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Binding of *N*-trifluoroacetyl-derivatized natural glycosphingolipids by uropathogenic *Escherichia coli* and *Neisseria subflava*

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Several neutral glycosphingolipids were hydrogenated and subjected to trifluoroacetylation in trifluoroacetic acid/trifluoroacetic acid anhydride under conditions leading to complete exchange of the *N*-acetyl groups of GalNAc for *N*-trifluoroacetyl. The derivatized glycosphingolipids were analyzed for binding by P-fimbriated uropathogenic *Escherichia coli,* recognizing the globo series of glycolipids (carrying Galα1-4Gal). Using *E. coli* it was shown that a GalNCO-CF_3 next to the minimum binding epitope Galα1-4Gal did not substantially influence the binding, as did not a trifluoro acetyl group on the ceramide. Exchange of *N*-acetyl of GalNAc in the receptor active gangliotetraosylceramide, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer, for *N*-trifluoroacetyl, did not change the binding of two out of the three strains tested of the bacterium *Neisseria subflava.* Discussion concerning the binding epitopes of the bacterial adhesins to carbohydrates is based on these results.

Keywords: **chemical modification, glycolipids, epitope dissection, P-fimbriated, Pap G**

Introduction

Binding studies with chemically derivatized glycolipids have been shown to give valuable information at the molecular level on the interaction between glycolipids and bacteria [1, 2], lectins [3, 4], or influenza virus [5–7]. We have used *N*-trifluoroacetylation to introduce fluorine at the ceramide head and the GalNAc residue and studied the influence on the binding of uropathogenic *Escherichia coli* and *Neisseria subflava* to glycosphingolipids derivatized in this manner.

The trifluoromethyl group was chosen because it is of approximately the same size as -CH_3, but in contrast to the methyl group, is strongly electronegative and a potential hydrogen bond acceptor. The fluoro group has also successfully been used in studies of carbohydrate-protein interactions [8-10]. Trifluoroacetolysis of glycosphingolipids [11, 12] leads to acid-catalyzed cleavage of the carbohydrate from the ceramide moiety when the glycolipid contains sphingosine, although carbohydrates linked to dihydrosphingosine or phytosphingosine are not released. A hydrophobic anchoring to thin-layer plates or microtiter wells is needed for biological assaying [13], and, therefore, saturation of sphingosine double bonds must be performed prior to trifluoroacetylation.

P-fimbriated *E. coli,* causing urinary tract infection, bind with high specificity to the globo series of glycosphingolipids, having in common the disaccharide Galα1-4Gal either in terminal and nonterminal positions [14-17]. Variant, genetically cloned adhesins of *E. coli* show preference for different members of the globoseries; class I for Gb_3Cer (glycolipids used, see Table 1), class II for Gb_4Cer, and class III for the Forssman glycolipid and Globo A (i.e., Gb_4 extended by a blood group A determinant) [15, 17–19].

A number of pathogenic and nonpathogenic *Neisseria* species have been shown previously to recognize and bind to LacCer [20]. These bacteria, as well as several other bacterial species, adhere to mucosa cell surfaces and the recognition of LacCer is dependent on the type of ceramide [21, 22]. For binding to occur to this two-sugar glycolipid, the structure of the ceramide must be phytosphingosine (carries one more OH-group than sphingosine) and/or contain 2-hydroxylated fatty acid as is the case for most glycolipids in epithelial cell membranes [23, 24]. No binding is observed to nonepithelial type LacCer, which

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Table 1. Structure of glycolipids and ceramides used.

Abbreviation	*Name*	*Structure*
LacCer	lactosylceramide	Galβ1-4Glcβ1-1Cer
Hex_2Cer	dihexosylceramide	Hex is either Gal or Glc
Gg_3Cer	gangliotriaosylceramide	GalNAcβ1-4Galβ1-4Glcβ1-1Cer
Gg_4Cer	gangliotetraosylceramide	Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer
Gb_3Cer	globotriaosylceramide	Galα1-4Galβ1-4Glcβ1-1Cer
Gb_4Cer	globoside	GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer
	globotetraosylceramide	
	Forssman glycolipid	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer
GM1		Galβ1-3GalNAcβ1-4(Neu5Acα2-3)Galβ1-4Glcβ1-1Cer
Cer OH		Cer with either phytosphingosine, or a hydroxylated fatty acid, or both
d18:1-24:0		ceramide with sphingosine and a saturated fatty-acid chain
t18:0-h24:0		ceramide with phytosphingosine and a saturated, hydroxylated fatty-acid chain
GalNTFA		*N*-trifluoroacetyl derivative of GalNAc
HexNTFA		*N*-trifluoroacetyl derivative of HexNAc

is composed of sphingosine and nonhydroxylated fatty acids. Other glycolipids, Gg_3Cer and Gg_4Cer, are also bound strongly by the bacteria but not in a ceramide dependent manner [20].

In the present articles we report conditions for derivatization by treatment with TFA/TFAA of two members of the globo series of glycosphingolipids, nonepithelial Gb_3Cer and Gb_4Cer, and to both epithelial and nonepithelial LacCer and Gg_4Cer. The products were characterized structurally and analyzed for binding by different clones of uropathogenic, P-fimbriated, *E. coli* or by commensal strains of *N. subflava.*

Materials and methods

Isolation of glycolipids

Preparation of glycosphingolipids was done as previously described [25]. Final purification of glycolipids were obtained by HPLC (Beckman Instruments, Inc., USA) on straight-phase silica gel. The columns used were either 22 mm i.d. × 300 mm, with a particle size of 10 μm and 120 Å pore diameter (Yamamura Chemical Lab Co. Ltd., Japan) or 50 mm i.d. × 500 mm, with a particle size of 25-40 μm and 120 Å pore diameter (only for globoside). Globoside was chromatographed also in its acetylated form to achieve good separation from paragloboside (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer), using an eluting gradient starting with chloroform and ending with chloroform/methanol 9:1 (all mixtures expressed as vol/vol unless stated otherwise). For Gb_3Cer and LacCer, as well as the *N*-TFA derivatives, the eluting gradient started with chloroform/methanol/water 65:25:4 and ended with 60:35:8. Eluted glycolipids were monitored by TLC.

The ceramide compositions of the glycolipids were determined by negative-ion fast-atom bombardment-mass spectrometry (FAB-MS) and in some cases by positive-ion electron impact (EI)-MS after methylation [26]. The source for Gb_3Cer, Gb_4Cer, and nonepithelial LacCer was human erythrocytes. All three contained almost exclusively sphingosine (d18:1). In LacCer 16:0, 22:0, and 24:0 fatty acids dominated. In Gb_3Cer 24:0 fatty acid dominated over 22:0, and in Gb_4Cer the fatty acid composition was 80% 24:0, 16% 22:0, and 4% 16:0. Epithelial-type Gg_4Cer and Hex_2Cer were prepared from feces of germ-free mice in which epithelial-type glycolipids are accumulated [27]. These glycolipids had sphingosine and hydroxylated fatty acid (d18:1-h16:0) as the dominating ceramide structure, but also phytosphingosine in combination with hydroxylated fatty acids was present (t18:0-h22:0 and t18:0-h24:0). Nonepithelial Gg_4Cer with d18:1 and d20:1 combined with 16:0, 18:0, and 20:0 was prepared by desialylation of GM1 isolated from calf brain (10 mg GM1, 10 ml 0.05 M HCl, 80°C, 2 h). Epithelial LacCer OH, used in the binding studies, was prepared from dog small intestine [28] and carried mostly t18:0 with hydroxylated fatty acids of different chain lengths.

Hydrogenation

Glycolipids (10–15 mg) were dissolved in 2-4 mL of tetrahydrofuran/water 30:12 and platinum (IV) oxide (PtO_2xH_2O, Merck, Germany) (1-2 mg) was added. The mixture was stirred under atmospheric hydrogen for 3 h at room temperature. The platinum oxide was removed by

filtration and the solution dried with a stream of N_2(g) at room temperature. The saturation of double bonds was confirmed by EI-MS of the permethylated products.

