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Selective binding by *Helicobacter pylori* of leucocyte gangliosides with 3-linked sialic acid, as identified by a new approach of linkage analysis

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The human gastric pathogen *Helicobacter pylori* has been shown to bind to glycoconjugates of human leucocytes in a sialic acid-dependent way. In order to improve the identification of the binding epitope, a new technique was developed to analyze the ketosidic linkage position between a terminal sialic acid and the consecutive monosaccharide. Permethylation and reduction with $LiAlH_4$ followed by trifluoroacetolysis in 1000:1 trifluoroacetic anhydride:trifluoroacetic acid (24 h, 100 °C) results in the cleavage of glycosidic but not ketosidic bonds. The disaccharide products were analyzed by gas chromatography-mass spectrometry and sialyl-3 or -6 position and NeuAc or NeuGc are identified by their separate retention times and mass spectra. The method was worked out on model saccharides and applied on five-sugar gangliosides (sialylparaglobosides) of human leucocytes. Radiolabeled *Helicobacter pylori* was shown to bind to the upper part, but not to the lower part, of the five-sugar interval of a mixture of gangliosides separated on a thin-layer chromatogram. Using a membrane blotting procedure the active and inactive bands were isolated and shown to be NeuAc*α*2-3- and NeuAc*α*2-6-paraglobosides, respectively.

***Keywords*: *Helicobacter pylori*, leucocytes, glycosphingolipids, trifluoroacetolysis, sialic acid, gas chromatography-mass spectrometry, linkage position**

***Abbreviations*: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; GC/MS, gas chromatography mass spectrometry; DMSO, dimethyl sulphoxide; ether, diethyl ether; TFA, trifluoroacetic acid; TFAA, trifluoroacetic anhydride; TFAc, trifluoroacetyl; MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; 6-SL, 6-sialyllactose; 3-SL, 3-sialyllactose; SPG, sialylparagloboside; Me, methyl; FAB MS, fast atom bombardment mass spectrometry; EI MS, electron ionization mass spectrometry; DHB, 2,5-dihydroxybenzoic acid; TLC, thin-layer chromatography.**

Introduction

Structurally varying sialic acids[†] [1] are involved in biological recognition events [2, 3], including the attachment of microbes to host cell glycoconjugates as an essential step in the infection process [4]. *Helicobacter pylori* is a bacterium causing stomach diseases including chronic gastritis and cancer and the infection affects more than half of the global population [5]. NeuAcα2-3Gal was proposed early on as a colonization factor for the bacterium [6], and this was recently confirmed and extended [7]. This binding specificity is expressed when bacteria are grown on agar. However, after growth in broth there is a second sialic acid-dependent binding specificity associated with polyglycosylceramides, complex *N*-acetyllactosamine-based glycosphingolipids of low abundance [8, 9]. The two sialyl-specificities being recognized are designated epitope 1 and 2, respectively. The complexity and microheterogeneity of polyglycosylceramides do not allow the identification of epitope 2 using presently available techniques. To improve this situation a method has been developed, where sialic acid and the first consecutive monosaccharide can be analyzed as a disaccharide derivative by gas chromatography mass spectrometry (GC/MS), providing information on the linkage position and the type of sialic acid. Traditional permethylation analysis [10] gives this information only indirectly, and fails for larger glycoconjugates. Enzymatic hydrolysis with available sialidases [11] lacks absolute specificity for the

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[†] Sialic acid includes both *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc). Tel: +46-31-7733492; Fax: +46-31-413190; E-mail: lena.johansson@medkem.gu.se

