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STRUCTURE ELUCIDATION OF BLOOD GROUP B-LIKE AND I-ACTIVE CERAMIDE EICOSA- AND PENTACOSA-SACCHARIDES FROM RABBIT ERYTHROCYTE MEMBRANES BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY; ELECTRON-IMPACT AND FAST-ATOM-BOMBARDMENT MASS SPECTROMETRY; AND TWO-DIMENSIONAL CORRELATED, RELAYED-COHERENCE TRANSFER, AND NUCLEAR OVERHAUSER EFFECT 500-MHz ^{1}H-N.M.R. SPECTROSCOPY*,†

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ABSTRACT

The structures of two glycosphingolipids, a ceramide eicosasaccharide BI_{rab}-3 and a ceramide pentacosasaccharide BI_{rab}-4 with "B-like" and distinct I blood-group activity, isolated in high yield from rabbit erythrocyte membranes, were investigated. The determination of their general structure, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→{4)-β-D-Glc*p*NAc-(1→3)-[α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→}$_n$4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-ceramide, with $n = 3$ for BI_{rab}-3 and $n = 4$ for BI_{rab}-4, was based on the results of methylation analysis, fast-atom-bombardment and electron-impact mass spectrometry, 1D and 2D COSY, RCT, and n.O.e. ^{1}H-N.m.r. spectra, and specific enzymic and chemical degradation.

*Dedicated to Professor Walter T. J. Morgan.

†This paper is part of a series concerning the Immunochemistry of I/i Active Oligo- and Poly-glycosylceramides from Rabbit Erythrocyte Membranes. This work was supported by the Deutsche Forschungsgemeinschaft (grants Ha 627/2-2 and Eg 39/9-6) and the Fritz Thyssen Stiftung.

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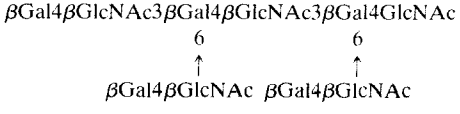
INTRODUCTION

The biological importance of the I/i blood-group active oligosaccharides can be referred to their target properties against human monoclonal autoantibodies resulting in a special kind of autoimmune hemolytic anemia known as cold agglutinin disease[1]. Furthermore, I/i antigens as biosynthetic precursors of ABH blood-group substances[2] alter their structure during fetal or postnatal development[3,4], and during cellular differentiation and malignant degeneration of certain epithelial tissues[4–6].

In a series of previous papers, the purification and structural elucidation of various rabbit erythrocyte membrane glycosphingolipids comprising up to 15 sugar units has been described[7–10]. These glycosphingolipids, or their partially degraded derivatives, or both, revealed distinct serological activity against different human cold-reacting or mouse monoclonal antibodies of I- or i-blood-group specificities[10–12]. Together with previous findings of other groups, the results allowed more insight into the complex nature of the different oligosaccharide epitopes corresponding to the I/i antigenicity. Thus far, the branched β-D-Gal*p*(1→4)-β-D-



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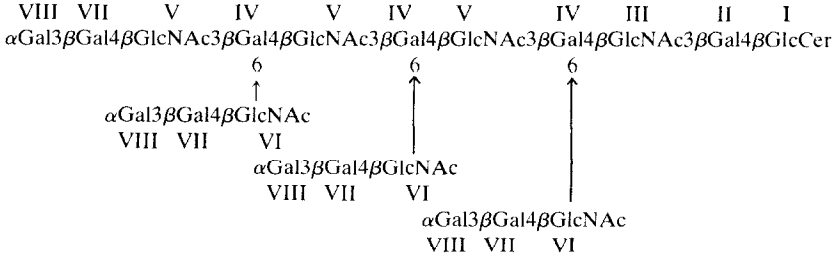
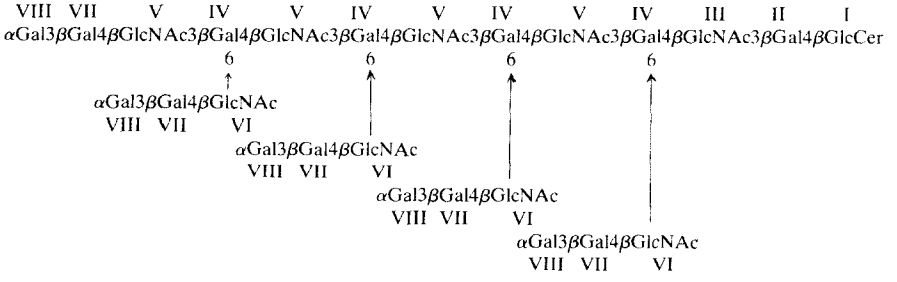


2 (BI_{rab}-3)



3 (BI_{rab}-4)

Glc*p*NAc-(1→3)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)]-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→ hexasaccharide region represents the basic unit of I blood-group antigenicity[13,14], whereas repetitive linear β-D-(1→3)-linked, unbranched *N*-acetyllactosamine units display i-blood-group activity[12,15]. The comparatively stronger I-activities of the rabbit red cell ceramide pentadecasaccharide and its α-D-galactosidase degradation product can mainly be referred to the doubly branched oligosaccharide-region[10,11] **1**.

