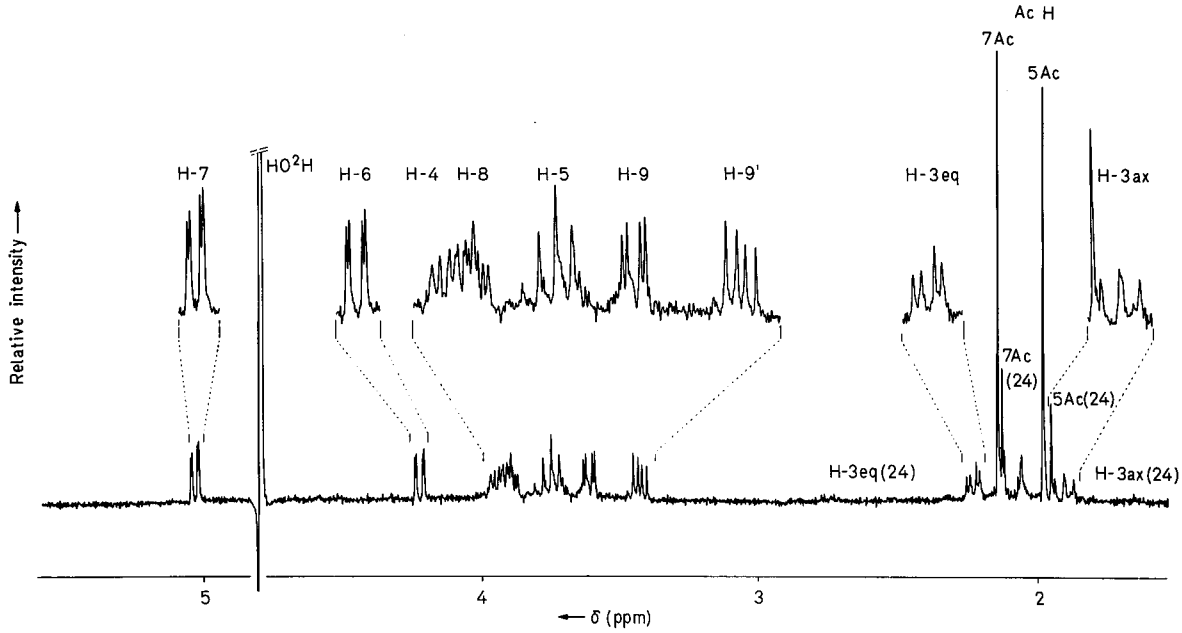

Fig. 1. *360-MHz ^{1}H-NMR spectrum of the anomeric mixture of βNeu5,7Ac$_2$ (compound 12) and αNeu5,7Ac$_2$ (compound 24)*