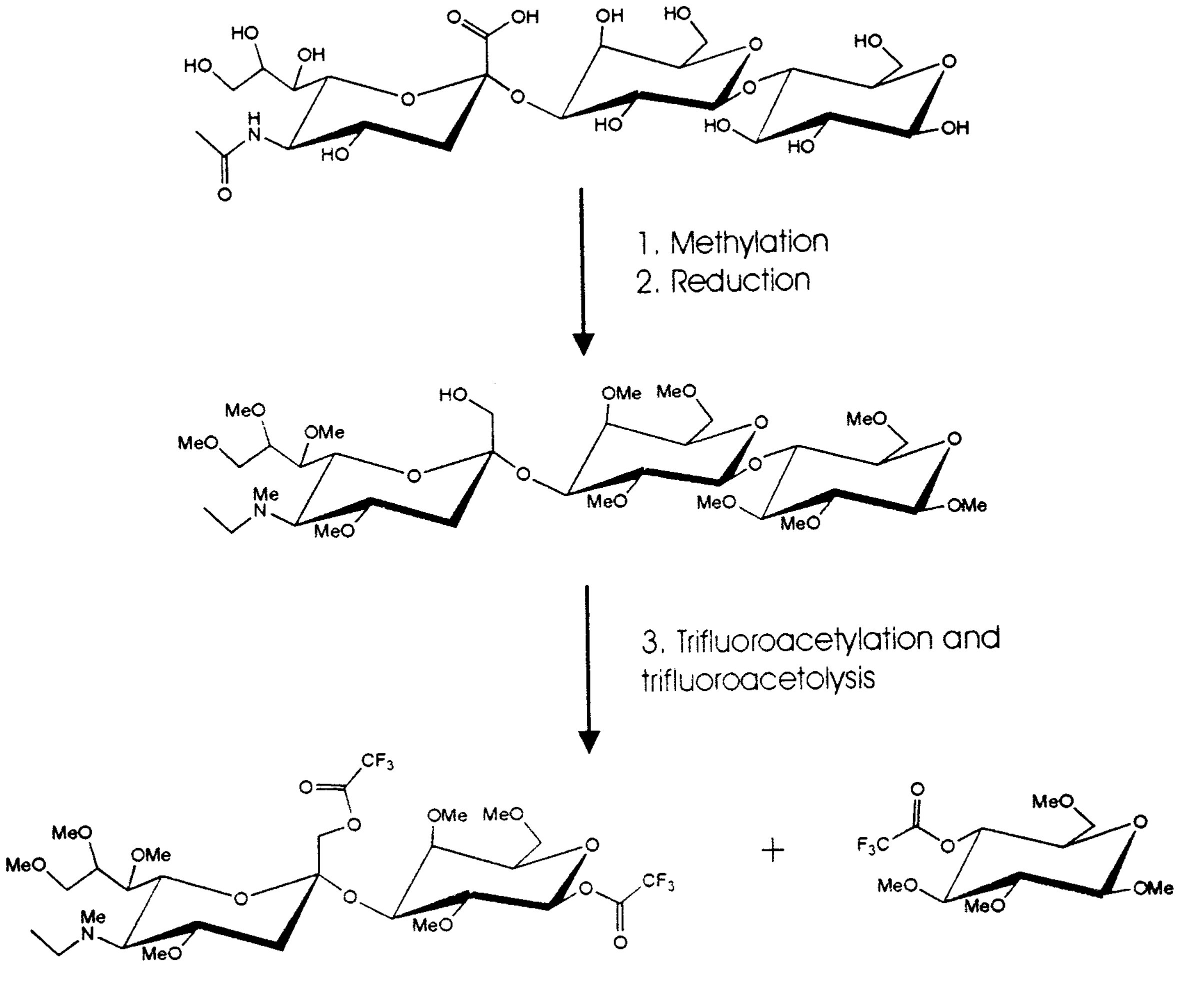


Figure 1. Reaction scheme for the methylation, reduction and trifluoroacetolysis.

two linkages. Although high-resolution nuclear magnetic resonance (NMR) spectroscopy is a potential alternative, we consider it essential to have a chemical analytical method which can be used also on complex fractions.

Theoretically, based on the provided mechanism of trifluoroacetolysis to release saccharides from glycoconjugates [12], the ketosidic linkage of sialic acids will be stable during trifluoroacetolysis if the carboxyl group is first reduced to an alcohol and trifluoroacetylated. Conditions to obtain disaccharides containing the terminal sialic acid were therefore designed as follows. Permethylation forms a methyl ester of the carboxyl group at C-1. After reduction, the primary alcohol obtained is trifluoroacetylated followed by trifluoroacetolysis of the glycosidic linkages (Figure 1). Retention times and mass spectra after GC/MS should then be able to establish the linkage position as well as the type of sialic acid, NeuAc or NeuGc.

In the present work, this approach was successfully tested on model saccharides and glycolipids (Table 1). The technique was then applied to simple leucocyte gangliosides carrying epitope 1 for binding by *H. pylori*. Leucocytes (neutrophils), in addition to gastric epithelial cells, are target cells for the bacterium, and on bacterial-neutrophil contact there is a rapid induction of the inflammatory response, which is the major cause of gastritis and ulcer formation [5]. On thin-layer chromatoghraphy (TLC) plates the bacterium (grown on agar) binds to the upper part of bands in the sialylparagloboside (SPG) interval (five-sugars), but not to the lower part. After extraction from blotting membranes the two fractions were shown to produce identical fast atom bombardment (FAB) spectra. However, the new procedure convincingly showed that the positive fraction contained 3-linked NeuAc and the negative fraction contained 6-linked NeuAc.

Materials and methods

Materials, reagents and solvents

Dimethyl sulphoxide (DMSO) (Merck, Germany) was distilled under reduced pressure and nitrogen atmosphere.