The degree of its I-activity, however, still essentially depends on the absence or presence of substituents linked to the nonreducing terminals of the sugar branches[10,11]. There is evidence, however, that in the complex, multibranched polyglycosylceramides, I-activity is less dependent on those substituents[16,17].

It is the main object of this paper to provide experimental evidence for the high-yield purification and complete structural elucidation of homogeneous, complex I-blood-group active glycosphingolipids. Since these multiantennary *neolacto*-(polyglycosyl)ceramides have appreciably more blood-group I-activity than their mono- or di-branched analogues, they may represent favored compounds for further detailed studies on the molecular basis of the I-antigenic determinants.

EXPERIMENTAL

General. — Starting material, reagents, and general isolation methods were essentially the same as described in a previous paper[10]. A similar procedure has also been applied to the isolation and purification of blood-group B-active glycosphingolipids from human erythrocytes[18].

Analytical procedures. — Analytical h.p.t.l.c. was performed as described[10] in 9100:900:1 and 4450:550:1 (v/v) chloroform–methanol–water, and 430:70:1 1,2-dichloroethane–methanol–water. Further methods used are the same as listed earlier[10]. After deacetylation[7], samples were treated with α-D-galactosidase from coffee beans[7], Smith degraded[10], serologically investigated by haemagglutination-inhibition tests[7,19,20], and permethylated[21–23]. Carbohydrate linkage analysis was performed by g.l.c.–m.s. of methylated alditol acetates[7,21,22].

Mass spectrometry. — F.a.b.m.s. was performed on a ZAB HF instrument (V.G., Manchester, U.K.) equipped with an ion-tech, fast-atom gun as described previously[10]. For the high mol. wt. samples, several consecutive scans, each covering ~1000 a.m.u. with readdition of matrix and sample, were combined into one spectrum. The resolution of the instrument was set to 250 p.p.m. giving a unit resolution up to ~4000 a.m.u. Spectra were evaluated manually by counting the spectral lines. Signals >4000 a.m.u. were determined by peak matching using $[Cs_{n+1}J_n]^+$ as standard. These signals, although incompletely resolved, are represented in Figs. 3 and 4 by vertical lines, one mass unit apart, following in height the intensity of the original u.v. plot. E.i.m.s. values were recorded on the same instrument. Samples were loaded into shallow quartz tubes that could be introduced into the ion source close to the electron beam. Samples were heated indirectly by

the heater of the ion source to 280–320° until relevant spectra were obtained. Acceleration voltage was 7 kV and electron energy 20 eV.

^{1}H-Nuclear magnetic resonance. — The ^{2}H-exchanged samples of BI_{rab}-3 and BI_{rab}-4 were dissolved in di($^2H_3$)methyl sulfoxide containing 2% D_2O and measured at 333 K. One-dimensional, and two-dimensional (2D) correlated[24] (COSY), relayed coherence transfer[25] (RCT), and NOESY[26] spectra of BI_{rab}-3 and -4 were measured at 500 MHz with a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer and an array processor. Bruker standard software 1986 was used. Further details are given in the legends to the figures.

Preparation of glycosphingolipids. — Fractions A III (234 mg) and A IV (345 mg), obtained by the 2nd silicic acid column chromatography of crude peracetylated glycosphingolipids[10] (11.8 g), were each further separated by silicic acid (Iatrobead) column chromatography (A III: 280 g, 2.4 × 200 cm; A IV: 830 g, 4.6 × 95 cm) using chloroform with increasing amounts of methanol up to 15%. Glycolipids migrating immediately after the ceramide pentadecasaccharide (BI_{rab}-2) were separated into the crude ceramide eicosasaccharide (BI_{rab}-3) and ceramide pentacosasaccharide (BI_{rab}-4) fractions. Purification of these peracetylated substances was achieved by silica gel h.p.t.l.c.[18].

RESULTS

Purification and yield of glycosphingolipids. — Two different glycosphingolipid fractions (crude BI_{rab}-3, 122 mg; and crude BI_{rab}-4, 205 mg) resulted from the extraction of 70 g of lyophilized ghosts (prepared from 6.2 L of packed rabbit erythrocytes) and subsequent fractionation of peracetylated glycolipid extracts by repeated silicic acid column chromatography[10]. These two fractions were further purified by preparative silica gel h.p.t.l.c. yielding two substances (peracetylated BI_{rab}-3, 87 mg; and BI_{rab}-4, 63 mg) (Fig. 1). Both substances were homogeneous in analytical h.p.t.l.c. when the above mentioned solvent systems were used. After deacetylation, 47 mg of BI_{rab}-3 and 34 mg of BI_{rab}-4 were obtained. The compounds did not migrate in analytical h.p.t.l.c. on silica gel, even in very polar chloroform–methanol–water.