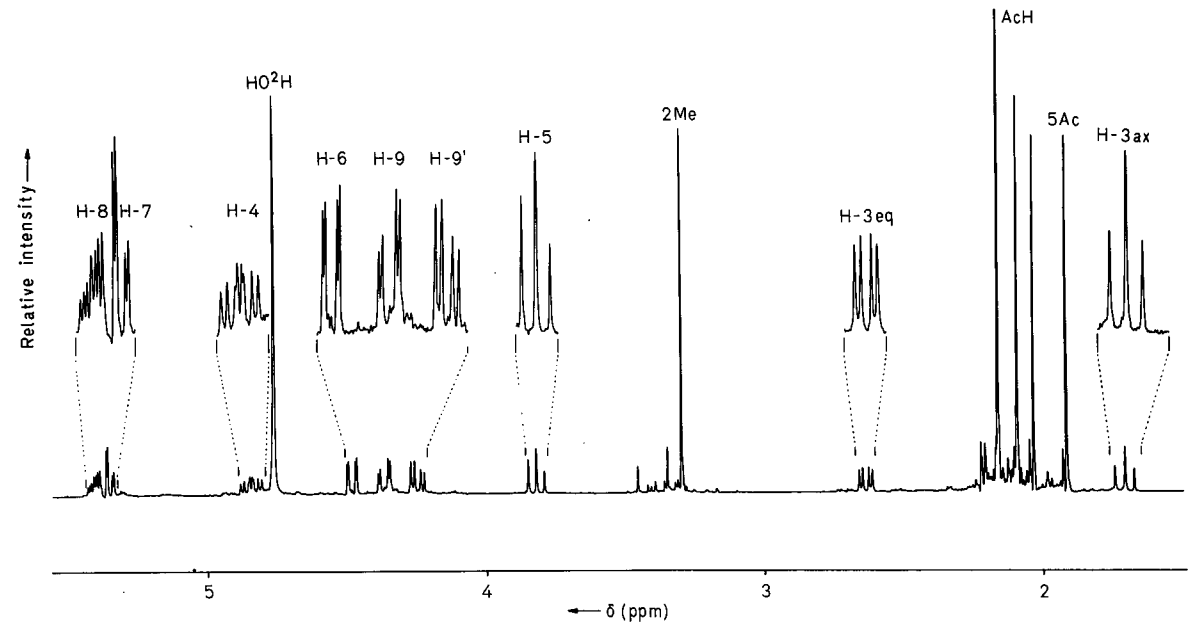

Fig. 2. *360-MHz ^{1}H-NMR spectrum of αNeu4,5,7,8,9Ac$_5$2Me (compound 26) showing the characteristic resonance positions and multiplet patterns for the various protons*

downfield (further denoted as positive shift increment values). The α effects (i.e. the effects on the proton(s) attached to the carbon atom which bears the *O*-acetyl group) observed for protons linked to secondary carbon atoms range from 1 – 1.5 ppm (i.e. 1.2 ppm for H-4, compare compounds 1a and 10 and 5 and 11; and 1.5 ppm for H-7, compare compounds 1a and 12). In the case of acetylation of the primary hydroxyl function at C-9 both H-9 and H-9′ are shifted much less: 0.5 – 0.6 ppm (compare compounds 1a and 7, 2 and 9 and 5 and 8). This difference in deshielding can in fact be ascribed to either the inductive effect of the *O*-acetyl group (due to sharing of the effect by the protons concerned) or the field effect of its carbonyl group (due to the increased distance between carbonyl group and protons), or to a combination of these effects. Based on the experiments of Narayanan and Parkar [29] on rigid steroid systems it can be concluded that the latter factor in general is the most important (provided their reasoning holds also for the less rigid carbohydrate molecules). The shift effects for acetylation at C-8 cannot be inferred in a direct way as specimens of Neu5,8Ac$_2$ or Neu8Ac5Gc were not available.

The β effects (i.e. the effects on protons at carbon atoms next to the *O*-acetylated carbon) in the cases of *O*-acetylation of C-4, C-7 and C-9 are about 0.2 ppm, except for the geminal protons H-3*ax.* and H-3*eq.* where shifts of 0.04 – 0.14 ppm were observed (compare compounds 1a and 10,

Table 1. *^{1}H-NMR chemical shift data for neuraminic-acid derivatives*

Chemical shifts, δ, are given for solutions in ^2H_2O at 25 °C and at the indicated p^{2}H values. Compound 6 was included to determine more accurately α anomers of compounds 10, 14a and 14b could not be obtained due to complication of the spectrum as a result of the presence of small non-carbohydrate *N*-acetylneuraminosyl-(α2 → 3)-lactose; Neu5Gc(α2 → 3)Gal(β1 → 4)Glc, *N*-glycoloylneuraminosyl-(α2 → 3)-lactose; Neu5Ac(α2 → 6)Gal(β1 → 4)Glc,