Treatment with TFA/TFAA

Saturated glycolipids (8–10 mg) were dissolved in 10 mL of TFA/TFAA (Merck, Germany), 1:100, in a sealed, thick-walled glass tube [11, 29] and heated in an oil bath at 100°C for 72 h (caution: highly corrosive mixture under pressure). The dark solutions were cooled to room temperature and dried with N_2(g). *O*-TFA groups were removed by treatment in methanol (2 mL) containing 1 drop of aqueous ammonia (25%). After 30 min, the methanol was evaporated off. The product mixtures were characterized by TLC and TLC/FAB-MS (see below). Products of Gb_4Cer, Gb_3Cer, Gg_4Cer, and LacCer, respectively, were separated by HPLC, and isolated fractions were analyzed by FAB-MS and EI-MS after permethylation.

Cleavage of N-TFA groups

N-Trifluoroacetylated globoside products were dissolved in 1.5 M ammonia in methanol/water 4:1 and left at room temperature for 48 h. No starting material could be detected by TLC analysis or TLC/FAB-MS.

Thin-layer chromatography

Isolated compounds and reaction mixtures (2-10 μg) were analyzed with TLC, on glass-backed silica gel 60 HPTLC nano plates of 0.2 mm layer thickness (Merck). Chemical detection was done by staining with anisaldehyde reagent [30]. Plates used for TLC/FAB-MS and bacterial overlay were aluminum-backed (Merck).

Mass spectrometry

Mass spectra were obtained with either a ZAB-2F/HF (VG Analytical, UK) or a Jeol SX 102A (JEOL, Japan) sector instrument. Negative-ion FAB MS was done with Xe-atom bombardment (6 keV), an accelerating voltage of 10 kV, and a linear magnetic scan. The matrix was triethanolamine (Fluka AG, Switzerland). For liberated monosaccharides, a Trio-II (VG Masslab, UK) quadropole instrument was used. For TLC/FAB-MS [31], the plates with separated glycolipids were cut into 6 mm wide strips and mounted on a movable FAB probe (VG Analytical). A thin layer of matrix was applied and 4 scans were collected per mm of plate. Mass determinations with the TLC/FAB-MS method may deviate one or two units from the calculated values, due to difficulties in obtaining exact mass calibration. Positive-ion EI-MS were obtained with the following instrument settings; 10 kV accelerating voltage, 70 eV electron energy, and 300 μA trap current. Spectra were acquired by a linear magnetic scan from *m/z* 100 to 2400 with a cycle time of 26 s and the temperature of the ion source was 370°C.

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian VXR 300 spectrometer (Varian, USA) operating at 7.05 T. Proton spectra were recorded with 0.4 Hz digital resolution using tetramethylsilane as shift reference. For analysis, approximately 1 mg of glycolipid was dissolved in 0.5 mL of dimethylsulfoxide-d_6D_2O 98:2, and samples were run at 30°C.

Growth and labeling of bacteria

Four different recombinant P-fimbriated *E. coli* strains were used, HB101/pPIL2GI-15 (a gift from Dr. I. van Die, Vrije Universitet, Amsterdam, Holland and Dr. B. Westerlund, University of Helsinki, Finland) [32], HB101/pPAP5 [33], HB101/pDC1[34], and HB101/pPAP601 (a gift from Dr. B. -I. Marklund, University of Umeå, Sweden, see [35]). The bacteria were cultivated in Luria broth supplemented with ampicillin (100 μg/mL) at 37°C overnight. The bacteria were collected by centrifugation, washed twice with phosphate-buffered saline (PBS, 0.2 M Na/KPO_4, pH 7.2, 0.14 M Na/KCl), and resuspended in PBS to approximately 1×10^9 colony-forming units/mL. ^{35}S-Labeled bacteria were obtained by addition of ^{35}S-methionine (Amersham Int., UK) to the culture medium (400 μCi/10 mL). The labeled bacteria were diluted to an activity of approximately 1×10^6 cpm/mL.

Three different strains of *N. subflava* 19243, 11076 (American Type Culture Collection, USA) and GN01 (Pharmacia, Sweden) were used. The bacteria were cultivated overnight at 37°C on brain heart infusion agar (Merck) supplemented with horse blood serum and labeled metabolically with ^{35}S-methionine.

Preparation of erythrocytes

Freshly collected human erythrocytes, blood group A, were washed three times with 0.14 M NaCl and suspended to 20% in PBS.

Chromatogram binding assay

Glycolipids (1–4 μg) were separated by TLC using chloroform/methanol/water 60:35:8. The TLC plate intended for bacterial binding was dipped in 0.5% (w/v) polyisobutylmethacrylate (Plexigum P28, Röhm GmbH, Germany) in diethylether for 1 min. After drying, the plate was soaked in PBS with 2% bovin serum albumin (BSA) and 0.1% NaN_3 in PBS for 2 h. The plate was then covered with a suspension of bacteria and incubated for 2 h at room temperature. After washing with PBS several times, the plates treated with *E. coli* were covered with erythrocyte suspension, incubated for 1 h, followed by careful washing with PBS [36]. For detection of *N. subflava* binding, however, the plates were subjected to autoradiography at room tem-

perature using Kodak XAR-5 film (Eastman Kodak, US). The films were developed after 16–72 h.

Microtiter well assay

The assay was performed as described earlier [36]. Briefly, serial dilutions of purified glycolipids in methanol were added to microtiter wells (Cooks M24, Nutacon, Holland), each dilution in triplicate, and dried at room temperature overnight. After coating with 2% BSA in PBS for 2 h, 50 µL of ^{35}S-labeled bacteria (2×10^3 cpmµL) were added per well. The incubation for 4 h at room temperature was followed by washing six times with PBS. After drying, the wells were cut out and the radioactivity measured.

Results

Derivatization reactions

The glycolipids were hydrogenated and subjected to trifluoroacetolysis with conditions of varying TFA/TFAA from 1:1 to 1:100 at 100°C (Scheme 1). After removal of the *O*-TFA groups in methanol/ammonia, the reaction mixture was analyzed by TLC, using detection with the anisaldehyde reagent. With TFA/TFAA 1:100, the reaction was complete after 72 h. Three bands appeared on TLC as the main products (exemplified with Gb_4Cer; Figure 1 left, lane 3, bands I, II, and III).