Methylation analysis of native and partially degraded substances. — Combined g.l.c.-m.s. of permethylated BI_{rab}-3 and BI_{rab}-4 yielded, after acetolysis, reduction, and acetylation, partially methylated alditol acetates indicating terminal, 3-*O*-, and 3,6-di-*O*-substituted galactose, 4-*O*-substituted glucose, and 4-*O*-substituted 2-acetamido-2-deoxyglucose residues in a molar ratio of 3.7:5:2,6:0.9:6.9 and of 4.7:6:3.6:0.9:9, respectively (Table I). All partially methylated alditol acetates were identified by their g.l.c. retention times and m.s. fragmentation patterns[7,20,21]. The results are in accordance with a ceramide eicosasaccharide BI_{rab}-3 and a ceramide pentacosasaccharide (BI_{rab}-4) having three or four 3,6-galactosyl branching points and four or five terminal galactosyl groups, respectively. All amino sugar residues are 2-acetamido-2-deoxyglucose (seven or nine mol) which are exclusively 4-*O*-substituted. Therefore, both glycosphingolipids belong to the *neo-lacto* series.

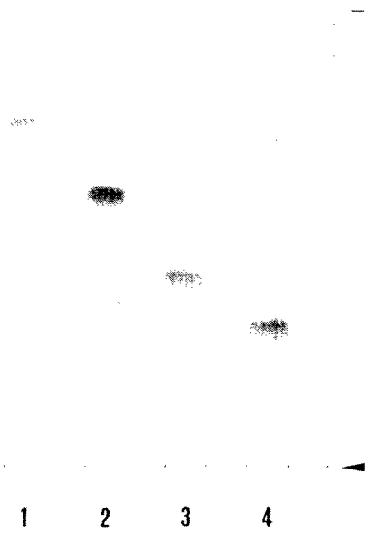


Fig. 1. Silica gel h.p.t.l.c. of peracetylated bi-, tri-, tetra-, and penta-antennary glycosphinglipids from rabbit erythrocyte membrane: (1) Ceramide decasaccharide (BI_{rab}-1); (2) ceramide pentadecasaccharide (BI_{rab}-2); (3) ceramide eicosasaccharide (BI_{rab}-3); and (4) ceramide pentacosasaccharide (BI_{rab}-4). Solvent system: 9100:900:1 chloroform–methanol–water.

Mass spectrometric analysis. — Neither the native nor the peracetylated compounds BI_{rab}-3 and BI_{rab}-4 furnished interpretable f.a.b. spectra. However, good spectra were obtained from the permethylated compounds. The ceramide eicosasaccharide BI_{rab}-3 showed, as expected, clusters of molecular-ion isotopes ($M + Na^+$) between 5040 and 5120 m.u., and the ceramide pentacosasaccharide BI_{rab}-4 between 6150 and 6200 m.u. The molecular-ion region of the ceramide pentacosasaccharide is shown in Fig. 2. Whereas the data of methylation analysis could be reconciled with several possible structures, the sequence data derived from the f.a.b. spectra revealed a very regular architecture that is shown in Schemes 1

TABLE I

MOLAR RATIOS OF PARTIALLY METHYLATED SUGARS FROM BLOOD-GROUP B-LIKE AND I-ACTIVE (POLYGLYCOSYL)CERAMIDES FROM RABBIT ERYTHROCYTE MEMBRANES AFTER PERMETHYLATION, ACETOLYSIS AND FURTHER DERIVATIZATION[a]

Glycolipid	*2,3,4,6-Me-Gal*	*2,4,6-Me-Gal*	*2,4-Me-Gal*	*2,4,6-Me-Glc*	*2(NMeNAc)3,4,6-Me-Glc*	*2(NMeNAc)3,6-Me-Glc*
BI_{rab}-3	3.7	5[b]	2.6	0.9		6.9
BI_{rab}-3_{α}	4.3	1	2.7	0.8		7.2
BI_{rab}-$3_{\alpha SD}$	0.8	1	2.7	1.1	3.4	2.8
BI_{rab}-4	4.7	6	3.6	0.9		9
BI_{rab}-4_{α}	4.6	1	3.7	0.8		9.5
BI_{rab}-$4_{\alpha SD}$	0.7	1	3.7	1.1	4.5	4.3

[a]Determined by g.l.c. and g.l.c.–m.s. of partially methylated alditol acetates. [b]Molar ratio based on the molar ratio of 3-*O*-substituted galactose to substance obtained by ^{1}H-n.m.r. analysis.