Compound	δ value						
	H-3*ax.*	H-3*eq.*	H-4	H-5	H-6	H-7	H-8
	ppm						
1a. βNeu5Ac	1.827	2.208	4.024	3.899	3.984	3.514	3.753
1b. βNeu5Ac	1.880	2.313	4.067	3.931	4.056	3.556	3.750
2. βNeu5Gc	1.840	2.243	4.127	4.002	4.106	3.549	3.777
3. βNeu5Ac1Me	1.913	2.315	4.067	3.916	4.067	3.552	3.731
4. βNeu5Ac2Me	1.645	2.337	4.009	≈3.88	3.785	3.532	≈3.88
5. βNeu5Ac1,2Me$_2$	1.784	2.392	4.044	3.917	3.84–3.88	3.581	3.84–3.88
6. βNeu5Ac1,2($^2H_3$)Me$_2$	1.783	2.391	4.040	3.914	≈3.87	3.582	≈3.87
7. βNeu5,9Ac$_2$	1.833	2.221	4.024	3.913	3.991	3.571	3.977
8. βNeu5,9Ac$_2$1,2Me$_2$	1.788	2.395	4.059	3.938	3.910	3.637	4.087
9. βNeu9Ac5Gc	1.842	2.234	≈4.14	4.006	4.109	3.570	3.970
10. βNeu4,5Ac$_2$	1.951	2.249	5.274	≈4.15	≈4.15	3.570	3.775
11. βNeu4,5Ac$_2$1,2Me$_2$	1.927	2.442	5.260	4.157	4.035	3.623	≈3.87
12. βNeu5,7Ac$_2$	1.905	2.236	3.950	3.767	4.246	5.045	3.911
13. βNeu5,7,9Ac$_3$	1.924	2.303	3.978	3.775	4.293	5.162	4.140
14a. βNeu5,8,9Ac$_3$	1.838	2.189	3.978	3.903	3.780	3.838	5.114
14b. βNeu5,8,9Ac$_3$	1.862	2.250	4.006	3.912	3.830	3.866	5.115
15. βNeu4,5,9Ac$_3$1,2Me$_2$	1.921	2.445	5.299	4.188	4.069	3.686	4.109
16. βNeu2,4,5,7,8,9Ac$_6$	1.862	2.467	5.276	3.934	4.105	5.438	5.182
17a. αNeu5Ac	1.621	2.730	n.d.	n.d.	n.d.	n.d.	n.d.
17b. αNeu5Ac	1.705	2.718	n.d.	n.d.	n.d.	n.d.	n.d.
18. αNeu5Gc	1.644	2.749	n.d.	n.d.	n.d.	n.d.	n.d.
19. αNeu5Ac1Me	1.732	2.723	n.d.	n.d.	n.d.	n.d.	n.d.
20. αNeu5Ac2Me	1.626	2.718	3.675	3.803	3.689	3.586	3.886
21. αNeu5Ac1,2Me$_2$	1.798	2.676	3.756	≈3.86	≈3.83	3.558	3.84–3.88
22. αNeu5,9Ac$_2$	1.624	2.720	n.d.	n.d.	n.d.	n.d.	n.d.
23. αNeu9Ac5Gc	1.649	2.751	n.d.	n.d.	n.d.	n.d.	n.d.
24. αNeu5,7Ac$_2$	1.649	2.757	n.d.	n.d.	n.d.	n.d.	n.d.
25. αNeu5,7,9Ac$_3$	1.686	2.751	n.d.	n.d.	n.d.	n.d.	n.d.
26. αNeu4,5,7,8,9Ac$_5$2Me	1.701	2.637	4.858	3.834	4.498	5.365	5.422
27. αNeu4,5,7,8,9Ac$_5$1,2Me$_2$	1.930	2.718	4.924	3.909	4.273	5.390	5.428
28. Neu5Ac(α2 → 3)Gal(β1 → 4)Glc	1.799	2.757	3.688	3.825	n.d.	n.d.	n.d.
29. Neu5Gc(α2 → 3)Gal(β1 → 4)Glc	1.816	2.777	n.d.	n.d.	n.d.	n.d.	n.d.
30. Neu5Ac(α2 → 6)Gal(β1 → 4)Glc	1.739	2.715	3.658	3.836	n.d.	n.d.	n.d.
31. Neu4,5Ac$_2$(α2 → 3)Gal(β1 → 4)Glc	1.926	2.768	4.955	4.088	n.d.	n.d.	n.d.

[a] Values may be interchanged.
[b] Also including the 2Ac signal.
[c] Individual acetyl signals could not be assigned.

6 and 11, 1a and 12, 6 and 8 and 2 and 9). The latter, relatively small, shift increments can be explained in a similar way to that suggested for the α effects on H-9 and H-9′; as a result of the rigid character of the C-3–C-4 part of the molecule the effects on H-3*ax.* and H-3*eq.* are unequal.

