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Specific Cleavage of the Glycosidic Bond between the Carbohydrate and Ceramide Portions in Glycosphingolipids Using Trifluoroacetolysis *

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A number of glycosphingolipids have been subjected to trifluoroacetolysis. Heating the glycosphingolipid at 100 °C for 48 h in mixtures of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) varying from 1:1 to 1:100 (v/v) resulted in complete cleavage of the glycosidic bond between the carbohydrate and the unsaturated base of the ceramide. The fatty acid(s) linked to the amino group of sphingosine were also released by transamidation. The carbohydrate portion was stable under these conditions due to stabilization of the glycosidic bonds by the electronegative *O*-trifluoroacetyl groups. The 2-acetamido-2-deoxy-hexose units in the glycosphingolipids were converted into their *N*-trifluoroacetyl analogues. The *N*-trifluoroacetyl functions could be removed and the 2-acetamido-2-deoxy-hexoses could be reconstituted by *N*-acetylation.

In previous publications we have demonstrated the versatility of the trifluoroacetolysis reaction. By trifluoroacetolysis it is possible to effect transamidation under conditions where most glycosides and reducing sugars are stable, being converted into their pertrifluoroacetylated derivatives.[1–5] By use of the trifluoroacetolysis reaction, procedures have been devised for the isolation of *O*- and *N*-glycosidically linked carbohydrate chains in glycoproteins.[6–9] Trifluoroacetolysis has also been used for releasing oligosaccharides from erythrocyte membranes of different blood group P phenotypes.[10] In this communication glycosphingolipid models have been subjected to trifluoroacetolytic degradation.

MATERIALS AND METHODS

Glycosphingolipids. Glucosylceramide, galactosylceramide, lactosylceramide and G_{M1}-ganglioside were purchased from Supelco Inc. (USA). Globotriaosylceramide was prepared from pig intestinal linings[11] and the glycolipids gangliotriaosylceramide, globotetraosylceramide and the Forssman antigen were prepared from guinea pig,[12] human[13] and sheep[14] erythrocyte membranes respectively. The identity of the glycosphingolipids was established by sugar analysis, methylation analysis and ^{1}H NMR spectroscopy.

Analytical methods. Sugar analyses were performed using gas-liquid chromatography[15] and mass spectrometry[16] after hydrolysis with 90 % aqueous formic acid at 100 °C for 5 h and also after subsequent hydrolysis with 0.25 M aqueous sulfuric acid at 100 °C for 18 h. Methylation analyses were performed as previously described.[17] Gas-liquid chromatography was carried out on a Perkin-Elmer 3920 gas chromatograph equipped with a flame ionization detector. Separations were performed on (a) an SE-30 W.C.O.T. vitreous silica capillary column (25 m×0.2 mm) at 170 °C (for partially methylated

* The principle and part of this work was presented at the C.N.R.S. Internat. Symp. on Stucture and Function of Gangliosides. Svensson, S. 1980 (Eds. Svennerholm, L., Mandel, P., Dreyfus, M. and Urban, P.-F., Plenum Press, New York) pp. 71–76.

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alditol acetates) and at 180–330 °C (for permethylated oligosaccharides alditols), (b) a Silar 10 W.C.O.T. glass capillary column (4 m×0.25 mm) at 190–230 °C (for alditol acetates). Gas-liquid chromatography-mass spectrometry was performed on a Finnigan 4021 instrument fitted with the appropriate column. The spectra were recorded at 70 eV, with an ionization current of 0.3 mA and an ion source temperature of 280 °C. The spectra were processed on an on-line computer system (Nova 3, Data General).

Trifluoroacetolysis of glycosphingolipids. The glycosphingolipids were treated with a mixture of trifluoroacetic acid (TFA) and trifluoroacetic anhydride (TFAA) at 100 °C in a sealed tube (caution: corrosive mixture under pressure). The reaction time was varied from 2 to 48 h and the proportions of TFA/TFAA (2 ml/mg glycosphingolipid) was varied from 1:1 to 1:100 (v/v). After cooling, the reaction mixture was concentrated to dryness, dissolved in methanol (5 ml) and again concentrated to dryness. The product was dissolved in glacial acetic acid (5 ml) and distilled water (5 ml) was added. After 30 min at 100 °C the solution was cooled and thereafter evaporated to dryness. The released oligosaccharides were freed from contaminating material by extraction with diethyl ether (10 ml) and distilled water (5 ml). The water phase was collected and the diethyl ether phase was shaken two more times with 5 ml water. The combined water phases were washed three times with 5 ml diethyl ether and evaporated to dryness.