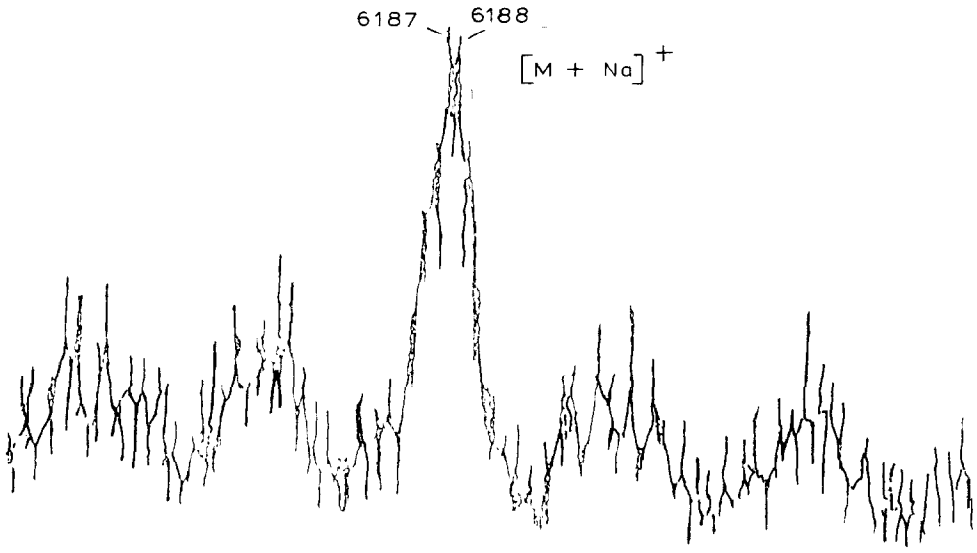
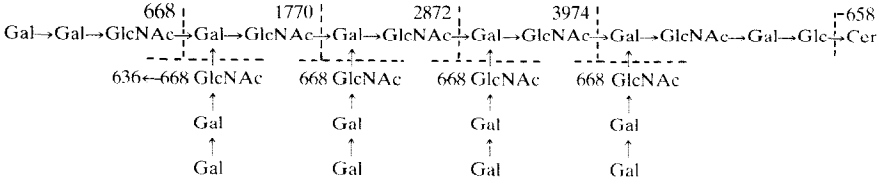


Fig. 2. Partial f.a.b. spectrum showing the u.v. plot of the molecular ion region of permethylated BI_{rab}-4 obtained at 4 kV acceleration voltage.

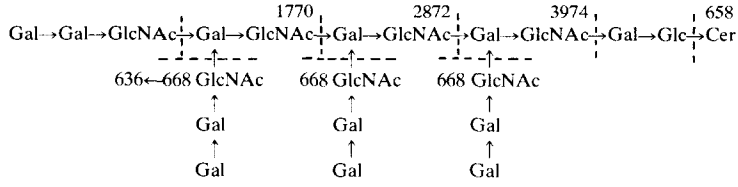
and 2. Besides *m/z* 668 for terminal Hex→Hex→HexNAc groups, intense sequence signals were seen at *m/z* 1770, 2872, and 3974 (nominal mass). The mass difference of 1102 a.m.u. corresponds to the addition of pentasaccharide units consisting of two lactosamine units plus an additional hexose residue (Figs. 3 and 4). These sequence ions are in agreement with the simplified structures **1** and **2**.

The major ceramide residue is represented in the f.a.b. spectra of all samples of this series by signals at *m/z* 658 and 660. In order to obtain more insight into the structures of the sphingosine and fatty acid residues, e.i. spectra were recorded for several samples. In Fig. 5, the e.i. mass spectrum of permethylated BI_{rab}-4 is presented up to *m/z* 1770. From the rearrangement ions at *m/z* 364, 404, and 406, it could be deduced that the ceramide residue is composed preponderantly of sphingosine, and tetracosenoic and tetracosanoic acids.

^{1}H-Nuclear-magnetic-resonance spectroscopy. — By analogy with the ceramide pentadecasaccharide[8,9] (BI_{rab}-2), it can be expected that ceramide eicosa-



Scheme 1. Fragmentation diagram of permethylated BI_{rab}-4; $C_{288}H_{514}N_{10}NaO_{128}$; mol. wt. 6188.26, nominal mass 6181.



Scheme 2. Fragmentation diagram of permethylated BI_{rab}-3; $C_{239}H_{428}N_8NaO_{103}$; mol. wt. 5058.03, nominal mass 5079.

saccharide (BI_{rab}-3) and pentacosasaccharide (BI_{rab}-4) contain several repeating fragments of practically identical primary structure. Correspondingly, much less of separate sugar proton resonances are expected than the theoretically possible number of 140 for BI_{rab}-3 or 175 for BI_{rab}-4, since a part of them will certainly coincide. As an example, five terminal α-D-galactopyranosyl groups of BI_{rab}-4 exhibited a common doublet of their anomeric protons (1D spectrum, Fig. 6a), and the connectivities of this H-1 resonance, found in the COSY and RCT spectra (Figs. 6b,c), yielded only one set of α-D-Gal*p* H-2,3,4,5,6,6′ resonances. The interpretation of crowded COSY spectra of complex oligosaccharides, including the peracetylated ceramide decasaccharide BI_{rab}-1, has been described in detail[27,28].