The γ and δ effects (defined analogously to the α and β effects) which can be derived for *O*-acetylation of C-4, C-7 and C-9 are about 0–0.2 ppm and 0–0.1 ppm, respectively. However, in contrast to the α and β effects these effects can be either downfield (4Ac, 9Ac) or upfield (7Ac) (compare compounds 1a and 7; 1a and 10; 6 and 8; 2 and 9 and 6 and 11 vs. 1a and 12). Furthermore, it should be noted that *O*-acetylation of C-7 influences the resonance positions of all sugar skeleton protons and of the 5Ac methyl protons. These wide-spread shift phenomena might be connected with the occurrence of a specific hydrogen bridge involving the substituent at C-7 [30, 31]. The relatively strong effect observed on the 5Ac methyl protons is an argument in favour of the hydrogen bridge between 7OH and the 5Ac amide proton, as was also proposed by Czarniecki and Thornton [30].

The *O*-acetylation effects observed for the various skeleton protons in the 7,9Ac$_2$ and 4,9Ac$_2$ derivatives, compounds 13 and 15, can be considered as the sum of the separate effects of *O*-acetylation at each position. This additivity is evident from comparison of the following pairs of compounds: 6 and 15, 1a and 10, 6 and 11, 1a and 7, 6 and 8, 2 and 9, and 1a and 12. Assuming a similar additivity of the separate *O*-acetylation effects in the 8,9Ac$_2$ derivative 14a, the downfield shifts for H-9, H-9′ (0.68 ppm), H-8 (1.36 ppm) and H-7 (0.32 ppm) and the small upfield shift for H-6 (−0.20 ppm) as compared with compound 1a indicate that *O*-acetylation at position 8 results in a downfield α effect (1.14 ppm) and β effect (0.16 and 0.10 ppm for H-9 and H-9′; 0.26 ppm for H-7), whereas the γ effect turns out to be upfield (−0.20 ppm). It should be noted that the latter effect is similar to the γ effect upon acetylation of position 7 and may also be

the resonance positions of H-6, H-8 and H-9 of compound 5 as these proton resonances coincide with that of the ester methyl group. Values for the contaminants in the samples. Compounds 12, 13, 24 and 25 were unstable at p^2H 7. n.d., value could not be determined. Neu5Ac(α2→3)Gal(β1→4)Glc, *N*-acetylneuraminosyl-(α2→6)-lactose; Neu4,5Ac$_2$(α2→3)Gal(β1→4)Glc, *N*-acetyl-4-*O*-acetylneuraminosyl-(α2→3)-lactose

δ value										p^2H
H-9	H-9′	5Ac	4Ac	7Ac	8Ac	9Ac	5Gc	2Me	1Me	
ppm										
3.835	3.608	2.050	–	–	–	–	–	–	–	7
3.841	3.619	2.053	–	–	–	–	–	–	–	2
3.821	3.613	–	–	–	–	–	4.143	–	–	7
3.834	3.619	2.051	–	–	–	–	–	–	3.838	7
≈3.85	3.662	2.047	–	–	–	–	–	3.200	–	7
3.84–3.88	3.667	2.050	–	–	–	–	–	3.274	3.868	7
3.841	3.664	2.049	–	–	–	–	–	–	–	7
4.365	4.187	2.057	–	–	–	2.119	–	–	–	7
4.433	4.201	2.057	–	–	–	2.128	–	3.278	3.878	7
4.365	4.183	–	–	–	–	2.115	4.144	–	–	7
3.844	3.619	1.992	2.065	–	–	–	–	–	–	7
3.846	3.667	1.990	2.059	–	–	–	–	3.304	3.868	7
3.629	3.444	1.976[a]	–	2.144[a]	–	–	–	–	–	4
4.106	4.106	1.981[a]	–	2.134[a]	–	2.106	–	–	–	2
4.528	4.287	2.057	–	–	2.089	2.105	–	–	–	7
4.545	4.287	2.059	–	–	2.091	2.107	–	–	–	2
4.443	4.214	1.999	2.059	–	–	2.125	–	3.302	3.885	7
4.446	4.212	1.940	← 2.047;	2.081;	2.089;	2.171;	2.179[b,c] →	–	–	–	7
n.d.	n.d.	2.030	–	–	–	–	–	–	–	7
n.d.	n.d.	2.036	–	–	–	–	–	–	–	2
n.d.	n.d.	–	–	–	–	–	n.d.	–	–	7
n.d.	n.d.	2.036	–	–	–	–	–	–	3.838	7
3.869	3.641	2.033	–	–	–	–	–	3.341	–	7
3.84–3.88	3.654	2.034	–	–	–	–	–	3.383	3.880	7
n.d.	n.d.	n.d.	–	–	–	n.d.	–	–	–	7
n.d.	n.d.	–	–	–	–	n.d.	4.123[d]	–	–	7
n.d.	n.d.	1.947	–	2.128	–	–	–	–	–	4
n.d.	n.d.	1.956	–	n.d.	–	n.d.	–	–	–	2
4.382	4.265	1.910	← 2.028;	2.089;	2.162;	2.162[c] →	–	3.305	–	7
4.356	4.243	1.922	← 2.041;	2.093;	2.167;	2.211[c] →	–	3.356	3.887	7
n.d.	n.d.	2.030	–	–	–	–	–	–	–	6
n.d.	n.d.	–	–	–	–	–	4.119	–	–	7
n.d.	n.d.	2.030	–	–	–	–	–	–	–	6
n.d.	n.d.	1.963	2.070	–	–	–	–	–	–	5