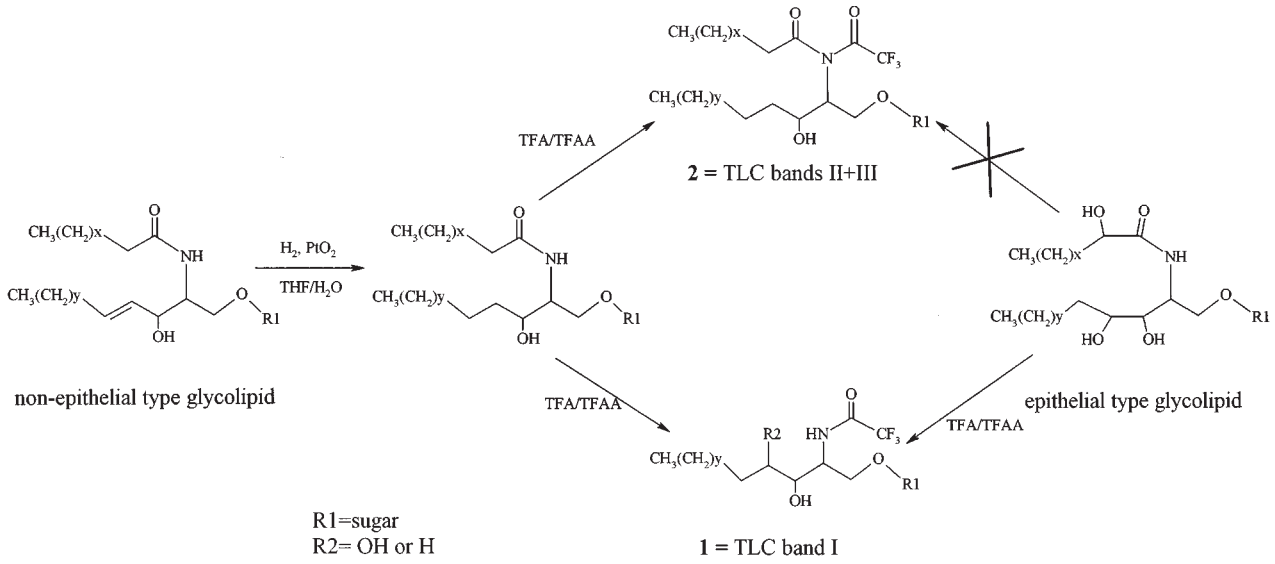
Chemical analysis of reaction products

TLC/FAB-MS analysis of the mixture of reaction products was performed, and structure 1 in Scheme 1 was suggested for the TLC band I in Figure 1, lane 3, and structure 2 for bands II and III based on the following results given in detail for Gb_4Cer. A prominent peak in the high-mass region of the negative-ion FAB spectrum from TLC/FAB-MS of band I appeared at *m/z* 1138 (Figure 2, scan 110). This is close to the theoretical $[M\text{-}H]^- = 1140$ for the expected product of globoside with substitution by *N*-trifluoroacetyl groups for the *N*-acetyl of GalNAc and the *N*-fatty-acyl chain, structure 1, Scheme 1. Abundant ions were also recorded at *m/z* 881, 719, 558, and 396, which all correspond to fragmentations at the glycosidic bonds (see top formula in Figure 2). Loss of the terminal HexNTFA is expected to give $[M\text{-}H\text{-}257]^-$ while loss of the hexoses will result in peaks 162 amu apart. The identity of the reaction product was further confirmed by EI-MS of HPLC-purified and permethylated band I (Figure 3). Peaks at *m/z* 314, 282, and 518, corresponding to HexNTFA and HexNTFA-Hex, were obtained. No peaks at *m/z* 260 or 464, corresponding to the underivatized *N*-acetyl analogues, were observed.

The TLC bands II and III (Figure 1, lane 3) gave identical mass spectra, when analyzed by TLC/FAB-MS (Figure 2, scans 95 and 75). The prominent peaks obtained at *m/z* 1487 and 1459 correspond to $[M\text{-}H]^-$ for structure 2 which differs from 1 in that it has the fatty-acyl moiety retained. This compound would have $[M\text{-}H]^- = 1490$, if the chain length of the acid was 24:0, and 1462 if it was 22:0. The relative intensities of *m/z* 1487 and 1459 were in agreement with the fatty acid composition determined for the underivatized glycolipid (see Materials and methods). The fragment at *m/z* 746 corresponds to ceramide *N*-substituted with TFA, and the fragments at 907, 1069, and 1231 to this ceramide linked to one, two, and three hexoses, respectively (see formula).

After HPLC fractionation and methylation, the mixture of bands II and III gave a EI-MS that supported structure 2. As for band I, no peaks were detected at *m/z* 260 or 464 (not shown).

Scheme 1



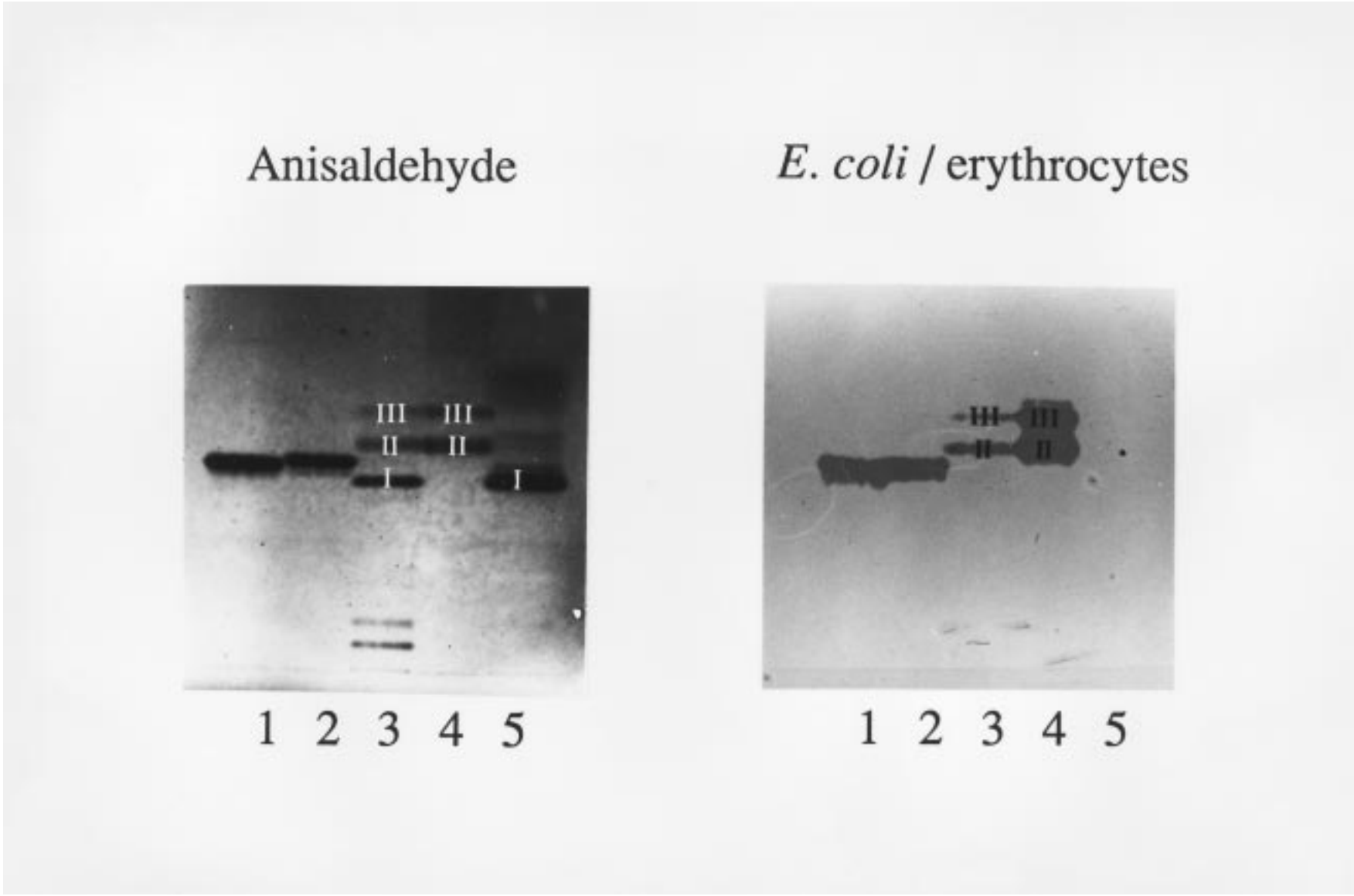


Figure 1. Thin-layer chromatograms after detection with anisaldehyde (left) and after binding of recombinant P-fimbriated *E. coli* pPIL2GI-15 (right). The binding assay was performed as described under Materials and methods, and bound bacteria were detected with human erythrocytes. The lanes on the chromatograms were 1, Gb_4Cer; 2, hydrogenated Gb_4Cer; 3, mixture of products obtained by TFA /TFAA treatment of Gb_4Cer; 4, purified structure 2; and 5, purified structure 1. On the left chromatogram 3 nmol was applied per lane, and on the right 0.7 nmol. TLC was developed in chloroform/met hanol/water 60:35:8.