The resonances of BI_{rab}-3 and -4 were assigned by combining the data obtained with the aid of COSY, RCT-2 and -3 (two- and three-steps relayed coherence transfer), and NOESY spectra. Since in each of these spectra each connectivity is

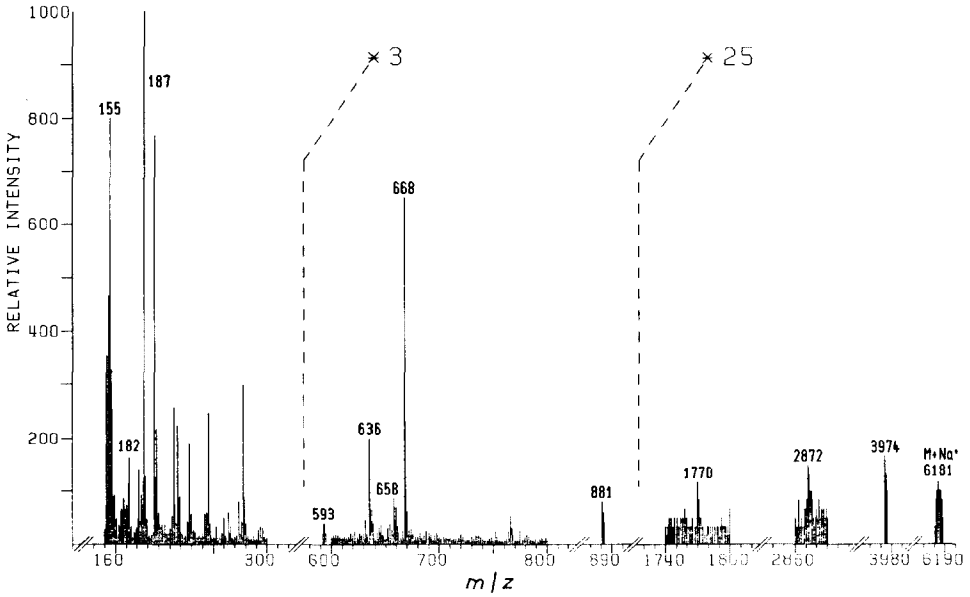


Fig. 3. Simplified f.a.b. mass spectrum of permethylated BI_{rab}-4 obtained at 4 kV acceleration voltage. Mass regions showing only background signals were omitted. Nominal masses are indicated in the spectrum. Further details are given in the Experimental section.

