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Structural Analysis of Naturally Occurring Sialic Acids

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Summary

Over the years several methodologies have been developed for the structural analysis of naturally occurring sialic acids (Sias), a family with more than 62 members. Currently there are two primary instrumental approaches: analysis of volatile Sia derivatives by gas-liquid chromatography (GLC) combined with electron-impact mass spectrometry (EI/MS), and analysis of fluorescently labeled Sias by high-performance liquid chromatography (HPLC) eventually coupled with electrospray mass spectrometry (ESI/MS). This chapter presents both approaches in detail. The volatile Sia derivatives are comprised of trimethylsilylated methyl ester derivatives, heptafluorobutylated methyl ester derivatives, or pertrimethylsilylated derivatives. The fluorescent Sia derivatives are prepared by reaction with 1,2-diamino-4,5-methylenedioxybenzene. For the identification of the different Sia derivatives, detailed GLC, HPLC, EI/MS, and ESI/MS data are included.

Key Words: Sialic acid; neuraminic acid; gas–liquid chromatography; high-performance liquid chromatography; mass spectrometry.

1. Introduction of Sialic Acids

Sialic acids (Sias) occur in nature in a great chemical diversity. These biologically important monosaccharides may be present in glycoprotein, glycolipid, and glycosylphosphatidylinositol membrane anchor glycans, and in oligosaccharides and homo- and heteropolysaccharides. They usually occur as terminal units, but examples where Sias have internal positions are well known (*see also* Chapters 7 and 11). Although the free forms have mainly the β-anomeric ring structure (>93%), glycoconjugate-bound Sias occur specifically in the α-anomeric form. In the literature, several recent reviews are available which focus on the biological sources of Sias and their significance in a variety of biological processes *(1–6)*.
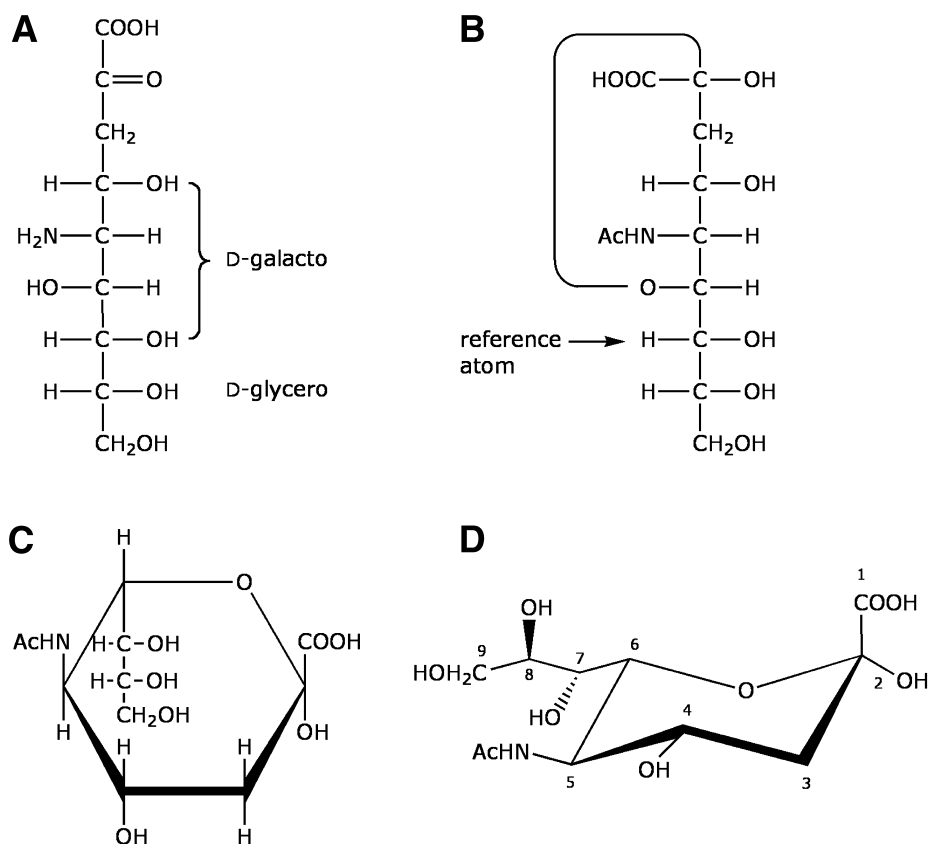
A

```
        COOH
         |
         C=O
         |
         CH₂
         |
    H —C—OH  ⎤
         |    |
 H₂N—C—H     ⎬  D-galacto
         |    |
 HO —C—H     |
         |    |
    H —C—OH  ⎦
         |
    H —C—OH      D-glycero
         |
        CH₂OH
```

B

```
   ┌──────────────────┐
   │   HOOC—C—OH       │
   │        |          │
   │        CH₂        │
   │        |          │
   │   H —C—OH         │
   │        |          │
   │  AcHN—C—H         │
   │        |          │
   └──── O—C—H         │
                |
 reference ──→  H —C—OH
 atom           |
           H —C—OH
                |
              CH₂OH
```

C

D

Fig. 1. Chemical structures for simple sialic acids in different views. **(A)** 5-Amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid (Neu, open chain, Fischer projection formula). **(B)** 5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosonic acid (α-Neu5Ac, Fischer projection formula, note that C-7 is the anomeric reference atom). **(C)** α-Neu5Ac (Haworth formula). **(D)** α-Neu5Ac ($^2C_5$ chair conformation).

In **Table 1**, 62 naturally occurring members of the Sia family currently identified are listed, together with their abbreviations *(3,5,7,8)*. The mother-molecule neuraminic acid, which does not occur in free form in nature owing to its immediate cyclization to form an internal Schiff base, is systematically named 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid. It is abbreviated as Neu, where the D-notation is implied in the trivial name (*see* **Fig. 1**). Chemically, a Sia is a 2-keto-carboxylic acid, a deoxysugar, and an amino sugar. In general, the amino group is *N*-acetylated (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulopyranosonic acid; *N*-acetylneuraminic acid; Neu5Ac) or *N*-glycolylated (5-hydroxyacetamido-3,5-dideoxy-D-*glycero*-

7-*O*-acetyl
8-*O*-acetyl
9-*O*-acetyl

8-*O*-sulfate

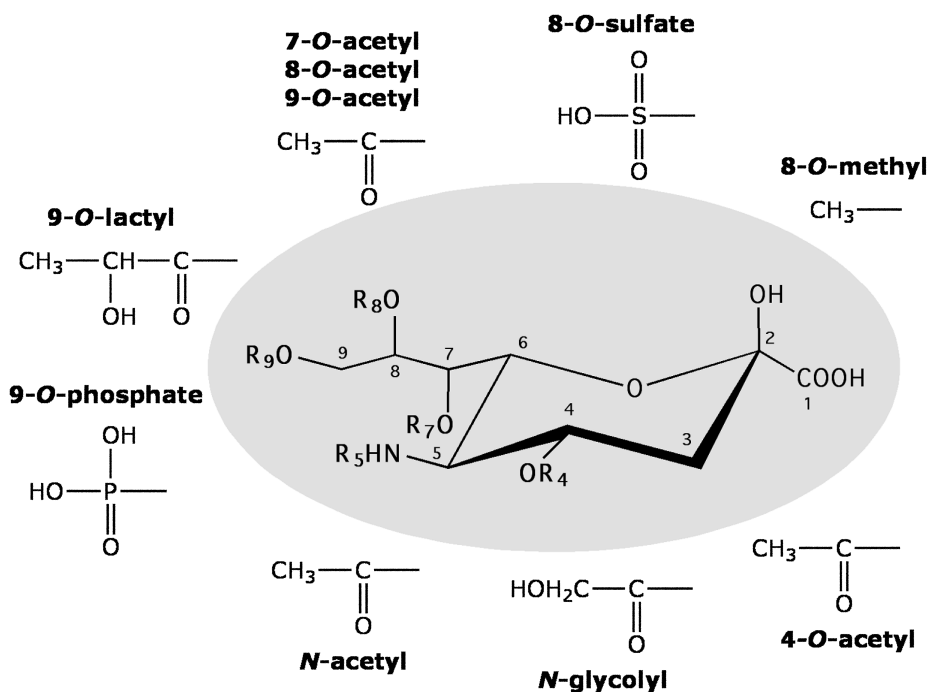**9-*O*-lactyl**

8-*O*-methyl

9-*O*-phosphate

Fig. 2. Schematic overview of the family of naturally occurring sialic acids, as detailed in **Table 1**.