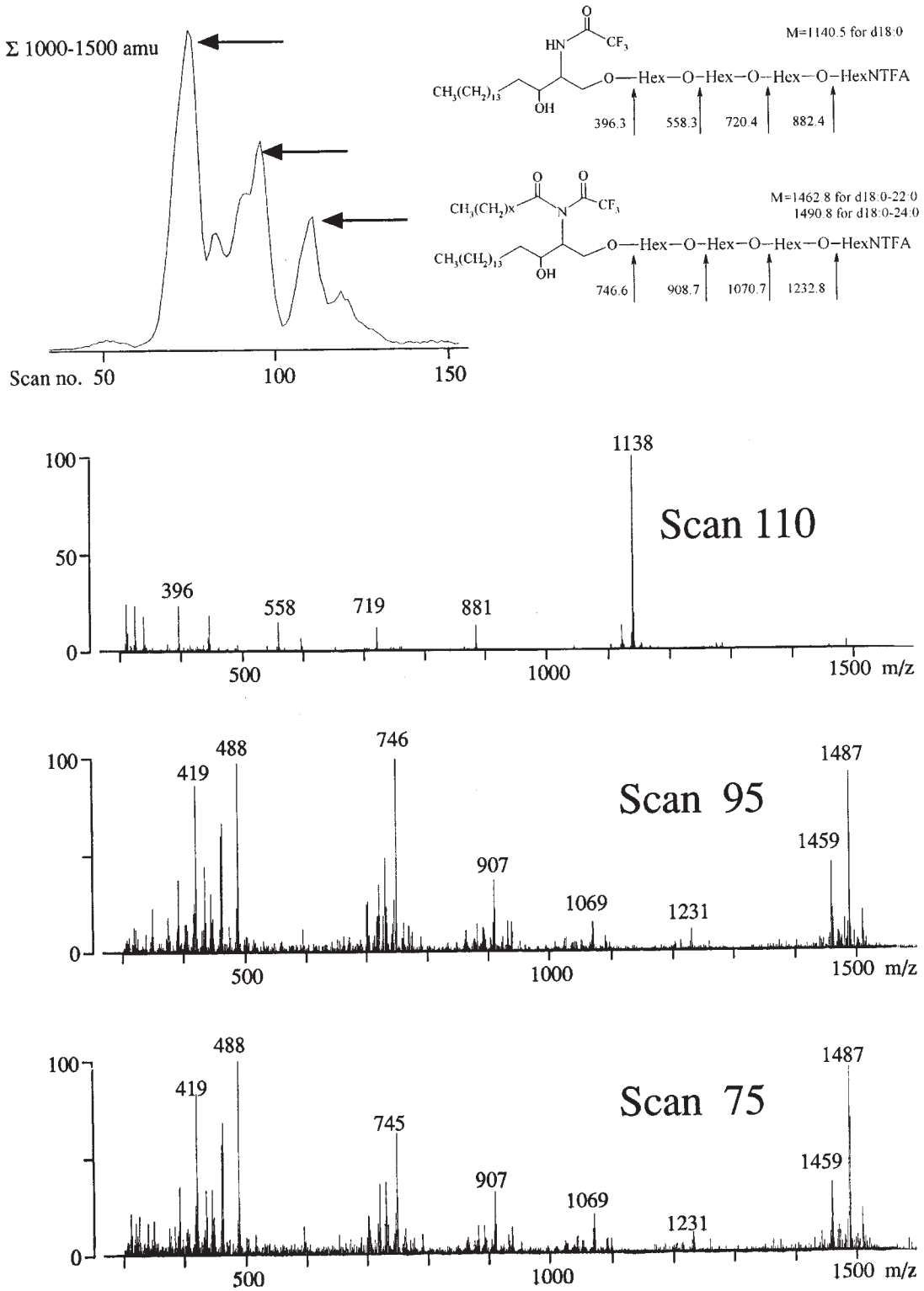


Figure 2. TLC/FAB-MS of the product mixture from globoside treated with TFA/TFAA. Summed ion current, 1000-1500 amu, is shown on the top left, and on the top right, the proposed MS fragmentation (arrows). Below are the mass spectra of scans 110, 95, and 75. These correspond to bands I, II, and III, respectively, of Figure 1 left, lane 3.

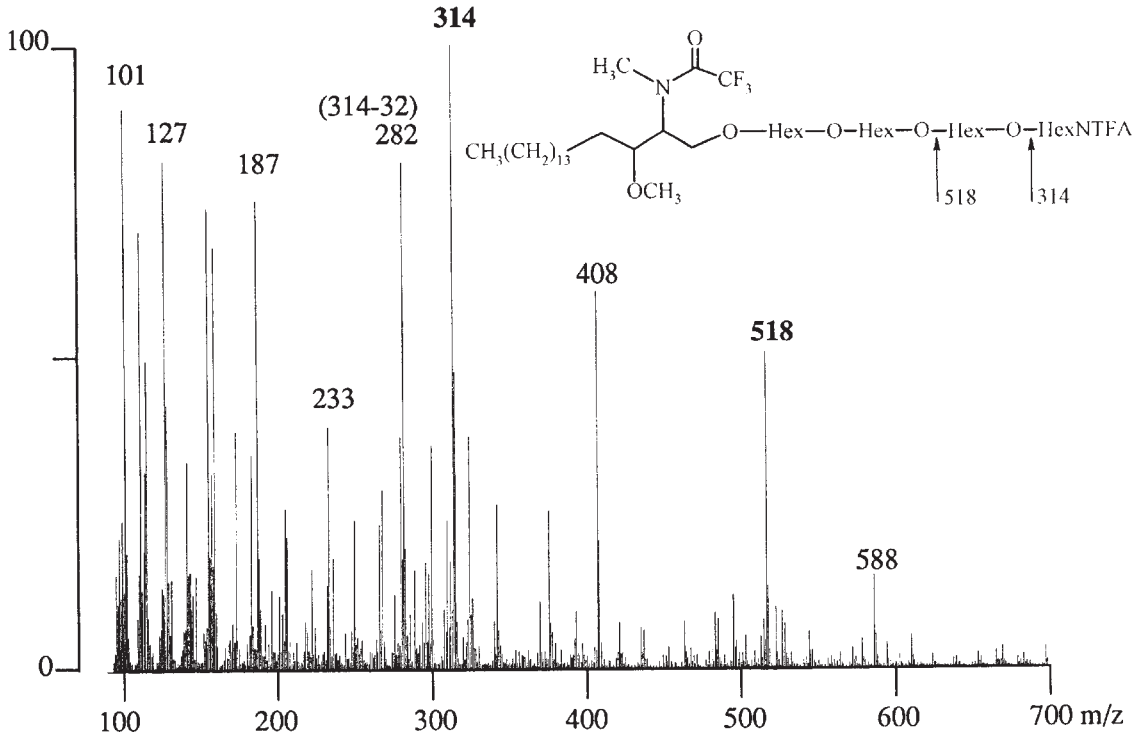


Figure 3. EI-MS of permethylated structure 1 of Gb_4Cer in the interval 100-700 amu. This part of the spectrum was identical to the one obtained for product 2 of Gb_4Cer.

Even though bands II and III (Figure 1, lanes 3 and 4) resolve well on TLC, we were unable to separate them by straight-phase HPLC although different eluents were tested. When TFA products of Gb_4Cer were analyzed by two-dimensional TLC, bands II and III gave rise to four spots of about equal intensity (Figure 4, bottom). The same eluent was used in both dimensions, and between the two separations, the plate was dried at 60°C for 3 h and then left overnight at room temperature. However, when the same analysis was repeated with a quick drying of the TLC plate, 15 min in vacuum, the two spots originating from bands II and III did not divide into four spots (Figure 4, top), suggesting that the separation of structure 2 into two bands in the former case was due to a slow conformational equilibrium. In both analyses, the product with structure 1 migrated as a single spot.

As further support for structures 1 and 2, the isolated TFA products of Gb_4Cer were treated with ammonia. This led to the appearance of TLC bands that migrated much slower than the starting material (chloroform/methanol/water 60:35:8). FAB-MS (not shown) of ammonolyzed 1 gave $[M\text{-}H]^-=1043$, which is 96 units lower than before ammonolysis, indicating that one TFA group had been released. Since the *m/z* 396 peak was present (cf., Figure 2) the TFA group of Cer was retained, while the TFA group of GalNTFA was released, giving a free amino group that would be consistent with a slower migration on TLC. Exact mass measurement with polyethyleneglycol as internal standard gave *m/z*=1043.4, which coincides with the theoretical value. The peaks obtained at 882.4 and 720.4 also agreed with the expected values (*cf.,* Figure 2). MS analysis of ammonolyzed 2 gave $[M\text{-}H]^-=1394$, as well as a large peak at 746, which again indicates that only the TFA group of the GalNTFA was released.