Oligosaccharides from glycosphingolipids containing 2-acetamido-2-deoxy-hexoses were reconstituted by reduction ($NaBD_4$) of the material and subsequent *N*-acetylation. The *N*-acetylation was performed in two steps, first acetylation by acetic anhydride/pyridine (1:1, (v/v), 5 ml, 100 °C, 30 min) and then de-*O*-acetylation with 2 M ammonia in 75 % aqueous methanol (10 ml) at room temperature for 20 h.

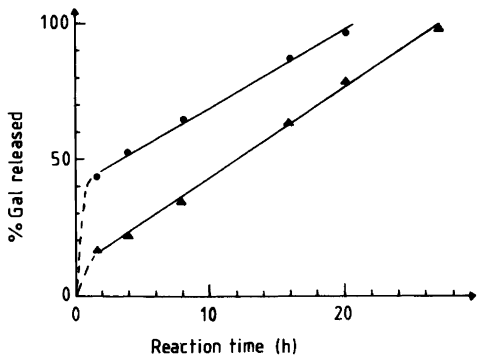


Fig. 1. Treatment of galactosylceramide with TFA/TFAA 1:1 (●) and 1:50 (▲) (v/v).

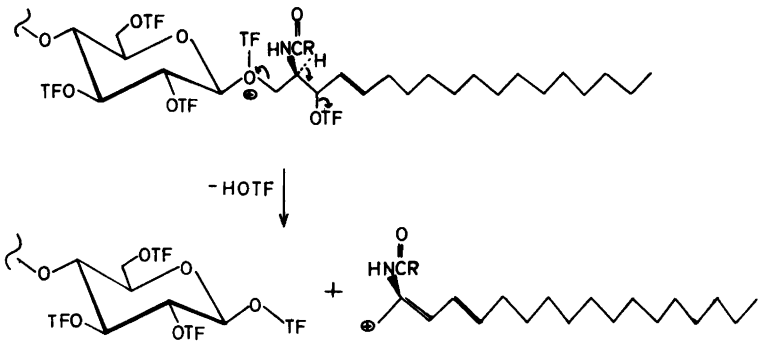
RESULTS AND DISCUSSION

Trifluoroacetolysis of the simple glycosphingolipids glucosylceramide, galactosylceramide, $LcOse_2Cer$ and $GbOse_3Cer$ proceeded well in mixtures of TFA/TFAA varying from 1:1 to 1:100 (v/v). After work up the undegraded oligosaccharides were recovered in high yield and analysed by GLC–MS as alditol acetates for the monosaccharides and as permethylated alditols for the oligosaccharides (Table 1). The reaction time needed for accomplishing complete cleavage of the glycosidic bond between the sugar moiety and the ceramide portion was 21 and 27 h

Table 1. Treatment of glycosphingolipids with TFA/TFAA at 100 °C.

Glycosphingolipid	Product[b]	Recovery %[a] TFA/TFAA 24 h 1:1	1:50	TFA/TFAA 48 h 1:1	1:50	1:100
Glc–Cer	D-Glc-ol-1-*d*[c]	97	89	98	98	98
Gal–Cer	D-Gal-ol-1-*d*	100	91	97	99	100
$LcOse_2Cer$	β-D-Gal-(1–4)-D-Glc-ol-1-*d*	95	87	97	98	100
$GbOse_3Cer$	α-D-Gal-(1–4)-β-D-Gal-(1–4)-D-Glc-ol-1-*d*	98	88	96	97	96

[a] Determined by GLC–MS, as alditol acetates for the monosaccharides and as permethylated oligosaccharide alditols for the others. [b] Product obtained after trifluoroacetolysis, de-*O*-trifluoroacetylation and reduction ($NaBD_4$). [c] D-Glc-ol-1-*d*, D-glucitol-1-*d*.