D-*galacto*-non-2-ulopyranosonic acid; *N*-glycolylneuraminic acid; Neu5Gc). The hydroxyl groups may be free, esterified (acetylated, lactylated, sulfated, phosphorylated), or etherified (methylated) (*see* **Table 1**). A schematic over-view is given in **Fig. 2**.

The structural analysis of naturally occurring Sias is currently carried out by the following two main approaches: analysis of volatile Sia derivatives by gas-liquid chromatography (GLC) combined with electron-impact mass spectrometry (EI/MS), and analysis of fluorescently labeled Sias by high-performance liquid chromatography (HPLC) eventually coupled with electro-spray mass spectrometry (ESI/MS).

Acid hydrolysis and enzymatic hydrolysis are the primary methods applied for the release of Sia from Sia-containing material. Each approach has advan-tages and disadvantages. Acid hydrolysis always gives rise to some de-*O*-esterification. Moreover, differences in rates of release are influenced by the sub-stitution patterns and the type of glycosidic linkages. In the case of enzymatic hydrolysis with sialidases, linkage specificity as well as a reduced or complete lack of susceptibility must be taken into account. It should be noted that in most

cases much lower amounts of Sias are released by sialidases than by acid hydrolysis. This chapter concentrates on acid hydrolysis protocols. In work-up procedures and analyses, pH values lower than 4.0 and higher than 6.0 should be avoided as much as possible to prevent solvolysis of *O*-acetyl groups and migration of *O*-acetyl groups at C-7/C-8 to C-9 *(9,10)*.

2. Materials

2.1. Release of Sias From Glycoconjugates

2.1.1. Hydrolysis With Acetic Acid

1. Acetic acid (Merck, Darmstadt, Germany).
2. Diethyl ether (Fluka Chemie AG, Buchs, Switzerland).

2.1.2. Hydrolysis With Propionic Acid

1. Propionic acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands).

2.2. Preparation of Volatile Sia Derivatives for GLC and GLC-EI/MS Analysis

2.2.1. Preparation and GLC (-EI/MS) Analysis of Trimethylsilylated Methyl Ester Derivatives

1. Phosphor pentoxide (Sigma-Aldrich).
2. Methanol (Biosolve BV, Valkenswaard, The Netherlands).
3. Dowex H^+ (Fluka).
4. Diazomethane in diethyl ether (Fluka), prepared using diazogen 99% (Acros, Geel, Belgium) according to the kit instructions.
5. Pyridine (Sigma-Aldrich).
6. Hexamethyldisilazane (Acros).
7. Trimethylchlorosilane (Merck).
8. MD800/GC8060 GLC-EI/MS instrument (Fisons Instruments/Interscience, Breda, The Netherlands).
9. AT-1 capillary column, 30 m × 0.25 mm (Alltech, Breda, The Netherlands).

2.2.2. Preparation and GLC (-EI/MS) Analysis of Heptafluorobutylated Methyl Ester Derivatives

1. Phosphor pentoxide (Sigma-Aldrich).
2. Methanol (Biosolve).
3. Diazomethane in diethyl ether (Fluka), prepared using diazogen 99% (Acros) according to the kit instructions.
4. Acetonitrile (Biosolve).
5. Heptafluorobutyric anhydride (Sigma-Aldrich).
6. Carlo Erba GC8000/Riber 10-10H GLC-EI/MS instrument (Riber, France).
7. CP-Sil 5CB capillary column, 25 m × 0.32 mm (Chrompack France, Les Ullis, France).

Table 1
Survey of Established Structures of Naturally Occurring Members of the Sialic Acid Family

Sialic acid	Abbreviation
Neuraminic acid	Neu[a]
Neuraminic acid 1,5-lactam	Neu1,5lactam
N-Acetylneuraminic acid	Neu5Ac
5-*N*-Acetyl-4-*O*-acetyl-neuraminic acid	Neu4,5Ac$_2$
5-*N*-Acetyl-7-*O*-acetyl-neuraminic acid	Neu5,7Ac$_2$
5-*N*-Acetyl-8-*O*-acetyl-neuraminic acid	Neu5,8Ac$_2$
5-*N*-Acetyl-9-*O*-acetyl-neuraminic acid	Neu5,9Ac$_2$
5-*N*-Acetyl-4,9-di-*O*-acetyl-neuraminic acid	Neu4,5,9Ac$_3$
5-*N*-Acetyl-7,9-di-*O*-acetyl-neuraminic acid	Neu5,7,9Ac$_3$
5-*N*-Acetyl-8,9-di-*O*-acetyl-neuraminic acid	Neu5,8,9Ac$_3$
5-*N*-Acetyl-4,7,9-tri-*O*-acetyl-neuraminic acid	Neu4,5,7,9Ac$_4$
5-*N*-Acetyl-7,8,9-tri-*O*-acetyl-neuraminic acid	Neu5,7,8,9Ac$_4$
5-*N*-Acetyl-4,7,8,9-tetra-*O*-acetyl-neuraminic acid	Neu4,5,7,8,9Ac$_5$
5-*N*-Acetyl-9-*O*-L-lactyl-neuraminic acid	Neu5Ac9Lt
5-*N*-Acetyl-4-*O*-acetyl-9-*O*-lactyl-neuraminic acid	Neu4,5Ac$_2$9Lt
5-*N*-Acetyl-7-*O*-acetyl-9-*O*-lactyl-neuraminic acid	Neu5,7Ac$_2$9Lt
5-*N*-Acetyl-8-*O*-methyl-neuraminic acid	Neu5Ac8Me
5-*N*-Acetyl-4-*O*-acetyl-8-*O*-methyl-neuraminic acid	Neu4,5Ac$_2$8Me
5-*N*-Acetyl-9-*O*-acetyl-8-*O*-methyl-neuraminic acid	Neu5,9Ac$_2$8Me
5-*N*-Acetyl-8-*O*-sulpho-neuraminic acid	Neu5Ac8S
5-*N*-Acetyl-4-*O*-acetyl-8-*O*-sulpho-neuraminic acid	Neu4,5Ac$_2$8S
5-*N*-Acetyl-9-*O*-phosphoro-neuraminic acid	Neu5Ac9P[b,c]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-neuraminic acid	Neu2en5Ac[c]
5-*N*-Acetyl-9-*O*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid	Neu2en5,9Ac$_2$[c]
5-*N*-Acetyl-2-deoxy-2,3-didehydro-9-*O*-lactyl-neuraminic acid	Neu2en5Ac9Lt[c]
5-*N*-Acetyl-2,7-anhydro-neuraminic acid	Neu2,7an5Ac[c]
5-*N*-Acetyl-4,8-anhydro-neuraminic acid	Neu4,8an5Ac[d]
5-*N*-Acetylneuraminic acid 1,7-lactone	Neu5Ac1,7lactone
5-*N*-Acetyl-9-*O*-acetyl-neuraminic acid 1,7-lactone	Neu5,9Ac$_2$1,7lactone
5-*N*-Acetyl-4,9-di-*O*-acetyl-neuraminic acid 1,7-lactone	Neu4,5,9Ac$_3$1,7lactone
N-Glycolylneuraminic acid	Neu5Gc
4-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu4Ac5Gc
7-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu7Ac5Gc
8-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu8Ac5Gc

(continued)