Table 1. Different substrates used during the development of the method

Substrate	*Abbreviation*	*Possible sequences after permethylation, reduction and trifluoroacetolysis according to Figure*[a]
Oligosaccharides		
NeuAcα2-3Galβ4Glc	3-SL	NeuAcα2-3GalβTFAc
NeuAcα2-6Galβ4Glc	6-SL	NeuAcα2-6GalβTFAc
NeuAcα2-3Galβ3(NeuAcα2-6)GlcNAcβ3Galβ4Glc	DSLNT	NeuAcα2-3GalβTFAc and NeuAcα2-6(TFAc3)GlcNAcβTFAc[b]
Galβ3(NeuAcα2-6)GlcNAcβ3Galβ4Glc	LST-b	NeuAcα2-6(TFAc)GlcNAcβTFAc[b]
Gangliosides		
NeuAcα2-3Galβ4GlcβCer	GM3 (NeuAc)	NeuAcα2-3GalβTFAc
NeuGcα2-3Galβ4GlcβCer	GM3 (NeuGc)	NeuGcα2-3GalβTFAc
Galβ3GalNAcβ4(NeuAcα2-3)Galβ4GlcβCer	GM1	NeuAcα2-3(TFAc4)GalβTFAc
NeuAcα2-3Galβ3GalNAcβ4(NeuAcα2-3)Galβ4GlcβCer	GD1a	NeuAcα2-3GalβTFAc and NeuAcα2-3(TFAc4)GalβTFAc
NeuAcα2-3Galβ4GlcNAcβ3Galβ4GlcβCer	3-SPG	NeuAcα2-3GalβTFAc
NeuAcα2-6Galβ4GlcNAcβ3Galβ4GlcβCer	6-SPG	NeuAcα2-6GalβTFAc

[a] Sialic acids are designated NeuAc or NeuGc and *N*-acetylhexosamines are designated GlcNAc or GalNAc, although the amides were reduced to amines.

[b] Not formed under the conditions used.

Diethyl ether (Merck) was distilled over lead alloy (Sigma-Aldrich, Germany) in a nitrogen atmosphere. Toluene (Labscan, Ireland) was distilled over sodium and benzophenon (Fluka, Switzerland) in a nitrogen atmosphere. CH_3I (Fluka) was distilled under nitrogen, and NaOH (EkaNobel, Sweden) was powdered, dried at 165 °C and stored in a desiccator. $LiAlH_4$ (Merck) was stored in a desiccator. Trifluoroacetic acid (TFA) (Sigma, USA) and trifluoroacetic anhydride (TFAA) (Fluka) were used without further purification and always kept under argon. All syringes, stirring magnets and lids were kept in a desiccator. Samples were lyophilized overnight before reduction and trifluoroacetolysis in reaction vials. The reaction vials were sealed with mininert valves (Supelco, USA) during the trifluoroacetolysis. TLC was performed on alumina backed sheets, coated with silica gel 60, 0.2 mm thickness (HPTLC nanoplates, Merck). The blotting membrane was a PVDF membrane (Millipore, USA).

The total glycosphingolipid fraction from leucocytes was prepared by methods already described [13]. The oligosaccharides were purchased from Dextra Laboratories Ltd (U.K.) and used without further purification. The model compounds used are listed in Table 1.

Permethylation and reduction

Permethylation was performed by the method of Ciucanu and Kerek [14] with the modifications described by Larson *et al.* [15]. The sample was dried by evaporation at 40 °C under a flow of nitrogen before permethylation. The reduction was performed with $LiAlH_4$ in ether as reported by Karlsson [16].

Trifluoroacetolysis

The sample was lyophilized over night and dissolved in 300 μl TFAA, sonicated for a few minutes and heated to 100 °C to trifluoroacetylate the sialic acid C-1 position. TFA (0.3 μl) was added to form a 1000:1 TFAA:TFA solution. The reaction mixture was left at 100 °C for 24 h. After the reaction mixture had cooled to room temperature, 50 μl of dry toluene was added as a top layer and the solvents were evaporated at 40 °C under a flow of nitrogen. The sample was then dissolved in 10 μl of toluene and analyzed by GC/MS and MALDI-TOF MS. To find the optimal temperature, concentration of TFA and reaction time, a series of samples was prepared from 100 μg of underivatized material. The samples were trifluoroacetolysed in 300 μl TFAA:TFA mixtures under different conditions, see Table 2. At different times 40 μl of the reaction solution was taken out from the reaction vial, poured into 40 μl of dry toluene and evaporated at 40 °C under a flow of nitrogen. The evaporated samples were dissolved in 10 μl of dry toluene and analyzed by MALDI-TOF MS.

Analysis by GC/MS and MALDI-TOF MS

GC/MS was performed on a JEOL SX-102A mass spectrometer (JEOL, Japan) interfaced to a Hewlett Packard

Table 2. Different reaction conditions of trifluoroacetolysis evaluated by the intensity of the fragment ion at m/z = 716 from GC/MS, and the $[M + H]^+$ ion at m/z = 748 from MALDI-TOF MS, produced by permethylated and reduced 3-SL and 6-SL

Temperature	*TFAA : TFA*	*Relative intensity after 24 h*
60 °C	100:1	20%
60 °C	1000:1	75%
80 °C	1000:1	70%
100 °C	100:1	15%
100 °C	1000:1	100%

5890 Series II gas chromatograph. A fused silica column (15 m × 0.25 mm inner diameter) coated with 0.1 µm of cross-linked PS264 (Fluka) was used, and 1 µl of the sample solution was injected on-column at 70 °C. After 1 min at 70 °C a temperature program was started with a rate of 10 °C min^{-1} up to 370 °C where it was held for 2 min. The mass spectrometer was operated in positive electron impact (EI) mode: GC/MS interface temperature 370 °C; ion source temperature 360 °C; trap current 300 µA; electron energy 70 eV; acceleration voltage + 10 kV; ion multiplier voltage 1.3 kV. The resolution was set to 1400 (10% valley definition).