Scheme 1.

respectively for the trifluoroacetolysis conditions 1:1 and 1:50 (v/v) (Fig. 1). The cleavage mechanism is possibly initiated by elimination of an *O*-trifluoroacetyl group from the sphingosine unit yielding an allyl cation (Scheme 1). This mechanism requires that the sphingosine moiety is unsaturated as has been previously suggested.[18]

Trifluoroacetolysis of $GgOse_3Cer$ and $GbOse_4Cer$ was performed in two mixtures of TFA/TFAA, 1:1 and 1:50 (v/v), and at both

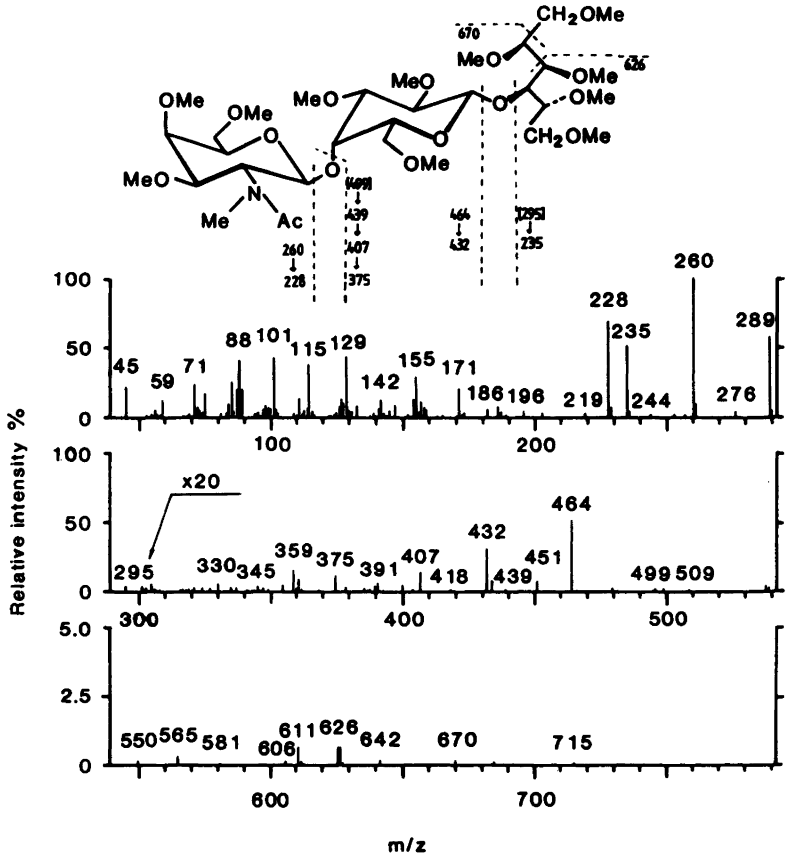


Fig. 2. Mass spectrum and some important primary fragments of β-D-GalNAc-(1−3)-β-D-Gal-(1−4)-D-Glc-ol as permethylated oligosaccharide alditol released from $GgOse_3Cer$ by trifluoroacetolysis.

Table 2. Treatment of glycosphingolipids with TFA/TFAA at 100 °C for 48 h.

Glycosphingolipid	Product [b]	Recovery %[a] TFA/TFAA 1:1	TFA/TFAA 1:50
$GgOse_3Cer$	β-D-GalNAc-(1−4)-β-D-Gal-(1−4)-D-Glc-ol-1-*d*[c]	98	100
$ObOse_4Cer$	β-D-GalNAc-(1−3)-α-D-Gal-(1−4)-β-D-Gal-(1−4)-D-Glc-ol-1-*d*	87	89

[a] Determined by GLC−MS, as permethylated oligosaccharide alditols. [b] Product obtained after trifluoroacetolysis, de-*O*- and de-*N*-trifluoroacetylation, reduction ($NaBD_4$) and *N*- acetylation. [c] D-Glc-ol-1-*d*, D-glucitol-1-*d*.

conditions the oligosaccharide portions were obtained in good yields as their pertrifluoroacetylated derivatives. After reconstitution (see Materials and Methods) the oligosaccharides were analyzed by GLC−MS as their permethylated oligosaccharide alditols (Table 2, Fig. 2 and 3).