[d] Small singlet peak in the spectrum of the anomeric mixture, identified on guidance of the resonance position of the 5Gc methylene protons of αNeu5Gc residues in oligosaccharides [28]; compare also compound 29.

connected with the occurrence of a hydrogen bridge involving the substituent at C-8 [30,31].

For the peracetylated derivative, compound 16, the resonance positions of H-5, H-6, H-7, H-8, H-9 and H-9′ can be derived on the basis of the various α, β and γ-effects of *O*-acetylation at positions 4, 7, 8 and 9, with a tolerance of ± 0.1 ppm. However, the chemical shifts of H-3*ax*., H-3*eq*. and H-4 cannot be calculated because the shift effects of the axially oriented 2Ac group are unknown. The proximity of the carboxylic acid function to the 2Ac group makes the shift effects in this region of the molecule unpredictable.

Acetylation shift effects on skeleton protons of the free sialic acid α anomers cannot be verified clearly due to the unfavourable α:β anomeric ratio. For the methyl glycosides, compounds 20 and 26, and the methyl ester methyl glycosides, compounds 21 and 27, the combined effects of tetra-*O*-acetylation can be observed and show trends for H-5, H-7, H-8, H-9 and H-9′ which are comparable with those observed in the β series going from compound 1a to 16. However, the rather strong downfield shifts for H-6 on tetra-*O*-acetylation (0.81 ppm for compound 20 → 26 and 0.44 ppm for compound 21 → 27) deviate from the shift observed in the β series (0.12 ppm for compound 1a → 16) and also from each other, due to different interactions of H-6 with the axial C-2 substituents (i.e. Ac in compound 16, COOH in 26 and COOMe in 27).

Coupling Constants of Skeleton Protons

The vicinal and geminal ^1H-^1H coupling constants of the various skeleton protons turn out not to be influenced significantly by the presence of *O*-acetyl substituents. From this observation it can be concluded that the (partially *O*-acetylated) neuraminic acid derivatives have the same $^2C_5$(D) ring conformation as the parent molecule. For average $J_{H,H}$ values we referred to the articles of Brown et al. [13], Jaques

et al. [14] and Beau et al. [17]. The various vicinal and geminal couplings between the neuraminic-acid skeleton protons bring about very characteristic proton multiplet patterns, which can be used for their identification.