MALDI-TOF MS was performed on a VG TofSpec E (Micromass, England) operated in the positive reflectron mode. The accelerating voltage was + 22.5 kV and the sampling frequency 500 MHz. The matrix chosen was DHB (Sigma-Aldrich) in acetone, 10 µg μl^{-1}. The samples were doped onto the DHB-film formed from 0.5 µl of matrix solution.

Separation and purification of SPG

The two SPG components were separated by TLC using 50:40:10 $CHCl_3$:CH_3OH:0.25% KCl in H_2O (by volume) and blotted to PVDF-membrane. The blotting-procedure developed by Taki *et al.* [17] was used. The membrane was cut in pieces containing the different products and extracted in 2 ml CH_3OH by ultrasonic treatment for 1 h. The samples were analyzed by FAB MS in the negative ion mode before degradation by the trifluoroacetolysis protocol.

Chromatogram binding assay

The binding by *H. pylori* of the total ganglioside fraction from leucocytes was examined on TLC-plates [18]. The leucocyte fraction was separated by using the solvent described above. *H. pylori* (NCTC 11637) was grown on agar plates, labeled with ^{35}S-methionine and used for binding as described [8, 19]. Anisaldehyde was used for chemical detection of the gangliosides [20].

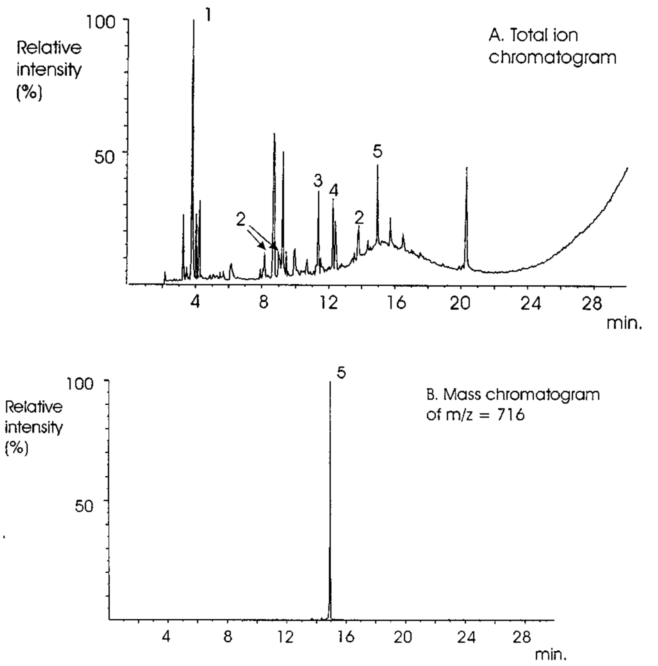


Figure 2. **A** Total ion chromatogram of the mixture after trifluoroacetolysis of permethylated and reduced 6-SL. The numbers indicate: (1) Di-trifluoroacetylated and permethylated monosaccharide residues that are formed as by-products in the trifluoroacetolysis; (2) Phthalate-containing contaminants; (3) Di- or tri-trifluoroacetylated saccharide of unsolved structure; (4) Mono-trifluoroacetylated disaccharide residues, here both TFAc6Galβ4Glcα and TFAc6Galβ4Glcβ; (5) The sialyl containing disaccharide (see Tables 1 and 2). The unmarked peaks are unidentified contaminants from the sample, the reagents or the reaction vial. **B** Mass chromatogram of the characteristic ion at m/z = 716 from the derivative of NeuAcα2-6GalβTFAc

Results

Model analysis

The total ion chromatogram (TIC) of the reaction mixture after trifluoroacetolysis contained different by-products, contaminants and unidentified components as shown in Figure 2A for 6-SL. The mass chromatograms of the characteristic ions were extracted from the complex TIC to detect the interesting ions, Figure 2B and Figure 3E–H. The characteristic ion of each product was the most intense ion in the higher mass range. The $[M\text{-}OMe]^+$ ion at m/z = 716 was due to an inductive cleavage of NeuAcα2-3GalβTFAc[‡] and NeuAcα2-6GalβTFAc and is used as characteristic ion (Figure 3A and B). The $[M\text{-}OMe]^+$ ion at m/z = 798 is used as characteristic ion for the NeuAcα2-3(TFAc-4)GalβTFAc[§]

[‡] Sequences are consistently written as β anomers since only one of two possible peaks was found.

[§] The sialic acid is consistently designated NeuAc (NeuGc) although the amide was reduced to amine.