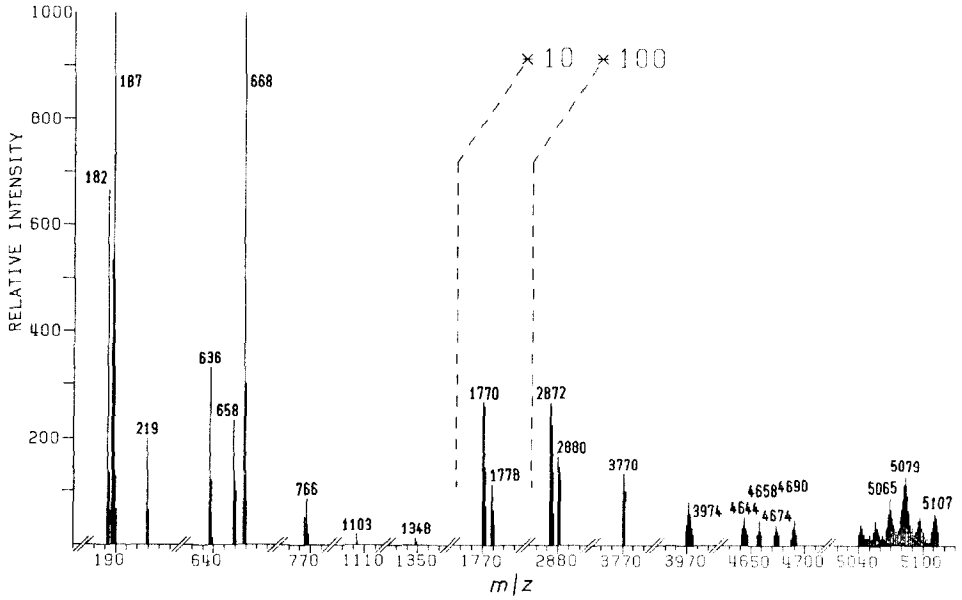
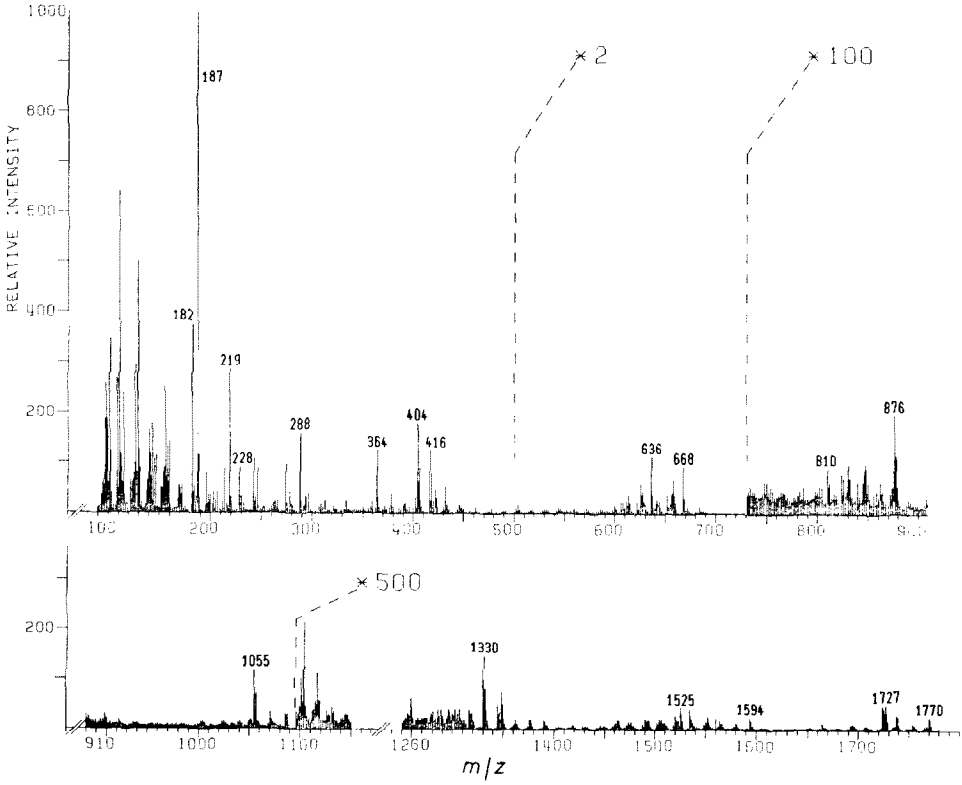


Fig. 4. F.a.b. mass spectrum of permethylated BI_{rab}-3 obtained at 5 kV acceleration voltage. For clarity, only the important sequence ions are shown together with nominal masses. For further details see the Experimental section.