Trifluoroacetylation was also performed with Gb_3Cer and LacCer, both with nonhydroxylated Cer. Analogous to the reaction of Gb_4Cer, three main products were formed that separated on TLC (Figure 5 left, lanes 2 and 4, respectively). FAB-MS (not shown) of the bands marked with I had their fatty-acyl groups exchanged for a TFA group whereas the bands marked with II and III had acquired a TFA group at the Cer. For LacCer and Gb_3Cer, the expected $[M\text{-}H]^-$ of band I coincided with peaks seen at 720 and 882 amu, respectively. Fragmentations at glycosidic bonds analogous to those of globoside were also obtained. The bands marked II and III showed identical FAB-MS. The M region of bands II and III of LacCer contained three peaks of about equal size, corresponding to fatty acids with 16, 22, and 24 carbons, at *m/z* 958, 1042, and 1070, respectively. In the M region of bands II and III of Gb_3Cer *m/z*, 1232 dominated, due to the major 24-carbon fatty acid.

Proton-NMR analyses of the TFA products of Gb_3Cer and Gb_4Cer were performed to measure the relative integrals of the methyl and methylene proton signals. For the

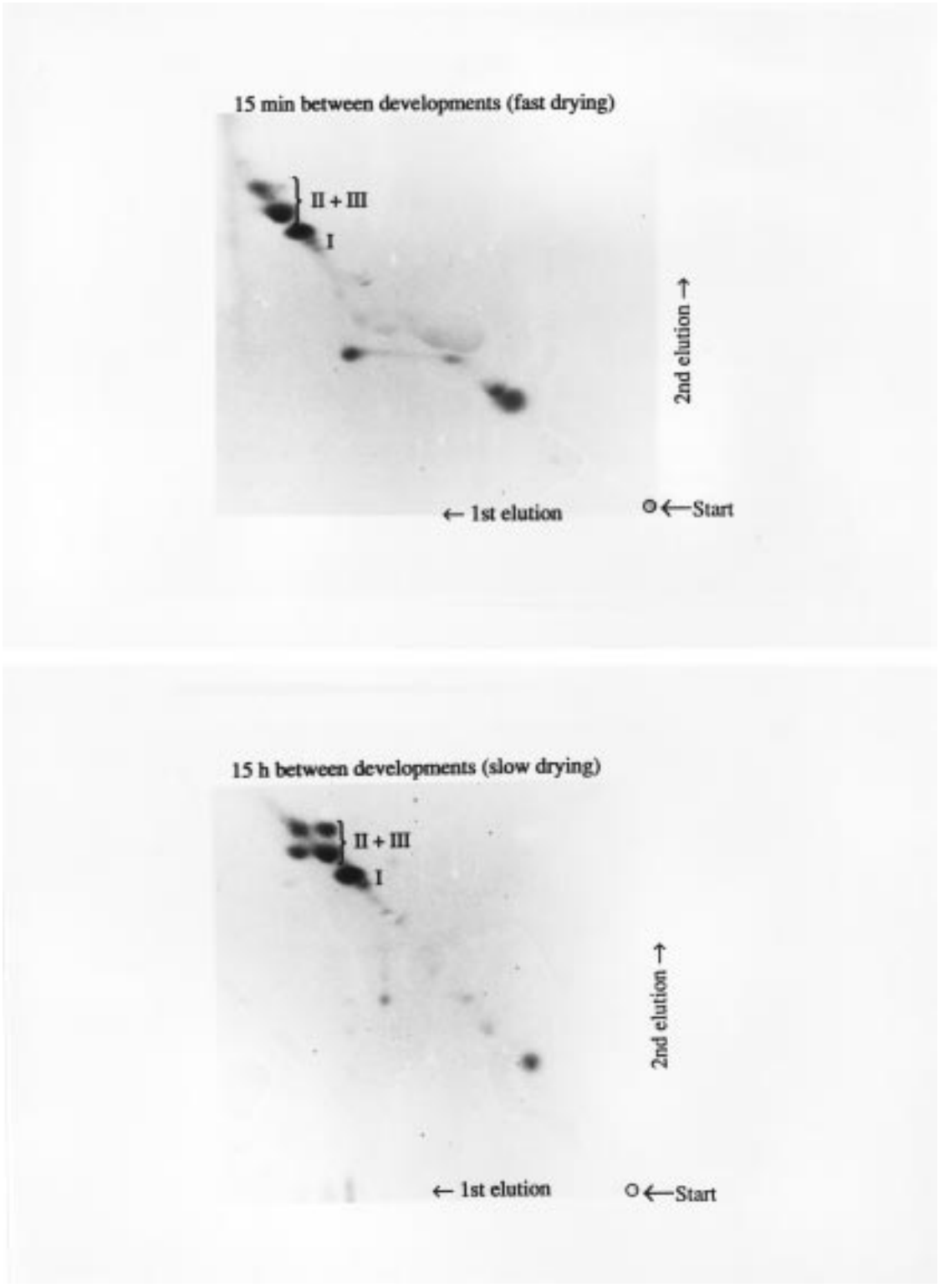


Figure 4. Two-dimensional TLC analysis of Gb_4Cer after treatment with TFA/TFAA. Both developments were done with chloroform/methanol/water 10:10:3, and visualization was done with anisaldehyde. On the top chromatogram, drying between the developments was obtained at 60°C (3 h), and then the plate was left at room temperature and athmosphere for 12 h, and in the bottom chromatogram, the plate was dried by keeping the plate for 15 min in a desiccator.