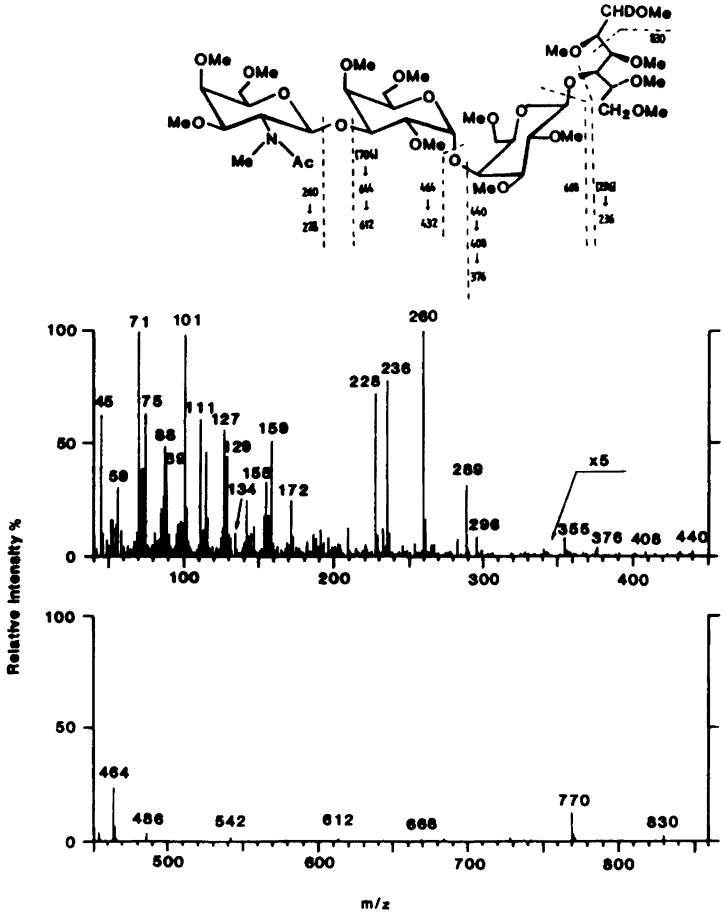


Fig. 3. Mass spectrum and some important primary fragments of β-D-GalNAc-(1−3)-α-D-Gal-(1−4)-β-D-Gal-(1−4)-D-Glc-ol-1-*d* as permethylated oligosaccharide alditol released from $GbOse_4Cer$ by trifluoroacetolysis.

Table 3. Treatment of Forssman antigen with TFA/TFAA at 100 °C for 48 h.

Product[b]	Recovery %[a] TFA/TFAA 1:1	TFA/TFAA 1:4	TFA/TFAA 1:15	TFA/TFAA 1:50	TFA/TFAA 1:100
α-D-GalNAc-(1−3)-β-D-GalNAc-(1−3)-α-D-Gal-(1−4)-β-D-Gal-(1−4)-D-Glc-ol-1-*d*[c]	28	26	47	67	83

[a] Determined by paper chromatography and also by GLC−MS, where the relationship terminal galactose and 3-linked galactose was calculated. [b] Product obtained after trifluoroacetolysis, de-*O*- and de-*N*-trifluoroacetylation, reduction ($NaBD_4$) and *N*-acetylation. [c] D-Glc-ol-1-*d*, D-glucitol-1-*d*.

The Forssman antigen was subjected to trifluoroacetolysis in different mixtures of TFA/TFAA varying from 1:1 to 1:100 (v/v). In mixtures of TFA/TFAA varying from 1:1 to 1:15 (v/v) some cleavage of the bond of the innermost D-GalNAc*p* residue was observed, yielding on