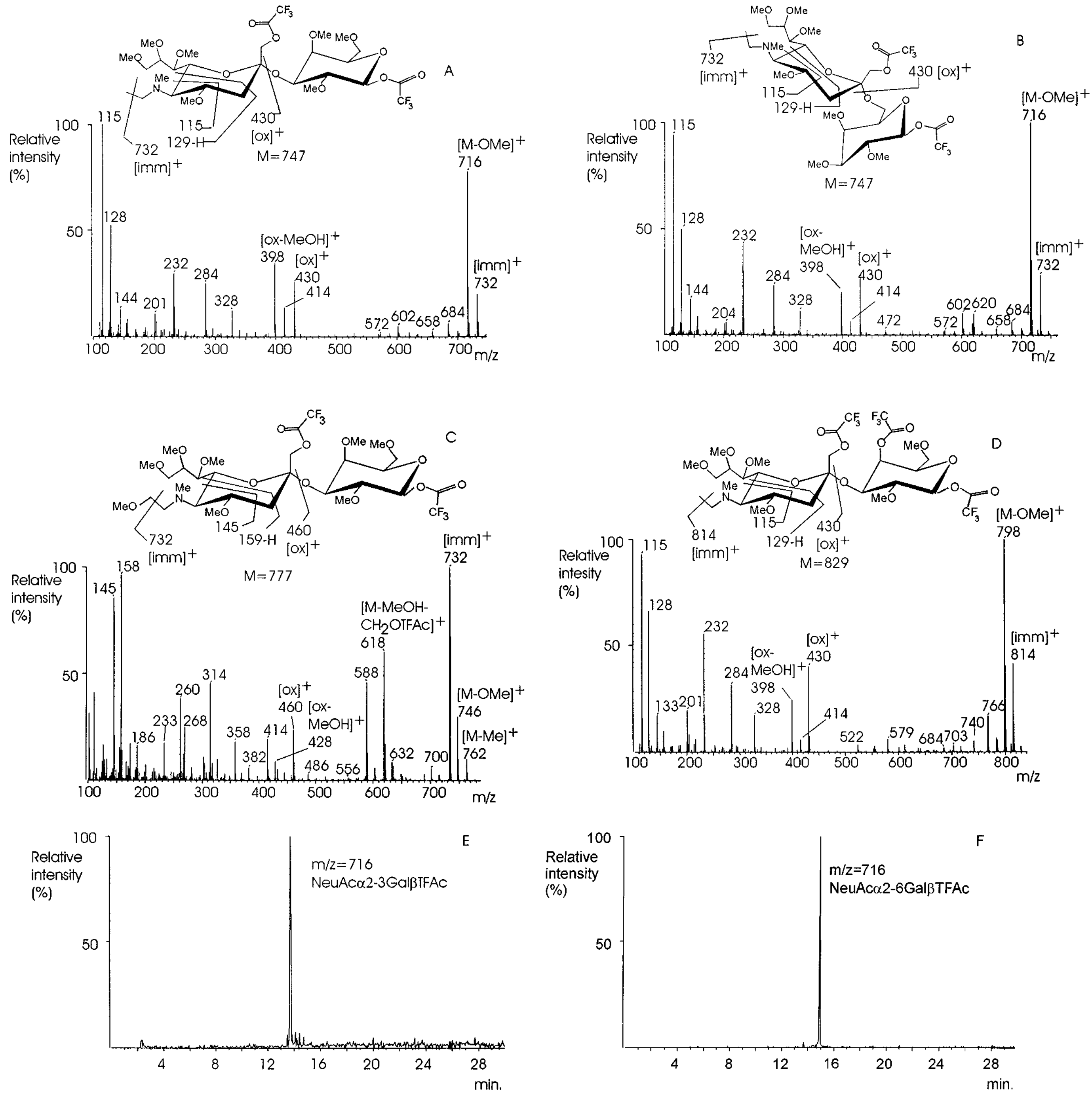


Figure 3. EI MS of the derivatized disaccharides. **A** NeuAcα2-3GalβTFAc; **B** NeuAcα2-6GalβTFAc; **C** NeuGcα2-3GalβTFAc; and **D** NeuAcα2-3(TFAc4)GalβTFAc. **E–H** are the corresponding mass chromatograms. Me = $-CH_3$, $[ox]^+$ = oxonium ion, and $[imm]^+$ = immonium ion.

(Figure 3D). The NeuGcα2-3GalβTFAc was cleaved into another pattern, producing the immonium ion $[M-CH_2OMe]^+$ at m/z = 732, that is used as characteristic ion (Figure 3C). The different products could be determined by their retention times, see Table 3.