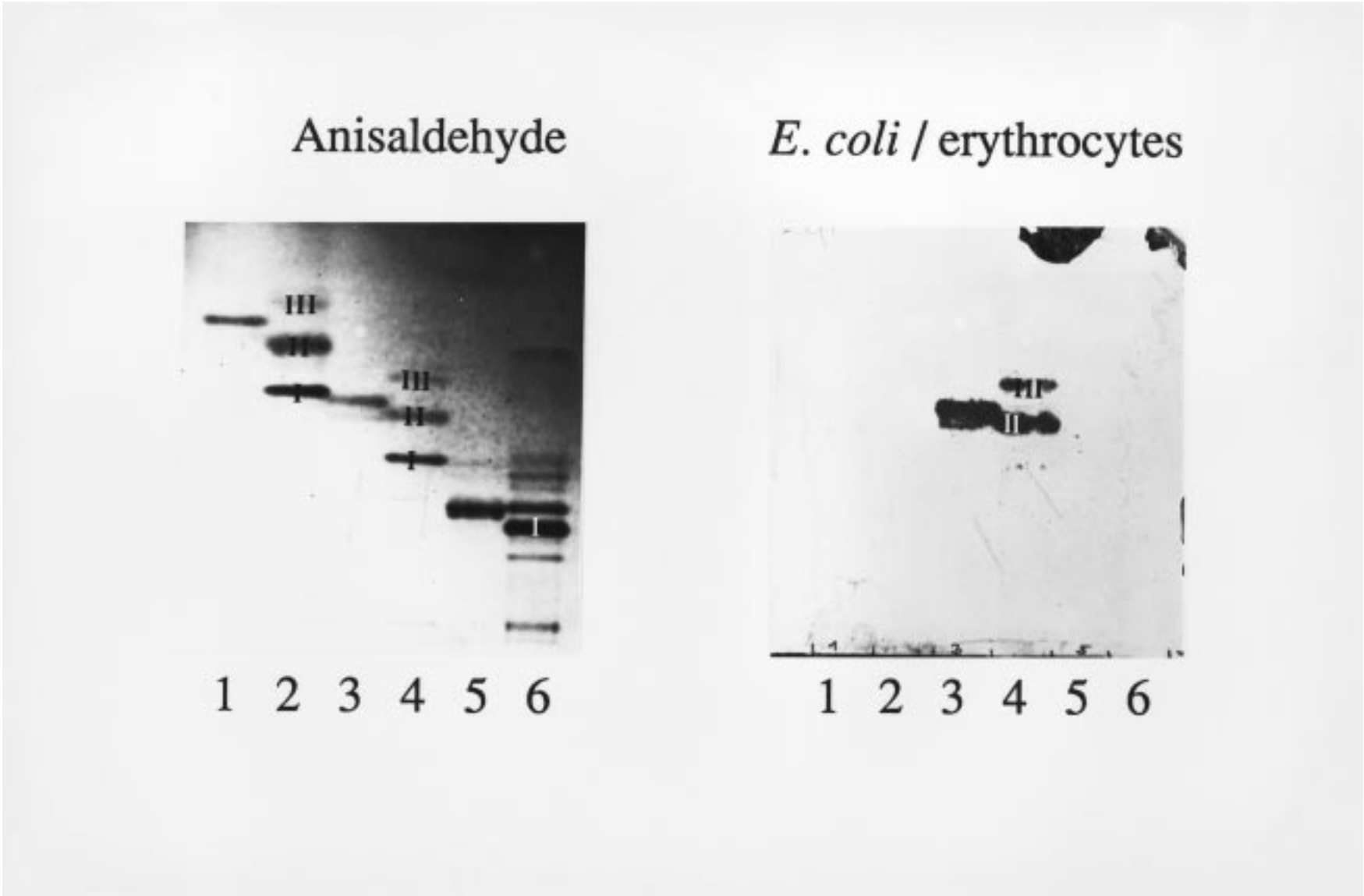


Figure 5. Thin-layer chromatograms after detection with anisaldehyde (left) and after binding of recombinant P-fimbriated *E. coli* pPIL2GI-15 (right). The lanes on the chromatograms were 1, LacCer; 2, mixture of products obtained by TFA/TFAA treatment of LacCer; 3, Gb_3Cer; 4, mixture of products obtained by TFA/TFAA treatment of Gb_3Cer; 5, Gg_4Cer; and 6, mixture of products obtained by TFA/TFAA treatment of Gg_4Cer. On the left chromatogram 3 nmol was applied per lane, and on the right 0.7 nmol. For further details, see legend to Figure 1.

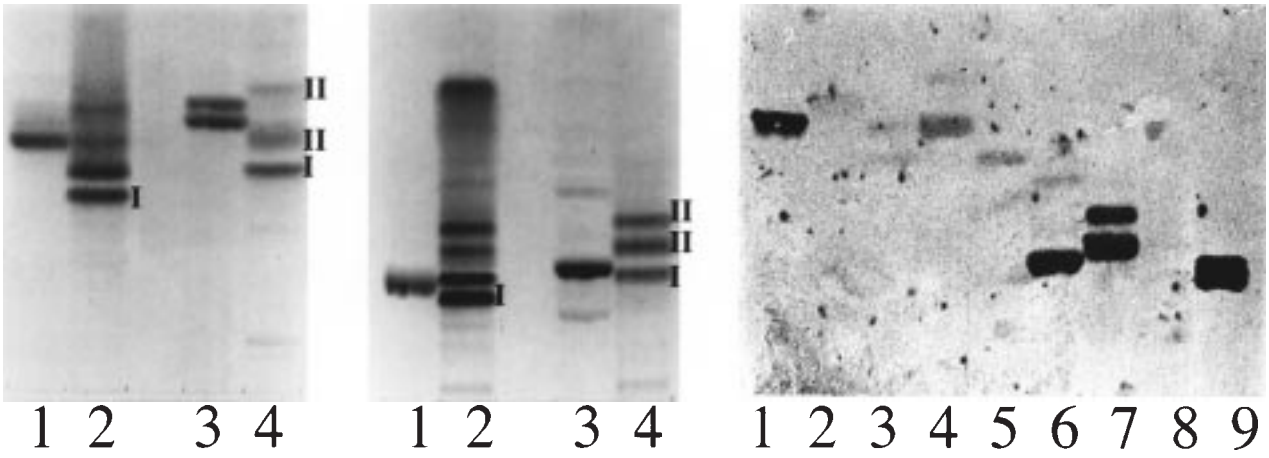


Figure 6. Thin-layer chromatograms after detection with anis aldehyde (left and middle) and after binding by *N. subflava* (right). In the left chromatogram is dihexosyl Cer from epithelial and none pithelial sources shown and in the middle is Gg_4Cer from epithelial and nonepithelial sources shown. The numbers I and II/III correspond to the same bands as in Figure 1 and 5 (Scheme 1). Binding of *N. subflava*, strain 19243, to 1-3 nmol/lane of native and derivatized glycolipids was detected after autoradiography. In the left chromatogram the lanes are 1, Hex_2Cer OH; 2, mixture of pro ducts obtained by TFA/TFAA treatment of Hex_2Cer; 3, LacCer; and 4, TFA/TFAA treated LacCer; in the middle chromatogram: 1, Gg_4Cer OH, 2 TFA/TFAA treated Gg_4Cer OH; 3, Gg_4Cer; and 4, TFA/TFAA treated Gg_4Cer; and in the right chromatogram: 1, LacCer OH; 2, LacCer; 3, TFA/TFAA treated LacCer; 4, HPLC purified 2 of LacCer; 5, HPLC purified 1 of LacCer; 6, Gg_4Cer; 7, 2 of Gg_4Cer; 8, 1 of Gg_4Cer; and 9, Gg_4Cer OH.

product corresponding to TLC band I, it was found that one of the lipid chains was missing. For the products of TLC bands II + III, however, the relative integrals of the methyl and methylene proton signals were roughly equal to those measured for the native glycolipids, confirming the presence of both lipid chains of Cer.

Glycosphingolipids with hydroxylated ceramide

When Gg_4Cer of epithelial origin was treated with TFA/TFAA, the TLC analyses (Figure 5, left, lane 6; Figure 6, middle, lane 2) gave a pattern different from those of LacCer, Gb_3Cer, and Gb_4Cer. According to FAB-MS, the two major bands, marked with I in Figure 6, had their fatty-acyl chains exchanged for trifluoroacetyl groups, corresponding to structure 1 in Scheme 1. In Figure 7, bottom, the TLC/FAB-MS analysis of the two partially separated bands I is shown. The more slow-moving band had sequence ions 16 mass units higher than the faster moving band corresponding to t18:0 and d18:0, respectively. However, no products with trifluoro acyl ceramide, structure 2, were detected [37]. When the TFA products from a dihexosyl Cer prepared from the same source as Gg_4Cer, and having the same hydroxylated Cer structure, was treated with TFA/TFAA, the same result was obtained (Figure 6, left, lane 2), which means only the deacylated product 1 was detected by MS (not shown). On the other hand, when Gg_4Cer prepared by desialylation of brain GM1, and therefore carrying sphingosine and nonhydroxylated fatty acids, was treated with TFA/TFAA, the TLC pattern (Figure 6, middle, lane 4) was identical to those of Gb_3Cer and Gb_4Cer of Figure 1 and 5. FAB-MS analyses (Figure 7, top and middle spectra) showed that both 1 and 2 had been formed.

To slow down the trifluoracetolysis reaction, milder reaction conditions were tested for the trifluoracetylation of Gg_4Cer having hydroxylated Cer. Reaction at 80°C and TFA/TFAA 1:1000 resulted in incomplete exchange of *N*-acetyl groups at the GalNAc residue. However, samples withdrawn after 24, 36, and 72 h showed no formation of trifluoroacyl ceramide (the imide). Together with unreacted starting material and structure 1, a more lipophilic product was identified, having only the *N*-acetyl group at the GalNAc residue exchanged for a trifluoroacetyl.

Binding of E. coli to TFA derivatives

The recombinant *E. coli* HB101/pPIL2GI-15 have P fimbriae that bind to the sequence Galα1-4Gal. When tested on TLC, this bacterium bound equally well to the untreated Gb_4Cer (Figure 1, right, lane 1) as to the same compound with saturated lipid chains (lane 2). Of the three major TFA products (lane 3–5), only the upper two bands, structure 2 of Gb_4Cer, were bound by the bacteria. After removal of the *N*-TFA-group of GalNTFA of structure 2 of Gb_4Cer, binding still occurred (not shown).

Figure 5 shows binding by *E. coli* to Gb_3Cer and its TFA products. Analogous to TFA-treated Gb_4Cer, the bacteria bound to structure 2 of Gb_3Cer, but not to the more slow-migrating structure 1. LacCer and Gg_4Cer, before and after trifluoroacetylation, were included in the binding assay as negative controls (Figure 5, lanes 1, 2, 5, and 6).