Table 1 *(continued)*

Sialic acid	Abbreviation
9-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid	Neu9Ac5Gc
4,7-Di-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu4,7Ac$_2$5Gc
4,9-Di-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu4,9Ac$_2$5Gc
7,9-Di-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu7,9Ac$_2$5Gc
8,9-Di-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu8,9Ac$_2$5Gc
4,7,9-Tri-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu4,7,9Ac$_3$5Gc
7,8,9-Tri-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu7,8,9Ac$_3$5Gc
4,7,8,9-Tetra-*O*-acetyl-5-*N*-glycolyl-neuraminic acid	Neu4,7,8,9Ac$_4$5Gc
5-*N*-Glycolyl-9-*O*-lactyl-neuraminic acid	Neu5Gc9Lt
4-*O*-Acetyl-5-*N*-Glycolyl-9-*O*-lactyl-neuraminic acid	Neu4Ac5Gc9Lt
7-*O*-Acetyl-5-*N*-Glycolyl-9-*O*-lactyl-neuraminic acid	Neu7Ac5Gc9Lt
8-*O*-Acetyl-5-*N*-Glycolyl-9-*O*-lactyl-neuraminic acid	Neu8Ac5Gc9Lt
4,7-Di-*O*-acetyl-5-*N*-Glycolyl-9-*O*-lactyl-neuraminic acid	Neu4,7Ac$_2$5Gc9Lt
7,8-Di-*O*-acetyl-5-*N*-Glycolyl-9-*O*-lactyl-neuraminic acid	Neu7,8Ac$_2$5Gc9Lt
5-*N*-Glycolyl-8-*O*-methyl-neuraminic acid	Neu5Gc8Me
7-*O*-Acetyl-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu7Ac5Gc8Me
9-*O*-Acetyl-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu9Ac5Gc8Me
7,9-Di-*O*-acetyl-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu7,9Ac$_2$5Gc8Me
5-*N*-Glycolyl-8-*O*-sulpho-neuraminic acid	Neu5Gc8S
5-*N*-Glycolyl-9-*O*-sulpho-neuraminic acid	Neu5Gc9S
N-(*O*-Acetyl)glycolylneuraminic acid	Neu5GcAc
N-(*O*-Methyl)glycolylneuraminic acid	Neu5GcMe
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-neuraminic acid	Neu2en5Gc[c]
9-*O*-Acetyl-2-deoxy-2,3-didehydro-5-*N*-glycolyl-neuraminic acid	Neu2en9Ac5Gc[c]
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-9-*O*-lactyl-neuraminic acid	Neu2en5Gc9Lt[c]
2-Deoxy-2,3-didehydro-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu2en5Gc8Me[c]
2,7-Anhydro-5-*N*-glycolyl-neuraminic acid	Neu2,7an5Gc[c]
2,7-Anhydro-5-*N*-glycolyl-8-*O*-methyl-neuraminic acid	Neu2,7an5Gc8Me[c]
4,8-Anhydro-5-*N*-glycolyl-neuraminic acid	Neu4,8an5Gc[d]
5-*N*-Glycolylneuraminic acid 1,7-lactone	Neu5Gc1,7lactone
9-*O*-Acetyl-5-*N*-glycolyl-neuraminic acid 1,7-lactone	Neu9Ac5Gc1,7lactone

[a]Present only in bound form.
[b]Biosynthetic intermediate.
[c]Present only in free form.
[d]Not occurring as such in nature, but sometimes isolated owing to hydrolytic conditions. For a survey of typical biological sources of the various sialic acids, *see* **refs. 3,5,7,8**.

2.2.3. Preparation and GLC (-EI/MS) Analysis of Pertrimethylsilylated Derivatives

1. Phosphor pentoxide (Sigma-Aldrich).
2. Pyridine (Sigma-Aldrich).
3. Hexamethyldisilazane (Acros).
4. Trimethylchlorosilane (Merck).
5. MD800/GC8060 GLC-EI/MS instrument (Fisons).
6. AT-1 capillary column, 30 m × 0.25 mm (Alltech).

2.3. GLC-EI/MS Data of Trimethylsilylated Sia Derivatives

Materials are as described in **Subheadings 2.2.1.** and **2.2.3.**

2.4. GLC-EI/MS Data of Heptafluorobutylated Sia Methyl Ester Derivatives

Materials are as described in **Subheading 2.2.2.**

2.5. Preparation of Fluorescent Sia Derivatives for HPLC and LC-ESI/MS Analysis

2.5.1. Preparation and HPLC (-ESI/MS) Analysis of 1,2-Diamino-4,5-Methylenedioxybenzene Sia Derivatives

1. Acetic acid (Merck).
2. 1,2-Diamino-4,5-methylenedioxybenzene (DMB) dihydrochloride (Sigma-Aldrich).
3. β-Mercaptoethanol (Sigma-Aldrich).
4. Sodium hydrosulfite (Sigma-Aldrich).
5. Spectroflow 400 HPLC solvent delivery system (ABI Analytical/Kratos Division, Ramsey, NJ).
6. Spectroflow 980 programmable fluorescence detector (ABI).
7. Cosmosil 5C18-AR-II reversed-phase column, 250 × 4.6 mm (Nacalai Tesque, Kyoto, Japan).
8. Acetonitrile (Biosolve).
9. Methanol (Biosolve).
10. Milli-Q water (Millipore BV, Etten-Leur, The Netherlands).
11. API-I simple quadrupole ESI/MS instrument (Perkin-Elmer Sciex Instruments, Thornhill, Canada).
12. Microbore ultrasphere octadecylsilane (ODS) column, 250 × 2 mm (Beckman, Fullerton, CA).

2.6. HPLC-ESI/MS Data of DMB Sia Derivatives

Materials are as described in **Subheading 2.5.1.**

3. Methods

3.1. Release of Sias From Glycoconjugates

   Several approaches have been reported for the effective acid hydrolysis of the labile glycosidic linkage between Sia and a neighboring monosaccharide. All these procedures are not optimal for giving the real spectrum of Sias originally present in the sialoglycoconjugate under study, especially in the case of a mixture of (*O*-acylated) *N*-acylneuraminic acids. The three most applied acid hydrolysis systems are formic acid/HCl *(11)*, acetic acid *(10)*, and propionic acid *(12)*. The use of H_2SO_4 is not recommended. The oldest formic acid/HCl protocol comprises a two-step hydrolysis, whereby glycoconjugate probes are incubated with aqueous formic acid (pH 2.0 for 1 h at 70°C), followed by incubation with HCl (pH 1.0 for 1 h at 80°C). After each step the liberated Sias are recovered by centrifugation, ultrafiltration, or dialysis. In the case of a spectrum of (*O*-acylated) *N*-acylneuraminic acids, the supernatant, ultrafiltrate, or diffusate of the formic acid hydrolysis contains the majority of the *O*-acylated *N*-acylneuraminic acids, while that of the HCl hydrolysis contains mostly Neu5Ac and Neu5Gc. In the case of low-molecular-mass substances, isolations can be carried out by gel-permeation chromatography. For purification of Sias, combined cation-anion exchange chromatography is often included. Although these conditions do not lead to significant de-*N*-acylation, de-*O*-acylation has been shown to occur to an extent of approx 30–50%. On the other hand, milder acidic conditions result in incomplete release of Sias. Most research groups currently use acetic acid or propionic acid protocols.

3.1.1. Hydrolysis With Acetic Acid

1. Glycoconjugate probes are incubated with 500 μL of 2 *M* acetic acid for 3 h at 80°C *(10)*. Because the exact hydrolysis time required can vary with different glycoconjugates, reported incubation times vary between 90 min and 4 h.
2. Solutions are cooled, concentrated *in vacuo* or lyophilized, or directly used in derivatization protocols.
3. Lipid impurities can be removed by diethyl ether extraction. If further purifications are necessary, different types of concentrators are used *(10,12)*.

3.1.2. Hydrolysis With Propionic Acid

1. Glycoconjugate probes are incubated with 500 μL of 2 *M* propionic acid for 4 h at 80°C (*see* **ref. *12***; 4 *M* propionic acid has also been proposed *[13]*).
2. Solutions are cooled, then concentrated *in vacuo* or lyophilized, or used directly in derivatization protocols.