Formation of different products of the reaction was monitored by MALDI-TOF MS. Figure 4A-D shows the resulting mass spectra for the trifluoroacetolysis of 6-SL at different times after the addition of TFA. Ions at m/z = 870, 892 and 908, corresponding to $[M + H]^+$, $[M + Na]^+$ and

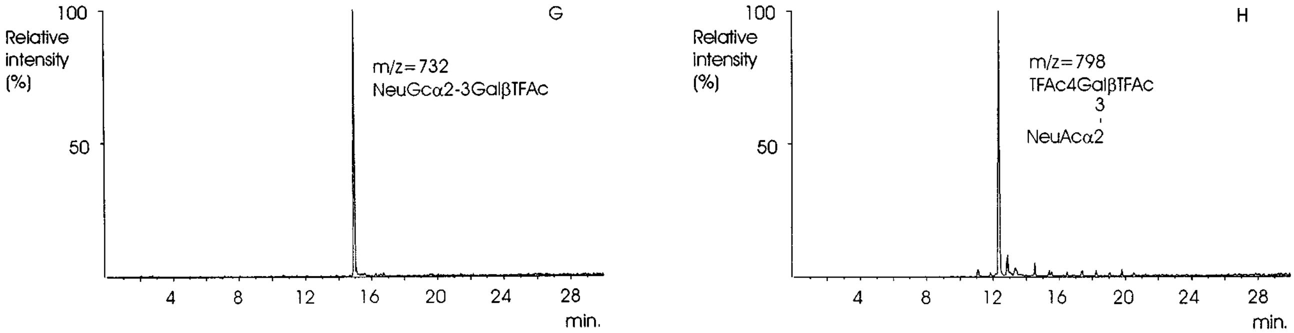


Figure 3. Continued

Table 3. Retention times and characteristic ions for the different detected products after permethylation, reduction and trifluoroacetolysis

Detected sequences[a]	*Retention time (min)*	*Characteristic ion (m/z)*
NeuAcα2-3GalβTFAc	13.90	716
NeuAcα2-6GalβTFAc	14.90	716
NeuGcα2-3GalβTFAc	15.10	732
NeuAcα2-3GalβTFAc 4 | TFAc	12.35	798

[a] Sialic acids are designated NeuAc or NeuGc although the amides were reduced to amines.

$[M + K]^+$, were abundant in the beginning of the reaction but as the reaction proceeded the peak intensity decreased while that of an ion at m/z = 748 increased. This ion is the H^+ adduct of the resulting disaccharide derivative of NeuAcα2-6GalβTFAc. Na^+ and K^+ adduct ions were not observed for disaccharides. The ion at m/z = 952 corresponds to the H^+ adduct after loss of a methoxy group. The unwanted by-product (NeuAcα2-6GalβTFAc) − MeO^- + $^-$OTFAc was formed, with a protonated ion at m/z = 830.

The trifluoroacetolysis method was successfully applied to different oligosaccharides and gangliosides (Table 1) containing the four different sialic acid sites shown in Table 3. Different temperatures, concentrations of TFA and reaction times were tested. The results from measuring the intensity of the ion at m/z = 716 from GC/MS and the $[M + H]^+$ ion at m/z = 748 from MALDI-TOF MS of 3-SL and 6-SL are given in Table 2.

Four samples with different concentrations of 6-SL were analyzed to evaluate the detection limit. First 400 µg as permethylated and reduced and 100 µg as taken and divided into 50, 25, 12.5 and 6.25 µg (65, 32.5, 16.25 and 8.1 nmol). The samples were trifluoroacetolyzed as described above, evaporated and dissolved in 10 µl of toluene before analysis by GC/MS. The mass chromatograms of the characteristic ion at m/z = 716 were diagnostic for all samples, but the mass spectrum of the 6.25 µg sample was too weak for a safe interpretation. Since the products can be determined by their retention time the detection limit was concluded to be in the low µg (nmole) range.

Helicobacter pylori and binding to leucocyte gangliosides

The mixture of leucocyte gangliosides as detected chemically on TLC is shown in Figure 5A, lane 1, and as bound by *H. pylori* and detected by autoradiography, Figure 5B. There is no binding to GM3 (Table 1), but binding from the 5-sugar region down to the origin of separation, with an increasing number of sugars and a successively stronger binding. The two bands prepared by membrane blotting are shown in Figure 5A, lanes 2 and 3, respectively. Only the first was bound by *H. pylori* as shown in the mixture (Figure 5B). The two bands were practically identical in their FAB MS spectra showing characteristic ions for SPG with a ceramide composed of sphingosine and 24:1 fatty acid ($[M - H]^-$ at m/z = 1626.9, and sequence ions at m/z = 1516.9, 1335.9, 1173.8, 970.8 808.7 and 646.6). Analysis by GC/MS using the new method showed the upper band (lane 2) to contain NeuAcα2-3 and the lower band (lane 3) NeuAcα2-6. Combined with earlier reported results [21] the two sequences therefore are: NeuAcα2-3Galβ4GlcNAcβ3Galβ4GlcβCer (3-SPG) and NeuAcα2-6Galβ 4GlcNAcβ3Galβ4GlcβCer (6-SPG). The double-band appearance of the two species was due to heterogeneity of the fatty acid (see indications at the left of Figure 5). Apparently, both bands of 3-SPG are positive and both bands of 6-SPG are negative.

Therefore, *H. pylori* is able to recognize 3-SPG but not 6-SPG and this is independent of fatty acid composition.