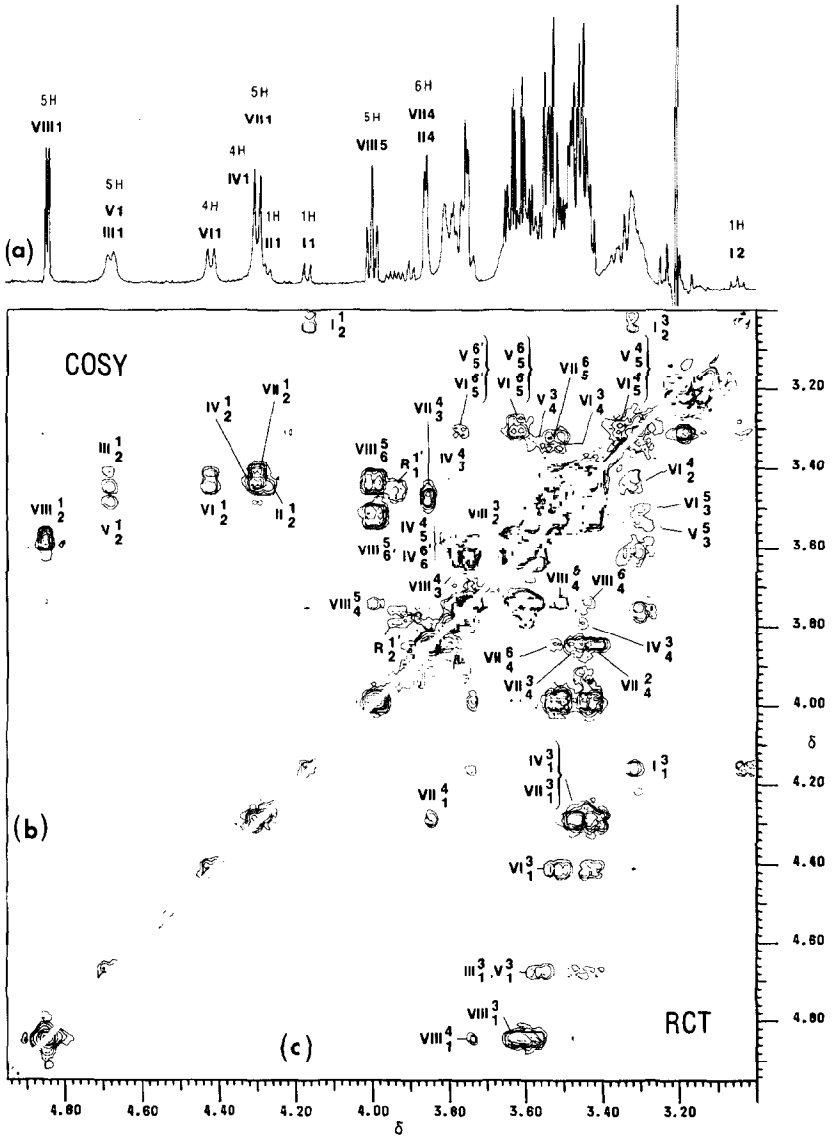


Fig. 6. Part of 500-MHz, ^{1}H-n.m.r. spectra of an 11mM solution of the deuterium-exchanged ceramide pentacosasaccharide (BI_{rab}-4) in di($^2H_3$)methyl sulfoxide containing 2% D_2O, at 333 K: (a) The resolution-enhanced 1D spectrum. The number of equivalent protons is indicated above each of the labelled signals. (b) Contour plot of the COSY spectrum recorded using the 90-t_1-90 puls sequence and saturation of HDO with two power levels. The correlation peaks are labelled, for example, for residue VI as VI^i_j, where the superscript i denotes the proton whose chemical shift defines the F_2 coordinate (horizontal scale) and the subscript j denotes the proton whose shift defines the F_1 coordinate (vertical scale). A spectral width of 2000 Hz in both dimensions was used with quadrature detection to collect a 256 × 1K data matrix with 112 transients for each t_1 increment. The recycle delay was 2 s. The matrix was zero-filled in the t_1 dimension and transformed in the magnitude mode using the sine-bell window function in both dimensions. Digital resolution in the resulting 512 × 512 matrix was 3.9 Hz per point. (c) Contour plot of the two-step relayed coherence transfer spectrum recorded by use of the 90-t_1-90-τ-180-τ-90 pulse sequence[25]. The τ delay was 30 ms, the recycle delay 2.1 s, and 224 transients for each t_1 were collected. Other details as in (b).

displayed twice, in form of two contours that are symmetrical with respect to the diagonal, we have adopted for space reduction the mode of presentation suggested by Wagner *et al.*[29], *i.e.*, we combined the upper triangle of a COSY spectrum with the lower triangle of a RCT or NOESY spectrum.

To illustrate the assignment procedure, the connectivities for GlcNAc-V of BI_{rab}-4 will be pursued in Fig. 6* (*cf.* Table II). The COSY V_2^1 contour clearly shows the connectivity from H-1 to H-2, but the further track gets lost in the thick knot around δ_{F_1,F_2} (H-2), *i.e.*, in the densely populated δ 3.4–3.6 region. However, the remote connectivity V_1^3 contour seen in the RCT-2 spectrum defines the V(H-3) chemical shift, δ_{F_2}(H-3), unequivocally. The ordinate through this contour leads to the V_4^3 cross-peak in the COSY spectrum, which, in turn, defines the V(H-4) chemical shift. The remaining connectivities for GlcNAc-V can also be found in the COSY spectrum. Although the V(H-4) and V(H-5) chemical shifts differ by 0.04 p.p.m. only, *i.e.*, their correlation contours V_5^4 and V_4^5 lie close to the diagonal, the clearly discernible V_5^6 and $V_5^{6'}$ cross-peaks confirm the location of the V(H-5) resonance. Besides, this resonance is confirmed by the remote connectivity V_3^5 contour in the RCT-2 spectrum. The location of the V(H-4) resonance is also directly indicated by the V_4^1 cross-peak in the RCT-3 spectrum (Fig. 7).

Other spin systems have been identified in a similar way, but, in addition, intraresidue n.O.e. was repeatedly used to assign 1,3,5 or 2,4 *syn*-axial ring protons[8,27,28] and other pairs of protons located in space closely, to each other, as H-6 or H-6', or both, on the one hand, and H-4 or H-5, or both, on the other hand. The I_4^3 cross-peak deserves special mention, since, due to the degeneracy of the H-3 and H-4 resonances of the D-glucose residue, the assignment of H-4 was inaccessible in any other way. There was a particular difficulty in assigning the Gal-IV H-4,5,6,6' resonances which constitute a "vicious circle" from the point of view of tracing scalar connectivities. Compared with the previous study[8] of the ceramide pentadecasaccharide (BI_{rab}-2), the assignment offered can be supported now by the intraresidue n.O.e. IV_1^5 cross-peak at δ_{F_2} 3.60–δ_{F_1} 4.31, which gives the H-5 assignment to the δ 3.60 resonance. Further arguments follow from the interresidue n.O.e. *(vide infra)*.