3. If further purifications are necessary, different types of concentrators are used. This more recent approach may significantly decrease the *O*-acyl group loss and migration when compared with the use of 2 *M* acetic acid or 0.5 *M* formic acid, while also providing better yields.

3.2. Preparation of Volatile Sia Derivatives for GLC and GLC-EI/MS Analysis

Starting with free Sia pools, mainly present in their β-anomeric form, volatile Sia derivatives are generated using mild derivatization procedures such as esterification with diazomethane followed by trimethylsilylation *(14)* or heptafluorobutylation *(7)*, or direct pertrimethylsilylation *(11)*.

3.2.1. Preparation and GLC (-EI/MS) Analysis of Trimethylsilylated Methyl Ester Derivatives

1. Lyophilized Sia samples (ng-μg amounts), dried over P_2O_5, are dissolved in anhydrous methanol (200 μL).
2. Dowex H$^+$ in 80 μL of methanol is added (Sias should be in the COOH form), and the mixture is filtered over cotton wool.
3. Diazomethane in diethyl ether is added until a faint yellow color remains for 5 min at room temperature. The solution is concentrated to dryness using a stream of nitrogen and dried over P_2O_5.
4. The residue is dissolved in 10 μL of trimethylsilylation reagent (pyridine∶hexamethyldisilazane∶trimethylchlorosilane, 5∶1∶1), and the mixture is kept for 2 h at room temperature.
5. Analyze 3-μL samples by GLC-EI/MS using an AT-1 capillary column. The temperature program is usually 220°C for 25 min, then 6°C/min to 300°C, and finally 6 min constant at 300°C. The injector temperature is 230°C, the source temperature is 200°C, and the electron voltage is 70 eV.

3.2.2. Preparation and GLC (-EI/MS) Analysis of Heptafluorobutylated Methyl Ester Derivatives (see **Notes 1** and **2**)

1. Lyophilized Sia samples (ng-μg amounts), dried over P_2O_5, are dissolved in anhydrous 200 μL of methanol, and diazomethane in 200 μL of diethyl ether is added. Samples are left for 4 h at room temperature without stirring.
2. The solution is concentrated to dryness using a stream of nitrogen, and 200 μL of acetonitrile and 25 μL of heptafluorobutyric anhydride are added. The mixture is heated for 5 min at 150°C (sand bath), cooled to room temperature, and concentrated to dryness using a stream of nitrogen.
3. The residue is dissolved in an appropriate amount of acetonitrile, and aliquots are analyzed by GLC-EI/MS using a CP-Sil 5CB capillary column. The temperature program is usually 90°C for 3 min, then 5°C/min to 260°C. The injector temperature is 260°C, the source temperature is 150°C, and the electron voltage is 70 eV.

3.2.3. Preparation and GLC (-EI/MS) Analysis
of Pertrimethylsilylated Derivatives

1. Lyophilized Sia samples (ng-µg amounts), dried over P_2O_5, are dissolved in 30 µL of trimethylsilylation reagent (pyridine:hexamethyldisilazane:trimethylchlorosilane, 5:1:1), and the solutions are kept for 2 h at room temperature.
2. 3-µL Samples are analyzed by GLC-EI/MS using an AT-1 column. The temperature program is usually 220°C for 25 min, then 6°C/min to 300°C, and finally 6 min constant at 300°C. The injector temperature is 230°C, the source temperature is 200°C, and the electron voltage is 70 eV.

3.3. GLC-EI/MS Data of Trimethylsilylated Sia Derivatives

A general EI/MS method has been developed for the identification of Sias isolated from biological material and derivatized as volatile trimethylsilylated (TMS) methyl ester derivatives or pertrimethylsilylated derivatives (PTMS). This method has also proved to be useful for the analysis of other isolated Sias, of (partially) *O*-methylated Sia methyl ester methyl glycosides as obtained in methylation analysis, and of synthetic Sia(s) (derivatives) *(3,14–16)*.

Figure 3 depicts a schematic survey showing the selected fragment ions A-H, which furnish the information (abundances and *m/z* values of the ions) necessary to deduce the complete structure of Sias. As typical examples, **Fig. 4** shows the EI mass spectra of the TMS methyl esters of β-Neu5Ac and β-Neu5Gc, and of the PTMS derivatives of β-Neu5Ac and β-Neu5Gc.

3.3.1 GLC-EI/MS Data of TMS Methyl Ester or PTMS Sia Derivatives

1. Fragments A and B indicate the molecular mass of the Sia derivatives (the molecular ion M is absent), and thus the number and type of substituents.
2. Fragments C–H contain the information concerning the position of the different substituents.
3. Fragments A–H are very useful for ion chromatogram screening of GLC effluents.
4. Fragment A is formed from the molecular ion M by elimination of a methyl group. In TMS (*O*-acylated/*O*-alkylated) *N*-acylneuraminic acid derivatives, the methyl group originates from a trimethylsilyl substituent.
5. Fragment B is obtained by elimination of the C-1 part of the Sia molecule. Eliminations of $OCOCH_3$ in *O*-acetylated Sia derivatives and of NH_2COCH_3 in Neu5Ac derivatives, which in principle give rise to the same *m/z* value as fragment B in the case of $R_1 = CH_3$, contribute little to the abundance of this ion. For *O*-trimethylsilylated *N,O*-acylneuraminic acids (β-anomers) it holds that, when compared with their methyl esters, in their trimethylsilyl esters the intensity of fragment A decreases relative to fragment B.
6. Fragment C is formed by elimination of the C-8,9 part, with localization of the charge on position 7. In general, cleavage occurs between two alkoxylated carbon

R_1: Me or SiMe$_3$
R_2: SiMe$_3$
R_4, R_7, R_8, R_9: Ac, L-lactyl, Me and/or SiMe$_3$
R_5: Ac, Gc (+ SiMe$_3$)

A: $M^{+\bullet}$ minus $^\bullet CH_3$

B: $M^{+\bullet}$ minus $^\bullet COOR_1$

C: $M^{+\bullet}$ minus $^\bullet CHOR_8 - CH_2OR_9$

D: C^+ minus R_2OH minus R_4OH

E: $M^{+\bullet}$ minus $^\bullet CHOR_7 - CHOR_8 - CH_2OR_9$ minus NH_2R_5

F: $R_8\overset{+}{O} = CH - CH_2OR_9$

G: $R_5\overset{+}{N}H = CH - {}^\bullet CHOR_4$

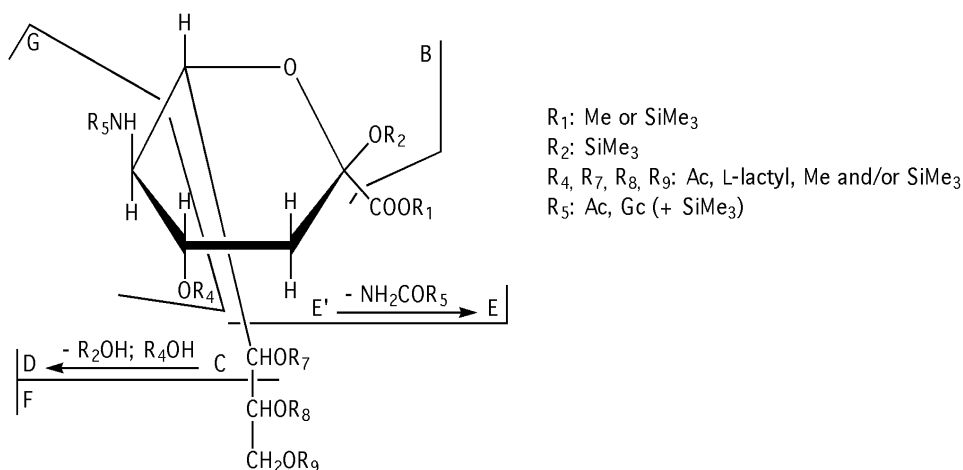H: $M^{+\bullet}$ minus $^\bullet CH_2OR_9$ minus R_4OH minus R_7OH

Fig. 3. Survey of the selected fragment ions A–H worked out for the trimethylsilylated methyl ester and the pertrimethylsilylated derivatives of *N*-acylneuraminic acids with *O*-acyl and/or *O*-alkyl substituents (*see* **Tables 2** and **3**).

atoms or between an acetoxylated and an alkoxylated carbon atom, rather than between two acetoxylated carbon atoms. Fragment C only has significant abundance if C-7 bears an ether group. When an ester group is present at C-7, this fragment ion is absent or hardly observable.