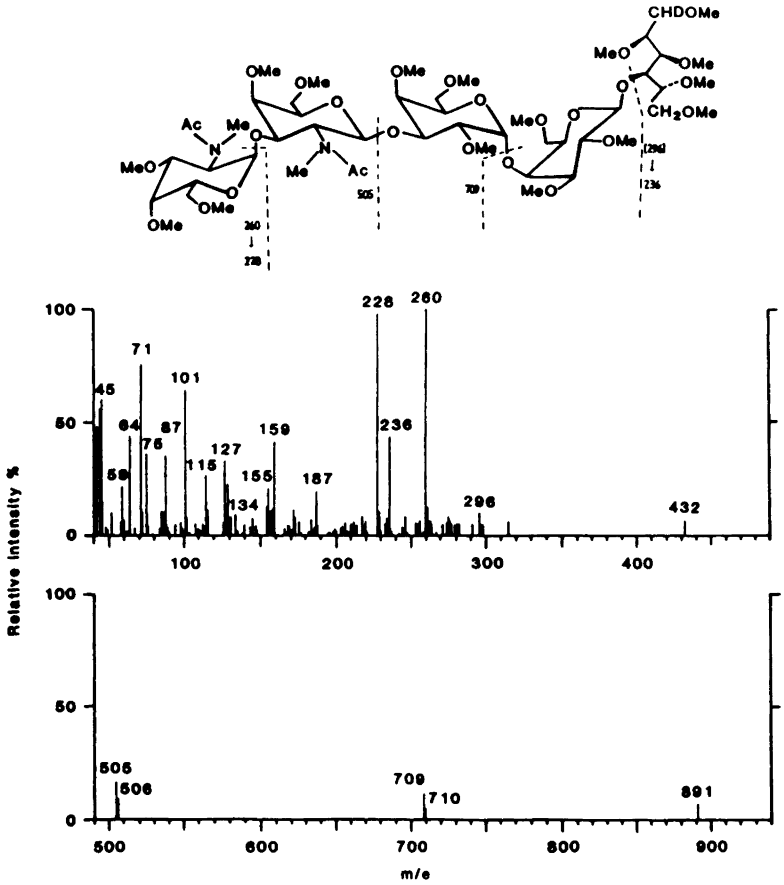


Fig. 4. Mass spectrum and some important primary fragments of α-D-GalNAc-(1−3)-β-D-GalNAc-(1−3)-α-D-Gal-(1−4)-β-D-Gal-(1−4)-D-Glc-ol-1-*d* as permethylated oligosaccharide alditol released from Forssman antigen by trifluoroacetolysis.

Table 4. Sugar and methylation analysis of the oligosaccharide released from Forssman antigen by trifluoroacetolysis.

Sugars	Relative molar proportions
Sugar analysis[a]	
D-Gal	1.85
D-Glc[b]	1.0
D-GalNAc	1.65
Methylation analysis[c]	
1,2,3,5,6-*O*-Me-D-Glc	0.97
2,3,6-*O*-Me-D-Gal[d]	1.0
2,4,6-*O*-Me-D-Gal	0.82
3,4,6-*O*-Me-D-GalN(Me)Ac 3,4,6-*O*-Me-D-GalNAc	0.85[e]
4,6-*O*-Me-D-GalN(Me)Ac 4,6-*O*-Me-D-GalNAc	0.80[e]

[a] Determined by GLC–MS after hydrolysis, reduction and acetylation. [b] D-Glc is set to 1.0. [c] Determined by GLC–MS after permethylation, hydrolysis, reduction and acetylation. [d] 2,3,6-*O*-Me-D-Gal is set to 1.0, [e] The relative molar proportions were calculated relative the amount of D-Gal from the sugar analysis, since the responsfactors for the hexosamine derivatives are not known.

analysis the disaccharide GalNAc*p*-(1–3)-GalNAc and the trisaccharide Gal*p*-(1–4)-Gal*p*-(1–4)-Glc as well as the undegraded pentasaccharide (Table 3). Using TFA/TFAA mixtures varying from 1:50 to 1:100 (v/v) the expected pentasaccharide was obtained in good yield as shown (Table 3) by paper chromatography after removal of trifluoroacetyl groups and subsequent *N*-acetylation. The pentasaccharide was isolated and the structure was confirmed by sugar- and methylation analysis (Table 4) and direct inlet MS (Fig. 4). The anomeric nature of the sugar residues as well as the number of *N*-acetyl groups were established by ^{1}H NMR spectroscopy. These results show that the glycosidic bond between a sugar residue and the ceramide portion with an unsaturated sphingosine in glycosphingolipids can be cleaved under conditions where most glycosidic bonds are stable and amides are transamidated into *N*-trifluoroacetyl functions. By de-*O*- and de-*N*-trifluoroacetylation (see Materials and Methods) followed by re-*N*-acetylation the carbohydrate portion of neutral glycosphingolipids can be recovered intact. The methodology is presently being explored for use in the development of a diagnostic procedure for the sphingolipidoses, as well as for preparative techniques for isolation of the oligosaccharide portions in glycosphingolipids.

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