When tested on TLC (not shown), three different recombinant *E. coli* expressing adhesins of either of the classes I,

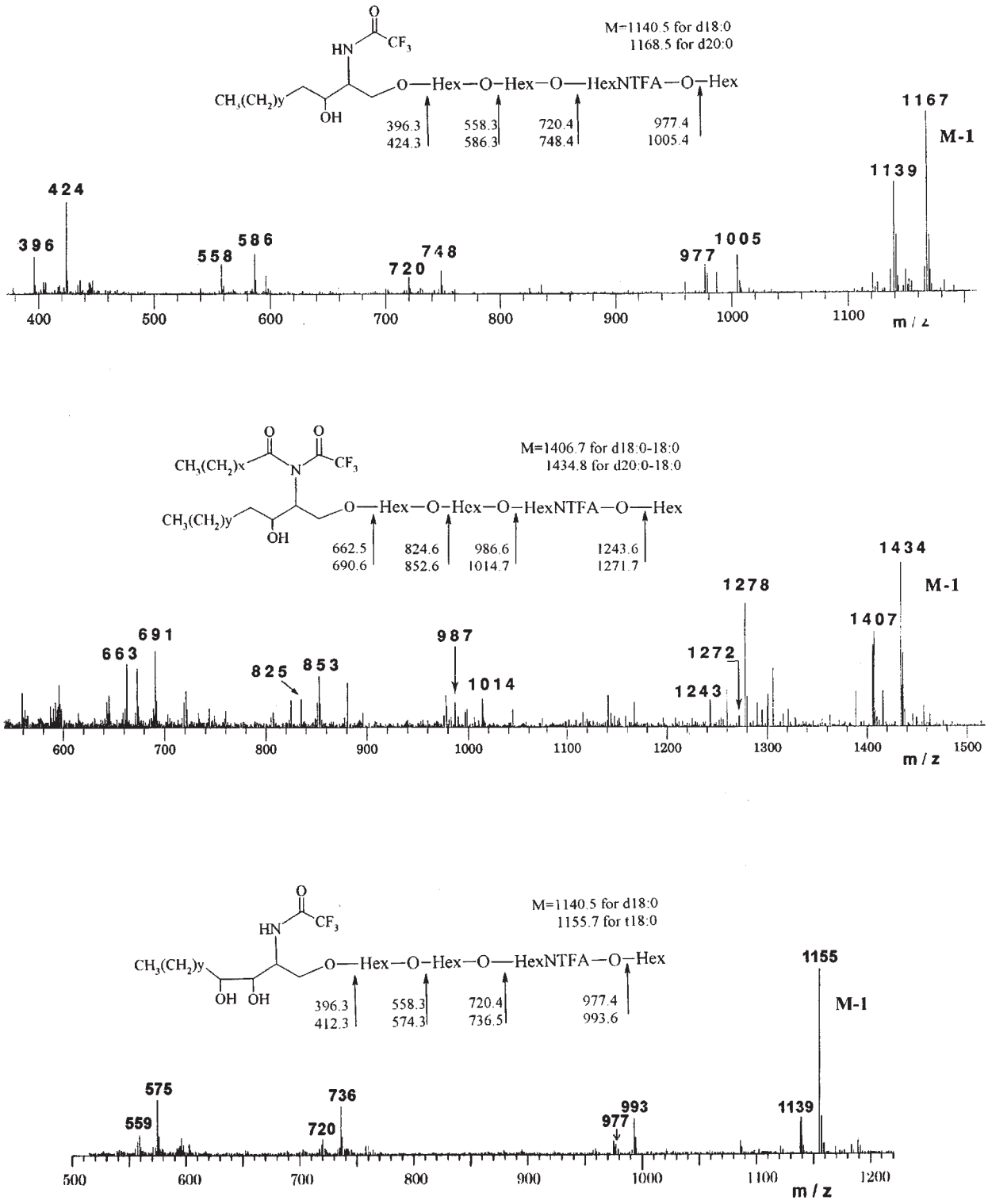


Figure 7. Negative-ion FAB MS of TFA products 1 (top) and 2 (middle) obtained from nonepithelial Gg_4Cer and TFA product 1 from epithelial Gg_4Cer (bottom). Tentative theoretical fragmentation patterns and molecular weight for different Cer species are shown above each spectrum.

II and III all bound similarly to structure 2 of Gb_3Cer and Gb_4Cer. Neither of these bacteria, however, bound to product 1 of Gb_3Cer and Gb_4Cer and apparently did not differ from the recombinant *E. coli* HB101/pPIL2GI-15.

The binding of *E. coli* HB101/pPIL2GI-15 to a dilution series of HPLC-purified structures 1 and 2 of Gb_3Cer and Gb_4Cer in microtiter wells was also studied. In contrast to the TLC assay (Figures 1 and 5), binding was found also to 1 of both Gb_3Cer and Gb_4Cer. This binding was of the same order as that to the corresponding native glycolipids.

Binding of N. subflava to TFA derivatives

Binding of *N. subflava* (strain 19243) to the native forms of LacCer and Gg_4Cer of both epithelial and nonepithelial types of Cer are shown in Figure 6, right. The binding to products 1 and 2 of nonepithelial type of LacCer and Gg_4Cer are also shown. As known before, the bacteria bound to Gg_4Cer irrespective of Cer structure but only to the epithelial type of LacCer [20] (Figure 6, right, lane 6 and 9). Exchange of the *N*-acetyl group of GalNAc in Gg_4Cer for an *N*-TFA group, structure 2 (Figure 6, right, lane 7), did not prohibit the binding while product 1 of Gg_4Cer was not bound when tested on TLC (Figure 6, right, lane 8). However, when the binding of strain 19243 to 1 and 2 of Gg_4Cer was tested in microtiter wells, 2 was bound while 1 was bound weakly (not shown).

Although LacCer of nonepitelial origin is not bound (Figure 6, right, lane 2) by *N. subflava*, the TFA-derivatives are weakly bound (Figure 6, right, lane 4 and 5). As a positive control, LacCer with hydroxylated Cer is included in Figure 6, right, lane 1.

Serial dilutions of some of the glycolipids were subjected to TLC and then incubated with *N. subflava*. In Table 2, the amounts of each glycolipid required to detect binding are shown. Strains 19243 and GN01 bound equally well to structure 2 of Gg_4Cer as to native Gg_4Cer, while strain 19243 bound 100 times stronger to native LacCer with hydroxylated Cer than to products 1 and 2 of LacCer.

Discussion

To investigate the role of the GalNAc residues of different glycolipids in the binding by bacterial adhesins, we set out to exchange the *N*-acetyl group of GalNAc in Gb_4Cer and Gg_4Cer for a *N*-trifluoroacetyl group. Studies at the molecular level of the binding between bacterial adhesins and a receptor would ideally require chemical synthesis of glycolipid analogues [9, 38, 39]. When considering extended isoreceptors, however, chemical modification of reactive groups of glycolipids isolated from natural sources may give important information on lectin-carbohydrate binding.

Table 2. Binding of three strains of *N. subflava* to glycosphingolipids separated on TLC plates.

	Binding by 3 strains of Neisseria subflava[a]		
Glycolipid	*19243*	*GN01*	*11076*
LacCer	− (n.d.)	− (n.d.)	−
LacCer-TFA, product 2	+ (40)	− (n.d.)	−
LacCer-TFA, product 1	+ (40)	− (n.d.)	−
LacCer OH	++ (0.3)	++ (0.3)	++
Gg_4Cer	++ (0.1)	++ (0.1)	++
Gg_4Cer-TFA, product 2	++ (0.2)	++ (0.04)	−
Gg_4Cer-FTA, product 1	− (n.d.)	− (n.d.)	−
Gg_4Cer OH	++ (0.8)	++ (0.8)	++

[a]Binding to 1-3 nmol of each glycolipid is marked with ++ if the staining of the film was black; +, for grey staining; −, for no detectable staining; and n.d., not determined. Figures within parentheses show the amount of glycolipid, expressed in nmol, required to give staining of auto radiograms when tested in serial dilutions (mean value of three separate tests).