Acetyl-Methyl Resonances

Comparison of the various Ac methyl singlets as compiled in Table 1 shows that distinct resonance regions can be indicated for *O*-acetyl groups at certain positions. The 9Ac protons (compounds 7, 8, 9, 13, 14a and 15) resonate in the narrow range of 2.10–2.13 ppm, the 4Ac protons (compounds 10, 11, 15) are found near 2.06 ppm and the 8Ac protons (compound 14a) near 2.09 ppm (very close to the 9Ac singlet). Apparently, the presence of a 4Ac (compounds 10, 11, 15) or a 7Ac (compounds 12, 13) substituent influences considerably the resonance position of the 5Ac methyl protons. For this reason the high-field acetyl signal ($\delta < 2.00$ ppm) in the peracetylated derivative, compound 16, and the tetra-*O*-acetyl derivatives, compounds 26 and 27, is assigned to the 5Ac group. Consequently, the 4Ac and 7Ac functions both have an upfield shift effect on the 5Ac protons, reinforcing each other.

It should be noted that the resonance positions of the acetyl signals in α anomers tend to differ just slightly with respect to corresponding resonances in β anomers (e.g. compare 1a and 17a, 12 and 24). In general, such differences between α and β anomers of free sialic acids cannot be verified clearly as most of the resonances of the α anomer are masked by the intense signals of the β anomer. In the glycosides, compounds 26 and 27, the four signals of the *O*-acetyl groups could not be assigned due to the lack of appropriate mono-*O*-acetyl and di-*O*-acetyl references.

N-*Acetyl-4*-O-*acetylneuraminosyl-(α2→3)-lactose from Echidna Milk*

The data for model compounds as described above can be used for identification of the substitution pattern of sialic acid residues in oligosaccharides and glycoconjugates. This will be demonstrated for the mono-*O*-acetylated compound 31, a derivative of compound 28 isolated from echidna milk [24]. Data for some reference sialyllactoses with different sialyl linkage types and 5Ac (compounds 28, 30) or 5Gc (compound 29) substituents but lacking *O*-acetyl groups are also included in Table 1. The structure elucidation of the *O*-acetylated compound by 500-MHz ^{1}H-NMR spectroscopy and mass spectrometry will be discussed elsewhere (unpublished results); it was concluded on the basis of the values for H-1 (4.53 ppm) and H-3 (4.13 ppm) of the galactose residue that the sialic acid residue in compound 31 is linked to galactose via an (α2→3) linkage [15]. The resonances of H-3*ax*, and H-3*eq*. of the sialic acid residue are found at 1.926 ppm and 2.768 ppm, respectively, whereas the signals at 4.955 ppm and 4.088 ppm are assigned to the sialic acid H-4 and H-5, respectively. The latter assignments are based on selective decoupling experiments. Two acetyl singlets are observed at 1.963 ppm and 2.070 ppm.

Comparison of compounds 28 and 31 reveals that the H-4 and H-5 signals have been shifted over 1.27 ppm (α effect) and 0.26 ppm (β effect), respectively, characteristic for the presence of an acetyl substituent at position 4 of the neuraminic acid unit of compound 31. The presence of the acetyl group at this position influences the H-3*ax*. and H-3*eq*. resonance positions by its β effect (0.13 ppm and 0.01 ppm, respectively) and also the 5Ac methyl resonance. The positions of the two acetyl singlets at 1.963 ppm and 2.070 ppm are in accordance with the positions of the 5Ac and 4Ac resonances, respectively, in the model compounds 10, 11 and 15.

Conclusions

The number of *O*-acetyl substituents in sialic acid residues can be inferred from the number of acetyl methyl singlets.

The α effects of *O*-acetylation of neuraminic acid residues cause skeleton protons to be strongly shifted downfield and to lie apart from the complex resonance pattern of other skeleton protons (and non-anomeric protons of other sugar residues). As the shifted proton resonances can be assigned on the basis of the characteristic proton multiplet patterns these α effects can be used for determination of the position(s) of *O*-acetylation. The resonance positions of the acetyl methyl singlets can be of help in this respect.

This NMR method can be used for determination of the position(s) of *O*-acetyl groups in underivatized oligosaccharides and glycopeptides obtained from glycoconjugates.

This investigation was carried out using a 360-MHz NMR instrument. However, the set of reference data presented can in principle be applied in ^{1}H-NMR studies of sialocarbohydrates at lower resolution, from 200 MHz on, provided that sensitivity and amount of material are sufficient.

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