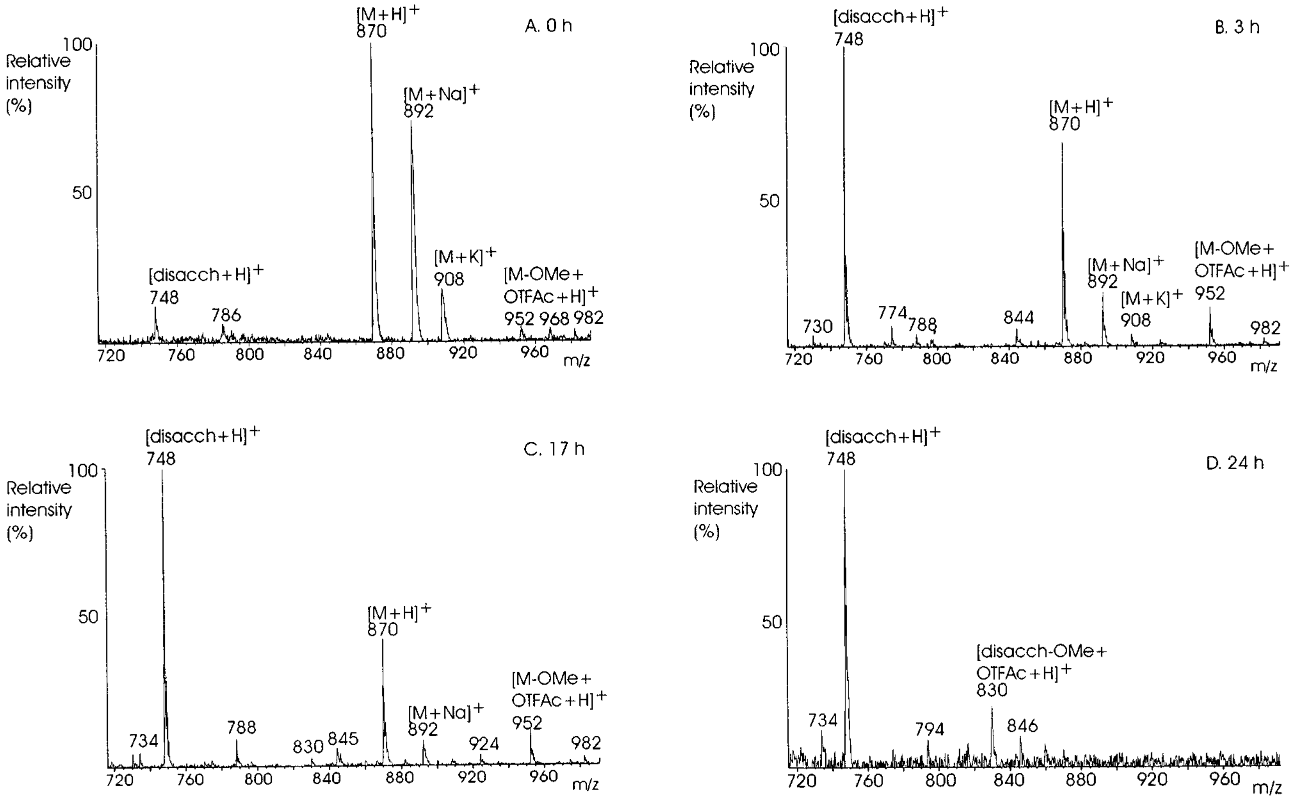
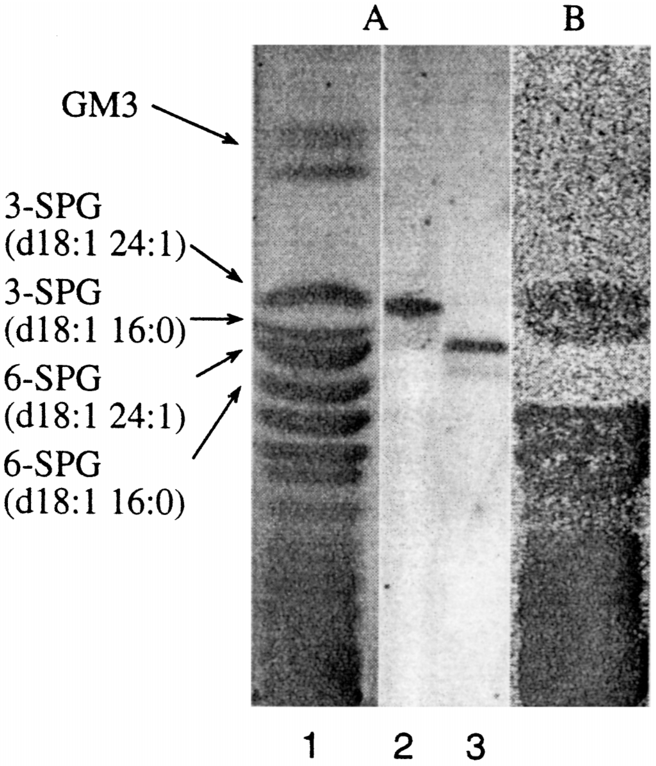


Figure 4. MALDI-TOF mass spectra of the reaction mixture during trifluoroacetolysis of 6-SL. Spectrum **A** is from a sample taken from the reaction mixture just before the addition of TFA. Spectra **B**, **C** and **D** are from samples taken from the reaction mixture 3, 17 and 24 h, respectively, after the addition of TFA. M in these spectra is the mass of the permethylated, reduced and trifluoroacetylated 6-SL before cleavage to the derivative of NeuAcα2-6GalβTFAc.