Structure of derivatized glycolipids

One major product was expected after trifluoroacetylation, but three bands in about equal intensities appeared on TLC. For band I of Gb_4Cer and Gb_3Cer, FAB-MS, EI-MS, and ^{1}H-NMR data were consistent with a loss of the fatty-acyl chain and substitution with two *N*-TFA groups, one at the GalNAc and another at the Cer. The MS analysis unambiguously showed that the product contained the hydrogenated long-chain base as reported also by ^{1}H-NMR. All data are therefore consistent with structure 1 (Scheme 1).

When mass spectra of the TLC bands II and III of Gb_4Cer and Gb_3Cer were recorded through desorption from a TLC chromatogram, they were identical. MS and ^{1}H-NMR analyses indicate two isomeric substances with retained fatty-acyl chain and two TFA groups, one as GalNTFA and the other as *N*-TFA on the intact ceramide, structure 2. We were unsuccessful in separating II and III by HPLC although both migrated as sharp bands on TLC. If the energy barrier between two expected conformers is low in water and high in nonpolar solvents, results like these may be rationalized. To test this, the mixture of reaction products was chromatographed on TLC in two dimensions, with different treatments of the plate between the two developments. In the case where the plate was dried quickly, the spots corresponding to II and III, respectively, appeared almost as one homogenous spot each along the diagonal, while prolonged incubation of the TLC plate at room temperature and humidity made each spot divide into two. This latter treatment was similar to the situation after the HPLC separation, where the products were eluted with chloroform/methanol/water and the solvent evapo-

rated off in a rotavapor at slightly elevated temperature (approximately 40–50°C).

The particular fraction of Gg_4Cer from mouse feces that was used for TFA derivitizations contained only Cer carrying 2-hydroxy fatty acid. Hydroxylation of the fatty acid seems to make the amide labile toward acetolysis as we could not isolate any TFA-product of this glycolipid with retained fatty acid (Scheme 1). When the products of the nonhydroxylated and hydroxylated Cer of either Hex_2Cer or Gg_4Cer were compared, the same results were obtained. The glycolipids with hydroxylated Cer did not give any 2, and the nonhydroxylated analogues of LacCer and Gg_4Cer gave the same relative amount of structures 1 and 2 as did Gb_3Cer and Gb_4Cer. These results indicate that trifluoroacetylation of the ceramide is always a complete transamidation when the fatty acid has a 2-hydroxy group and that product 2, the imide, is not formed as an isolatable intermediate even when milder reaction conditions and shorter reaction times are used. The Gal-*N*-acetyl residues of Gg_4Cer and Gb_4Cer also undergo complete transamidation. The incomplete hydrolyses of nonepithelial Cer may be due to steric effects or solvation effects. There is also a possibility of neighboring group participation in the hydrolyses of the more highly hydroxylated ceramides. No further attempts were made to find a mechanistic explanation for the different product compositions obtained from nonepithelial type and epithelial type glycolipids, respectively.

Bacterial binding to derivatized glycolipids

Uropathogenic *E. coli* is a good model system for studying lectin-carbohydrate interaction, since the binding specificity to natural glycolipids has been studied in detail [14, 15, 17], and the genetics of the P fimbriae, including adhesin expression, is well worked out [18, 35, 40]. Clinical isolates of uropathogenic *E. coli* express variant adhesins, with slightly different preferences for isoreceptors. Thus, class II adhesin [15, 18, 19, 41] is often found in isolates from humans with pyelonephritis [42–44] and has Gb_4Cer as an optimal receptor. Isolates from dog infections or human cystitis, however, carry a variant adhesin [19, 45, 46], the class III adhesin, which prefers the Forssman glycolipid or a blood group A-substituted Gb_4Cer, GalNAcα1-3(Fucα1-2)Galβ1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer [14, 15, 17, 18]. A clone expressing a class I adhesin, which preferentially binds Gb_3Cer is also available [14, 18]. For this adhesin (class I), all of the Galα1-4Galβ1-4 disaccharide and part of the Glc has been suggested to constitute the binding epitope [47], whereas for the class II adhesin an epitope shift along the saccharide chain results in the Galα1-4Galβ1-4 disaccharide and part of the terminal GalNAcβ1-3 residue as the suggested epitope of Gb_4Cer [48]. In the case of the class III adhesin, a further shift in the specificity occurs, resulting in the GalNAcα1-3GalNAcβ1-3Galα1-4 trisaccharide of the Forssman glycolipid as the binding epitope [15]. The selectivity afforded by a membrane is in part explained by conformational restrictions around the Glcβ1-1Cer linkage due to the bilayer plane [21, 46, 49]. However, consideration of other natural membrane constituents and the effects on the dynamic behavior of glycolipid receptor structures is at present poorly understood but is most likely just as crucial for the observed selectivity. The lack of binding observed for product 1 of both Gb_3Cer and Gb_4Cer on the TLC plate is probably due to insufficient hydrophobic anchoring, necessary for proper binding epitope presentation, and hydrophilic interactions augmented by the TFA groups, despite treatment with polyisobutylmethacrylate, whereas in the microtiter well interactions with the remaining lipid tail are predominant. However, effects to the contrary have been observed for Vero toxin-1-binding to Gb_3Cer lacking the fatty-acyl chain or having a very short such chain in that binding was obtained on the TLC plate but not in the microtiter wells [50]. It is thus obvious that a very delicate balance between hydrophobic and hydrophilic interactions with the solid matrix determines whether binding will be obtained and a more relevant experimental situation would therefore be needed.

The class II adhesin from recombinant *E. coli* (HB 101/pPIL2GI-15) was primarily used for testing the reaction products for receptor activity in the present study. The affinity of this adhesin for bands II and III of Gb_3Cer and Gb_4Cer, identified as conformational isomers of product 2, on the TLC plate was very similar to the native glycolipids. Furthermore, no difference in binding strength between bands II and III was discernible. The conditions in the TLC overlay assay allow, moreover, for a slow interconversion between the isomeric forms, rendering any conclusions about their relative binding strength invalid. One firm conclusion that may be reached, however, is that replacement of the acetamido moiety of the terminal GalNAcβ1-3 residue of Gb_4Cer by an *N*-TFA group does not influence binding significantly. Thus, the absence of any appreciable effect in exchanging a hydrophobic methyl group for a highly electronegative trifluoromethyl group strongly indicates that at least this part of the GalNAcβ1-3 residue does not participate in the binding epitope of the class II adhesin mentioned above nor the representatives of the other two adhesin classes. This conclusion is contrary to the findings of Striker *et al.,* who concluded that the acetamido moiety most likely would have to be included in the binding epitope of class II adhesins [48].

Since the binding of *N. subflava* to Gg_4Cer is independent of the degree of ceramide hydroxylation (Table 2, [20]), it would suggest that the binding epitope for this receptor is not lactose-based but, in analogy with other ganglio series-binding bacteria [22, 51], rather should have the Galβ1-3GalNAc or GalNAcβ1-4Gal disaccharide as the optimal epitope. However, when *N*-acetyl in Gg_4Cer

was exchanged for *N*-trifluoroacetyl, the bacteria (strains 19243 and GNO1) bound just as well to product 2 on TLC as to the native glycolipids. This implies that the *N*-acetyl of GalNAc does not directly interact with the adhesins from these two strains, whereas for strain 11076 a direct participation of this moiety most likely is at hand.

Although LacCer of nonepithelial origin was not bound by the bacteria, strain 19243 surprisingly revealed an affinity for both 1 and 2 of LacCer being approximately 100-fold less than for LacCer OH. This unexpected result may stem from a different distribution of conformations around the Glc-Cer linkage and/or additional interactions provided by the TFA moiety.

One goal in studies of pathogen binding to carbohydrates is the development of methods to inhibit the pathogenesis. A few cases have been reported where natural receptor-active glycoconjugates have been modified, leading to an increase in the binding of a microorganism [3, 2, 52]. This approach may therefore be useful for pharmaceutical applications [53, 54], especially if combined with multivalent exposure, carbohydrate receptors may be extremely effective [55]. To acquire useful knowledge in this respect, epitope mapping is an important step, and derivatizations with fluorine have several times proven valuable in investigations of carbohydrate-protein interactions [8-10, 56].

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