7. Fragment D is formed from fragment C by consecutive elimination of R_2OH and R_4OH. It is evident that the occurrence of this fragment ion is dependent on the presence of fragment C.

8. Fragment E is formed by elimination of the side-chain C-7,8,9 and the substituent at C-5. This fragment ion is not seen if an *O*-acyl group is attached to C-4,
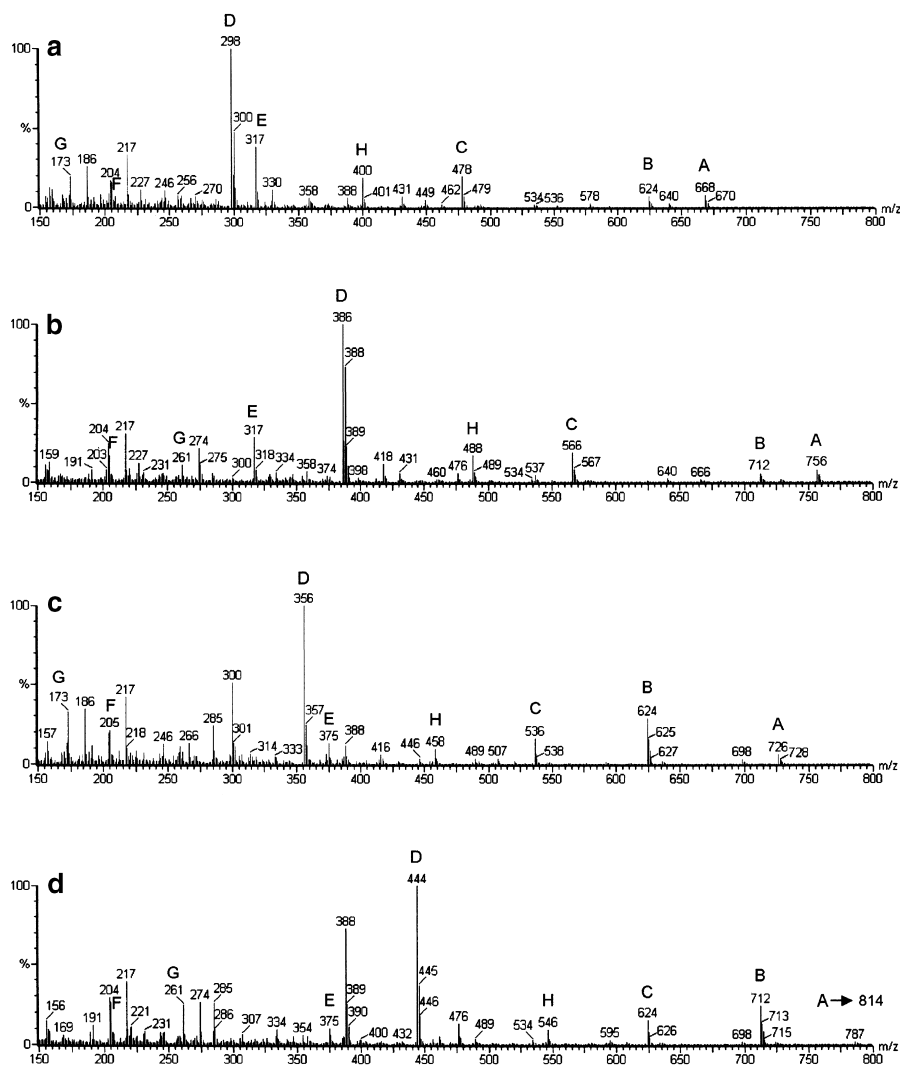
Fig. 4. Electron impact mass spectra of β-Neu5Ac and β-Neu5Gc. **(a)** Trimethyl-silylated methyl ester of β-Neu5Ac; **(b)** trimethylsilylated methyl ester of β-Neu5Gc; **(c)** pertrimethylsilylated β-Neu5Ac; **(d)** pertrimethylsilylated β-Neu5Gc.

illustrating that the transition state in the McLafferty rearrangement is not favored when the substituent at C-4 is an ester group rather than an ether group. For *O*-trimethylsilylated *N,O*-acylneuraminic acids (β-anomers) it holds that, when compared with their methyl esters, in their trimethylsilyl esters the intensity of fragment E is greatly reduced but still present; instead, an additional fragment derived from fragment E by loss of Me$_3$SiOH is clearly present.

9. Fragment F contains the C-8,9 part. Based on the same fragmentation rules as mentioned for fragments C and D (**steps 6** and **7**), this ion can be readily formed only if an ether group is connected at C-8.
10. Fragment G consists of the C-4,5 part of the Sia molecule.
11. Fragment H is formed by elimination of the C-9 part of the molecule, followed by elimination of R_4OH and R_7OH; this fragment is only useful in the case of *O*-alkyl substituents.

    Table 2 presents the GLC-EI/MS data of a series of naturally occurring Sias, derivatized as TMS methyl esters. **Table 3** presents the GLC-EI/MS data of a series of naturally occurring Sias, derivatized as PTMS derivatives. Sias predominantly occur in the β-anomeric form as previously discussed. However, occasionally the minor amount of α-anomer can be detected separately from the β-anomer as a shoulder. Inspection of the EI/MS data in **Tables 2** and **3** shows that each Sia gives rise to a unique series of A−G fragment ions. The H fragment is also used in case of substitution patterns of *O*-trimethylsilyl and *O*-methyl groups. For EI/MS spectra of members of the Sia family presented in **Tables 2** and **3** but not depicted in **Fig. 4**, *see* **refs. *11,14–18***.

3.3.2. Detailed Analyses of GLC-EI/MS Data of TMS Methyl Ester or PTMS Sia Derivatives

1. Fragment G requires more detailed discussion. The occurrence of an *O*-acetyl group instead of an *O*-trimethylsilyl group at C-4 as in the derivatives of Neu4,5Ac$_2$ (acetyl [AC]), Neu4,5,9Ac$_3$, Neu4,5Ac$_2$9Lt (lactyl [LT]), and Neu4Ac5Gc (glycolyl [GC]) leads to a negative shift of 30 atomic mass units (amu) for this fragment. Therefore, in the mass spectrum of 4-*O*-acetylated *N*-acetylneuraminic acids the peak at *m/z* 173 is absent: *m/z* 173 shifts to *m/z* 143. However, in all mass spectra a peak at *m/z* 143 with a general formula $C_6H_{11}O_2Si$ is observed. But in the mass spectra of Neu4,5Ac$_2$, Neu4,5,9Ac$_3$, and Neu4,5Ac$_2$9Lt, the main contribution to the abundance of *m/z* 143 originates from fragment G ($C_6H_9NO_3$). In the mass spectrum of Neu4Ac5Gc the peak at *m/z* 261 is not observed. For this compound, fragment G shifts to *m/z* 231. Using high-resolution mass spectometry, this fragment ion could not be distinguished from other generally occurring fragment ions in Sia, which also contributes to the intensity of the peak at *m/z* 231.
2. Using the fragment ions A−G, Neu5,8Ac$_2$ and Neu5,9Ac$_2$ are distinguished solely on the basis of the intensity of the peak at *m/z* 175 (fragment F). Of course, the mass spectra of these compounds also differ in other aspects. For example, the side chain CH_2OCOCH_3-$CHOSi(CH_3)_3$-$CH=O^+Si(CH_3)_3$ in Neu5,9Ac$_2$ clearly eliminates CH_3COOH, giving rise to the fragment ion at *m/z* 217. In Neu5,8Ac$_2$, the side chain $CH_2OSi(CH_3)_3$-$CHOCOCH_3$-$CH=O^+Si(CH_3)_3$ eliminates CH_3COOH (*m/z* 217) as well as $HOSi(CH_3)_3$ (*m/z* 187).
3. The fragment ion at *m/z* 103 ($CH_2=O^+Si(CH_3)_3$) is not characteristic for a primary trimethylsiloxyl group in the Sia derivatives, but can also be formed along other routes.