Discussion

The aim of this study was to develop a chemical method for the investigation of the sialic acid binding position to the consecutive saccharide residue in complex glycoconjugates.

The procedure involves permethylation, reduction and trifluoroacetolysis, so far tested on gangliosides and oligosaccharides. The permethylation and reduction result in a primary alcohol at C-1 of sialic acid. The alcohol is trifluoroacetylated, which stabilizes the ketosidic linkage between the sialic acid and the consecutive hexose residue during the following trifluoroacetolysis of the glycosidic linkages. The trifluoroacetyl group attracts the activating lone pair electrons from the ketosidic oxygen

Figure 5. **A** TLC of (1) the fraction of gangliosides from leucocytes; (2) the 3-SPG fraction; and (3) the 6-SPG fraction; which where subjected to the trifluoroacetolysis method. These lanes were developed chemically with anisaldehyde. **B** Autoradiogram after binding of *H. pylori* to the same fraction as in lane 1. As indicated in lane 1, 3-SPG and 6-SPG in fact are double-bands. The upper of each double-band as isolated for analysis (lanes 2 and 3) and shown to contain 24:1 fatty acid. The lower bands contained 16:0 fatty acid.

and makes this less nucleophilic, which explains the stabilization.

The reaction conditions gathered in Table 2 were based on the conditions reported by Gunnarsson [12] for the release of oligosaccharides from ceramide of glycolipids. In this method the underivatized glycolipid is treated with 1 : 1 to 50 : 1 TFAA : TFA at 100 °C. The glycolipid is trifluoroacetylated at all positions, causing a stabilization of all glycosidic bonds against trifluoroacetolysis. The less stabilized bond between the ceramide and the first saccharide residue is however cleaved. Permethylation and reduction prevent trifluoroacetylation of all positions, except the sialic acid C-1 position, and the glycosidic bonds are therefore not stabilized against trifluoroacetolysis. Less TFA and lower temperatures were expected to be the appropriate reaction conditions. Thus, the reaction temperature at 100 °C combined with 1000 : 1 TFAA : TFA was observed to be the best reaction conditions for a 24 h reaction time.

The method is useful for gangliosides and oligosaccharides, both in the case of NeuAc and NeuGc. For sialic acid bound to *N*-acetylhexosamine no disaccharides have been observed, suggesting that stabilisation of the ketosidic bond does not occur in this case. When acidic hydrolysis (HCl) of permethylated gangliosides and oligosaccharides is performed, complete hydrolysis of the glycosidic linkages is usually observed. Acidic hydrolysis of permethylated and reduced gangliosides and oligosaccharides gives complete hydrolysis between hexoses, but the glycosidic bond between an *N*-ethylhexosamine and the consecutive hexose residue is stable against hydrolysis [22, 23]. The same result was expected during trifluoroacetolysis. However, no such stabilization has been observed, neither in DSLNT (Table 1) where NeuAcα2-6(TFAc3)GlcNAcβ3GalβTFAc could be formed, nor in the gangliosides, GM1 and GD1a, where NeuAcα2-3(TFAc3GalNAcβ4)GalβTFAc were expected products. Other conditions for trifluoroacetolysis of the sialic acid bound to a hexosamine were tested, such as (a) lower reaction temperatures, (b) addition of different Lewis acids (BF_3 and BCl_3) and (c) different concentrations of TFA, but without success.

If a sample is left in too strong TFAA : TFA solution or at too high temperature the wanted reaction products will continue to decompose.

The products are unstable in water and can not be stored, which is why the analysis has to be performed within one hour after the trifluoroacetolysis. To prevent detrifluoroacetylation of the products during evaporation and concentration, dry toluene is added to form a top layer over the reaction solution. The toluene will also prevent concentrated TFA from being left in the sample tube during the evaporation.

Unfortunately, there is a series of by-products or contaminants from the procedure (see Figure 2A). This is the case regardless of source of sample or careful handling. However, the mass chromatograms obtained appear conclusive for linkage position and type of sialic acid.

Sialic acid in position 3 or 6 of Gal, is readily deduced. This is of importance in case of binding by *H. pylori* to leucocyte SPG (epitope 1), since the 3-isomer, and not the 6-isomer, was found to be recognized by the bacterium on TLC plates. In separate communications we will document the use of the present method in the detailed characterization of various sialic acid-containing glycoconjugates being recognized by the bacterium. One particular challenge is epitope 2, apparently restricted to human polyglycosylceramides, where sialic acid is essential but where the pronounced microheterogeneity is prohibiting rapid progress [8, 9].

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