Table 2
GLC and Characteristic EI/MS Fragment Ions (70 eV) of Trimethylsilylated Methyl Ester Derivatives of Naturally Occurring Sialic Acids (β-Anomers)

Sialic acid	R_{Neu5Ac}	Fragments (*m/z*)							
		A	B	C	D	E	F	G	H
Neu5Ac	1.00	668	624	478	298	317	205	173	400
Neu4,5Ac$_2$	1.18	638	594	448	298	—	205	143	400
Neu5,7Ac$_2$	1.04	638	594	—	—	317	205	173	400
Neu5,8Ac$_2$	1.05	638	594	478	298	317	—	173	—
Neu5,9Ac$_2$	1.13	638	594	478	298	317	175	173	400
Neu4,5,9Ac$_3$	1.31	608	564	448	298	—	175	143	400
Neu5,7,9Ac$_3$	1.14	608	564	—	—	317	175	173	400
Neu5,8,9Ac$_3$	1.19	608	564	478	298	317	—	173	—
Neu5,7,8,9Ac$_4$	1.15	578	534	—	—	317	—	173	—
Neu5Ac9Lt	2.55	740	696	478	298	317	277	173	400
Neu4,5Ac$_2$9Lt	3.01	710	666	448	298	—	277	143	400
Neu2en5Ac	1.09	578	—	388	298	227	205	—	—
Neu5Gc	1.81	756	712	566	386	317	205	261	488
Neu4Ac5Gc	2.02	726	682	536	386	—	205	231	488
Neu7Ac5Gc	1.83	726	682	—	—	317	205	261	488
Neu9Ac5Gc	2.04	726	682	566	386	317	175	261	488
Neu7,9Ac$_2$5Gc	2.01	696	652	—	—	317	175	261	488
Neu8,9Ac$_2$5Gc	1.99	696	652	566	386	317	—	261	—
Neu7,8,9Ac$_3$5Gc	1.93	666	622	—	—	317	—	261	—

R_{Neu5Ac} values on 3.8% SE-30 (packed column) at 215°C are given relative to β-Neu5Ac. The R_{Neu5Ac} values are given as a directive to set up an in-house set of GLC values with reference sialic acids. (Data taken from **ref. 3**.)

4. The fragment ions at *m/z* 186 (CH$_3$CON$^+$H=CH-CH=CHOSi(CH$_3$)$_3$ and CH$_3$CON$^+$H=CH-C(OSi(CH$_3$)$_3$)=CH$_2$) in *N*-acetylneuraminic acids and at *m/z* 274 in *N*-glycolylneuraminic acids only give information about the type of substitution at C-5 (amino group).

3.4. GLC-EI/MS Data of Heptafluorobutylated Sia Methyl Ester Derivatives

In a more recent EI/MS method for the identification of Sias isolated from biological material, heptafluorobutylated (HFB) methyl ester derivatives are used *(7)*. HFB derivatives are rather stable in contrast to the TMS derivatives. It should be noted that in the derivatization procedure the anomeric HO-2 group remains free. The molar ratio between the α- and β-anomers changes slowly if

Table 3
GLC and Characteristic EI/MS Fragment Ions (70 eV) of Pertrimethylsilylated Derivatives of Naturally Occurring Sialic Acids (β-Anomers)

Sialic acid	R_{Neu5Ac}	Fragment ions (m/z)							
		A	B	C	D	E	F	G	H
Neu5Ac	1.00	726	624	536	356	375	205	173	458
Neu4,5Ac$_2$	1.05	696	594	506	356	—	205	143	458
Neu5,9Ac$_2$	1.02	696	594	536	356	375	175	173	458
Neu5,7,9Ac$_3$		666	564	—	—	375	175	173	458
Neu5,8,9Ac$_3$	1.04	666	564	536	356	375	—	173	—
Neu5,7,8,9Ac$_4$		636	534	—	—	375	—	173	—
Neu5Ac9Lt		798	696	536	356	375	277	173	458
Neu5Ac8Me	0.98	668	566	536	356	375	147	173	400
Neu5,9Ac$_2$8Me	1.00	638	536	536	356	375	117	173	400
Neu2en5Ac	1.01	636	—	446	356	285	205	—	
Neu2,7an5Ac		564	462	374	—	—	205	173	
Neu5Gc	1.19	814	712	624	444	375	205	261	546
Neu4Ac5Gc		784	682	594	444	—	205	231	546
Neu9Ac5Gc	1.21	784	682	624	444	375	175	261	546
Neu7,9Ac$_2$5Gc		754	652	594	414	375	175	261	546
Neu5Gc8Me	1.14	756	654	624	444	375	147	261	488
Neu9Ac5Gc8Me	1.17	726	624	624	444	375	117	261	488
Neu5GcAc	1.21	784	682	594	414	375	205	231	
Neu2en5Gc		724	—	534	444	285	205	—	
Neu2,7an5Gc		652	550	286	—	—	205	261	

R_{Neu5Ac} values on CP-Sil 5 (capillary column), using the program 5 min/140°C; 2°C/min up to 220°C; 15 min/220°C, are given relative to β-Neu5Ac. The R_{Neu5Ac} values are given as a directive to set up an in-house set of GLC values with reference sialic acids. (Data taken from **ref. 3**.)

samples are kept in the acylation mixtures for a long time, because of mutarotation of the free HO-2 group.

Table 4 presents the GLC-EI/MS data of a series of naturally occurring Sias, derivatized as HFB methyl esters. For EI/MS spectra of members of the Sia family presented in **Table 4**, *see* **ref. 7**.

1. The EI spectra of the HFB Sia methyl ester derivatives do not show molecular ion peaks.
2. These EI spectra are rather complex owing to the presence of only *O*-acylated groups in most of the naturally occurring Sias (*N,O*-acylated neuraminic acids). No use can be made of the preferences in cleavage comparing two neighboring alkoxylated carbon atoms, a neighboring acetoxylated and alkoxylated carbon

Table 4
GLC and Reporter EI/MS Fragment Ions (70 eV) of Heptafluorobutylated Methyl Ester Derivatives of Naturally Occurring Sialic Acids (α,β-Anomers)

Sialic acid	R_{Neu5Ac}'s	Reporter ions (m/z)
Neu	1.14/1.15	815-801-765-731-550-534-505-334-294-253
Neu5Ac	0.99/1.00	801-773-757-704-543-490-330-264-238
Neu4,5Ac$_2$	1.14/1.15	862-833-801-756-704-606-560-493-491-453-320-154-84
Neu5,7Ac$_2$	1.16/1.16	862-790-773-757-543-493-453-347-279-238
Neu5,8Ac$_2$	n.d./1.17	862-832-778-776-734-704-605-520-492-307
Neu5,9Ac$_2$	1.11/1.14	862-813-756-648-620-560-543-519-490-393-347-264-73
Neu4,5,9Ac$_3$	n.d./1.32	708-648-595-490-335-223-150-84-73
Neu5,7,9Ac$_3$	1.31/1.33	708-604-494-452-379-238-166-153-73
Neu5,8,9Ac$_3$	n.d./1.27	708-648-561-351-347-264-238-145-103-73
Neu4,5,7,9Ac$_4$	n.d./1.96	580-507-281-241-209-205-191-135-73
Neu4,5,7,8,9Ac$_5$	2.43/2.44	427-368-352-260-213-145-73
Neu5Ac9Lt	1.10/1.11	971-842-776-755-562-514-451-349-238-112
Neu4,5Ac$_2$9Lt	1.45/1.46	962-919-872-748-679-562-451-348-112
Neu5,7Ac$_2$9Lt	n.d./1.32	903-832-562-451-349-238-153-112-73
Neu5Ac8Me	1.09/1.14	833-804-790-693-622-578-364-265
Neu4,5Ac$_2$8Me	n.d./1.47	721-694-678-621-578-424-407
Neu5,9Ac$_2$8Me	n.d./1.47	721-678-620-578-424-96-73
Neu5Ac8S	1.38/1.40	831-688-619-407-365-322-295-122
Neu4,5Ac$_2$8S	1.52/1.53	694-619-534-467-365-295-207-122
Neu5Ac1,7lactone	1.08/1.11	861-841-647-620-533-407-380-350-347-320-252-194-136
Neu5,9Ac$_2$1,7lactone	1.31/1.35	706-604-494-452-450-379-347-306-252-166-136-73
Neu4,5,9Ac$_3$1,7lactone	1.53/1.53	494-450-438-407-394-347-339-254-166-136-83
Neu5Gc	1.25/1.26	859-831-815-761-618-548-404-348-298-294-227
Neu9Ac5Gc	n.d./1.24	903-832-777-647-562-542-349-238-112
Neu4,7Ac$_2$5Gc	n.d./1.48	900-802-706-664-620-559-405-227-211-166-84
Neu4,9Ac$_2$5Gc	n.d./1.37	899-848-703-624-518-366-304-276-238-227-84
Neu7,9Ac$_2$5Gc	n.d./1.40	920-876-847-706-664-620-548-510-406-350-227-153-134-73
Neu8,9Ac$_2$5Gc	n.d./1.30	903-859-744-703-648-594-532-277-145-103
Neu4,7,9Ac$_3$5Gc	n.d./1.31	792-777-703-518-491-304-238-227-73

Table 4 *(continued)*

Sialic acid	R_{Neu5Ac}'s	Reporter ions (*m/z*)
Neu4,7,8,9Ac$_4$5Gc	n.d./1.48	703-624-537-518-304-277-238-227
Neu5Gc9Lt	n.d./1.26	859-857-831-829-815-761-618-548-348-227
Neu4Ac5Gc9Lt	1.25/1.26	901-830-773-686-620-509-407-350-296-227-112
Neu7(8)Ac5Gc9Lt	n.d./1.25	903-861-789-688-619-562-474-408-350-255-195-153-112
Neu4,7Ac$_2$5Gc9Lt	n.d./1.41	961-917-848-677-620-509-407-296-227-153-112-84
Neu7,8Ac$_2$5Gc9Lt	1.40/1.44	920-902-876-748-704-662-632-560-548-255-227-195-112
Neu5Gc1,7lactone	n.d./1.30	859-745-619-519-350-347-277-227-136
Neu9Ac5Gc1,7lactone	n.d./1.41	920-873-815-662-548-388-299-294-227

R_{Neu5Ac} values on CP-Sil 5CB (capillary column), using the program 3 min/90°C; 5°C/min up to 260°C, are given relative to β-Neu5Ac. The R_{Neu5Ac} values are given as a directive to set up an in-house set of GLC values with reference sialic acids. (Data taken from **ref. 7**.)

  atom, and two neighboring acetoxylated carbon atoms, as this is the key of the fragmentation scheme of the TMS derivatives.
3. Characteristic ions from the HFBs are found at *m/z* 69, 119, and 169.
4. Most of the Neu5Ac and Neu5Gc derivatives give rise to peaks at *m/z* 238 and 227, respectively.
5. A peak at *m/z* 84 is specific for Neu4,5,xAc$_3$ derivatives, a peak at *m/z* 73 for 9-*O*-acetylated Sias, peaks at *m/z* 103 and 145 for 8,9-di-*O*-acetylated Sias, and a peak at *m/z* 136 for 1,7 intramolecular lactones.
6. All 9-*O*-lactyl derivatives contain a peak at *m/z* 112.
7. The peak at *m/z* 505 is specific for Neu, and at *m/z* 122 and 295 for 8-*O*-sulfated Sias.

3.5. Preparation of Fluorescent Sia Derivatives for HPLC and LC-ESI/MS Analysis

  By starting from free Sia pools, fluorescent Sia derivatives can be generated by reaction with the fluorogenic reagents DMB (*see* **Fig. 5** and **ref. 19**) or *o*-phenylenediamine (OPD; *see* **ref. 20**). Most research groups currently use the DMB derivatives for HPLC analysis, and therefore the focus will be on these compounds. A typical HPLC chromatogram is presented in **Fig. 6**.
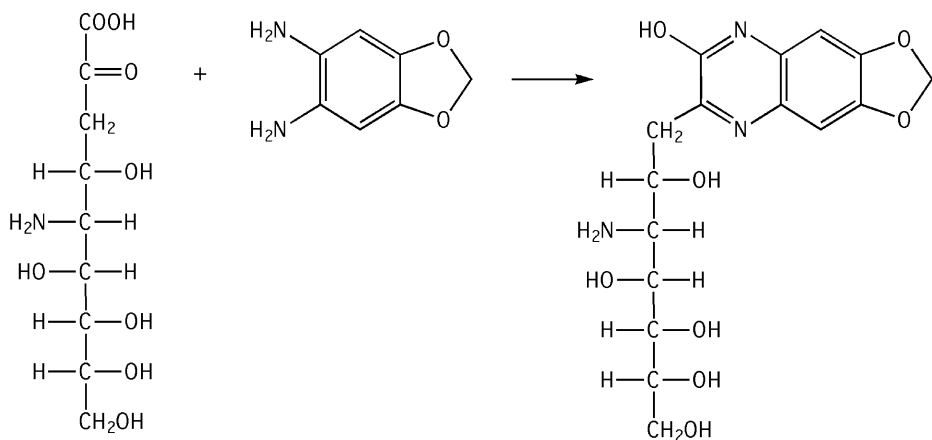
Fig. 5. Conversion of Neu5Ac into the corresponding fluorescent DMB derivative.
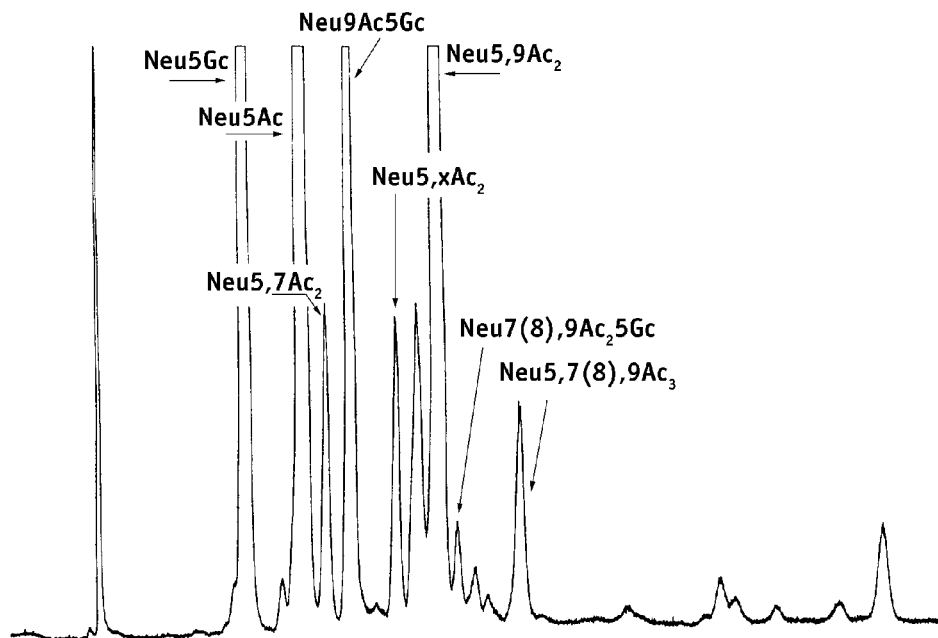

Fig. 6. Typical HPLC pattern of a mixture of fluorescent DMB derivatives of sialic acids on an octadecylsilane column. For elution system details, *see* **Table 5**. (Data taken from **ref. 8**.)

3.5.1. Preparation and HPLC (-ESI/MS) Analysis of DMB Sia Derivatives

1. Lyophilized Sia samples (pg-ng amounts) in 200 µL of 2 *M* acetic acid are mixed with 200 µL of 7 m*M* DMB dihydrochloride in 1.4 *M* acetic acid, containing 0.75 *M* of β-mercaptoethanol and 18 m*M* of sodium hydrosulfite. The DMB stock solution can be used for more than 1 wk when stored in a refrigerator. It should be noted that the derivatization can often be carried out directly on the acetic acid hydrolysate of a glycoconjugate without intermediate purification steps.
2. The solution is heated for 2.5 h at 50°C in the dark (1 h at 56°C has also been proposed *[21]*), and after cooling on ice, a fixed volume (2–20 µL) is used for analysis by HPLC.
3. The DMB Sia derivatives are analyzed on a reversed-phase Cosmosil 5C18-AR-II column, using acetonitrile:methanol:water (9:7:84) as the solvent system at a flow rate of 1 mL/min at room temperature. Authentic Sias are used as reference compounds. Fluorescence detection occurs at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. The DMB derivative of Neu5Ac is used for quantifications as an internal standard.
4. For a combination with ESI/MS, a microbore ultrasphere ODS column has been applied. Here, elutions have been carried out with a linear two-step gradient of acetonitrile:methanol:water (3.85:7.00:89.15) to 7.15:7.00:85.85 in 40 min, and then to 11.00:7.00:82.00 in 10 min at a flow rate of 0.2 mL/min. A survey of HPLC retention times on an ODS column is presented in **Table 5** (*see* **refs. 8** and *22*).

3.6. HPLC-ESI/MS Data of DMB Sia Derivatives

A sensitive LC-ESI/MS methodology has recently been worked out using the fluorescent DMB Sia derivatives, which is highly suitable for the identification of Sias with free anomeric centers *(8)*. Intermediate purification steps are not always necessary owing to the fluorescent label. Best results can be obtained with a microbore reversed-phase HPLC column (with split for separate fluorescent detection), whereby in the ESI/MS system molecular ion species can be detected on the 5-pmol level, and typical CAD (collisional activation decomposition) fragments on the 10- to 15-pmol level.

Table 5 summarizes ESI/MS data of a series of naturally occurring Sias. Speculative MS fragmentation pathways for several of them have been reported *(8)* (for a series of ESI/MS spectra, *see* **refs. 8** and *23*). From the information available to date it is clear that a combination of retention time and spectral characteristics allows the identification of the number, type, and position of the various substituents in Sias that can be converted into DMB derivatives.

Table 5
HPLC Relative Retention Times (R_{Neu5Ac}) on an Ultrasphere
ODS Column and Characteristic Ions Generated
by LC-ESI/MS of DMB Sialic Acid Derivatives

Sialic acid	R_{Neu5Ac}	$[M+H]^+$	CAD fragments	
			$[M+H-H_2O]^+$	Fragments (m/z)
Neu5Ac	1.00	426	408	313-295-283-229
Neu4,5Ac$_2$	1.60	468	abs.	408-313-283-229
Neu5,7Ac$_2$	1.08	468	450	313-295-283-229
Neu5,8Ac$_2$	1.31	468	450	313-295-229
Neu5,9Ac$_2$	1.51	468	450	313-295-229
Neu4,5,9Ac$_3$		510	abs.	450-313-295-229
Neu5,7,9Ac$_3$[a]	1.74	510	492	313-295-229
Neu5,8,9Ac$_3$[a]	1.57	510	492	n.d.
Neu5,7,8,9Ac$_4$	2.16	552	534	313-295-229
Neu5Ac9Lt	1.45	498	480	313-295-229
Neu5Ac8S	0.17	506	abs.	426
Neu4,8an5Ac	1.39	408	abs.	313-283-229
Neu5Gc	0.78	442	424	313-295-283-229
Neu4Ac5Gc	1.18	484	abs.	424-313-283-229
Neu7Ac5Gc	0.99	484	466	n.d.
Neu8Ac5Gc	1.04	484	466	313-295-229
Neu9Ac5Gc	1.19	484	466	313-295-229
Neu7,9Ac$_2$5Gc[a]	1.66	526	508	313-295-229
Neu8,9Ac$_2$5Gc[a]	2.10	526	508	313-295-229
Neu5Gc9Lt	1.14	514	496	n.d.
Neu5Gc8Me	0.98	456	438	313-295-229
Neu7Ac5Gc8Me	1.41	498	480	430-316-313-229
Neu9Ac5Gc8Me	1.90	498	480	316-313-295-229
Neu5Gc8S	0.16	522	abs.	464-442-424-313-229
Neu4,8an5Gc	1.21	424	abs.	313-283-229

[a]Assignments may be interchanged.

Elution with a linear two-step gradient of acetonitrile:methanol:water: 3.85:7.00:89.15 to 7.15:7.00:85.85 in 40 min, and then to 11.00:7.00:82.00 in 10 min, at a flow rate of 0.2 mL/min. The R_{Neu5Ac} values are given as a directive to set up an in-house set of HPLC values with reference sialic acids. (Data taken from **refs. 8** and **23**.)

abs., absent; n.d., not determined.

1. The molecular mass of the Sia derivative, and thereby the number and type of substituents, is reflected by the pseudomolecular ion $[M+H]^+$ and its sodium adduct $[M+Na]^+$.
2. An important fragment ion is the result of H_2O elimination $[M+H-18]^+$.

3. The ions [M+H]⁺, [M+Na]⁺, and [M+H-18]⁺ are very useful for molecular ion chromatogram screening of HPLC effluents.

4. When focusing on *N,O*-acylneuraminic acids, the [M+H-18]⁺ ion is suggested to correspond with a 4,8-anhydro ring structure in case both HO-4 and HO-8 are not substituted. Pathways starting from this ring structure fragmentation have been formulated for non-*O*-acylated, 7-*O*-acylated, and 9-*O*-acylated *N*-acylneuraminic acids.

5. Fragment A at *m/z* 313 (elimination of acylamide followed by 2X H_2O, 1X H_2O plus 1X HOAc, or 2X HOAc from the ring structured [M+H-18]⁺ ion) is present in all Sia derivatives.

6. Fragment D at *m/z* 229 (fragmentation of the ring of the [M+H-18]⁺ ion; C-3,4,5 containing fragment R-C_3H_3, with R = C-1,2 part) is present in all Sia derivatives.

7. Fragment B (further loss of H_2O from fragment A) at *m/z* 295 is relatively more important compared with fragments A and D when the Sia is substituted at O-9.

8. Fragment C (elimination of C-9 as formaldehyde from the [M+H-18]⁺ ion) at *m/z* 283 is only present when the Sia is not substituted at O-8 or O-9.

9. It should be noted that the ESI/MS data do not differentiate between 8- and 9-*O*-acylations.

10. 4-*O*-acetylated Sias, missing a [M+H-18]⁺ ion, are characterized by a major [M+H-HOAc]⁺ ion.

11. Focusing on *N*-acyl-8-*O*-alkyl-neuraminic acids, it is suggested that etherification of HO-8 (Neu5Ac/5Gc8Me) blocks the cyclization between C-4 and C-8. Furthermore, an ion [M+H-18]⁺ is present, as well as *m/z* 229 and the fragments arising from the loss of acylamide and two or three molecules of water. The elimination of a formaldehyde part does not occur.

4. Notes

1. It has been stated that in the case of preparing HFB methyl ester derivatives *(7)*, no purification of the liberated Sia pool is needed (in the case of glycoproteins, a short-term centrifugation step is recommended).

2. The report on preparing HFB methyl ester derivatives *(7)* mentions that treatment of standard Neu5Ac with diazomethane reagent up to 15 d at room temperature did not produce Neu5Ac8Me. Experience shows that HO-4 is especially sensitive for *O*-methylation in a diazomethane protocol, and short incubation times are advised.

Acknowledgments

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