The chemical shifts assigned by the combination of the aforementioned methods are gathered in Table II. This analysis discriminated no more than eight closed spin-systems which could only mean that many of the 25 sugar units reside in similar environments, as noted above for the terminal α-D-galactopyranosyl groups. The residues that are equivalent from this point of view are labelled by the same Roman number (structures **2** and **3**).

The 2D spectra of BI_{rab}-3 (*cf.* COSY, Fig. 8b) were almost identical and all assignments derived from them were identical too, hence there is no need to list them. These two compounds were distinguished with the aid of integrals of several

*Arabic numerals denote proton positions in the given sugar residue specified by a Roman numeral (see Structures **2** and **3**).

TABLE II

CHEMICAL SHIFTS (δ) FOR BI_{rab}-4[a] AS OBTAINED BY TWO-DIMENSIONAL ^{1}H-N.M.R. SPECTROSCOPY[b]

Proton	*α*-D-*Gal*	*β*-D-*Gal*	*β*-D-*GlcNAc*-(*1→6*)	*β*-D-*GlcNAc*-(*1→3*)	*β*-D-(*1→3,6*)-*Gal*	*β*-D-*GlcNAc*-(*1→3*)	*β*-D-*Gal*	*Glc*	R[c]
	VIII	*VII*	*VI*	*V*	*IV*	*III*	*II*	*I*	
H-1	4.84	4.29	4.42	4.68	4.31	4.68	4.27	4.17	3.46; 3.93
H-2	3.59	3.42	3.44	3.47	3.43	3.43	3.44	3.04	3.78
H-3	3.63	3.48	3.51	3.57	3.45	3.57	3.45	3.32	3.89
H-4	3.74	3.86	3.33	3.35	3.80	3.35	3.86	3.32	5.37
H-5	4.00	3.47	3.31	3.30	3.60	3.30	3.47		5.55
H-6	3.44	3.53	3.62	3.62	3.59	3.62	3.53		
H-6′	3.52	[d]	3.77	3.77	3.80	3.77	[d]		

[a]For a solution in di($^2H_3$)methyl sulfoxide at 333 K; chemical shifts from the signal of Me_4Si.The assignments for BI_{rab}-3 are identical (see text). [b]COSY, RCT, and NOESY. [c]Numbering for the ceramide residue R: –CH_2(-*1,1′*)–CH(-*2*)–[NHC(O)Alk]–CH(-*3*)(OH)–CH(*4*)=CH(*5*)–Alk. [d]Not found (probably degenerate with H-6′).

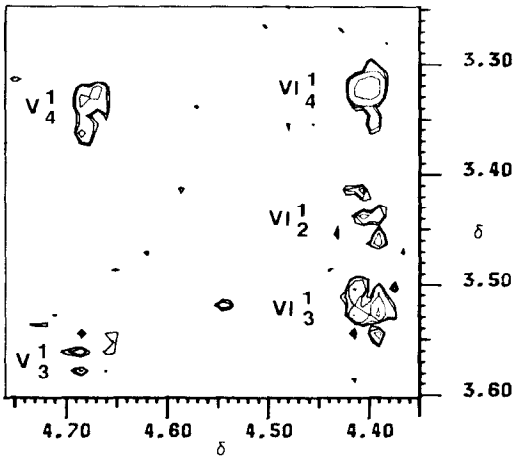


Fig. 7. Part of the three-step relayed coherence transfer spectrum of BI_{rab}-4 recorded by applying the 90-t_1-90-τ_1-180-τ_1-90-τ_2-180-τ_2-90 pulse sequence and saturation of HDO with two-power levels[25]. The τ_1 and τ_2 were set equal to 30 ms. Other details as in Fig. 6c.

well-separated peaks in their 1D spectra (Figs. 6a and 8a, and Table III), a unit integral intensity being set for the H-1 signal of the D-glucose residue. For comparison, the intensities of the one-proton signals of Glc (H-2) and ceramide (H-5) were also measured and confirmed the aforementioned conclusion within reasonable error limits.

The final stage in the determination of the primary structure of oligosaccharides by n.m.r. spectroscopy is the sequence and linkage analysis. Interresidue n.O.e. originating from the interaction between anomeric protons and protons at the glycosylation site, or sometimes in its vicinity, proved extremely useful for this purpose[7,8,28,30–32]. The interresidue cross-peaks are distinctly seen in Fig. 8c, along with a greater number of intraresidue ones. The sequence could be elucidated as follows. The II→1R through-space connectivity reflected in the δ_{F_2} 4.17–δ_{F_1} 3.46 cross-peak was evident. The identification of the III→4I connectivity was possible on account of the independent assignment of the Glc H-4 resonance from the I_4^2 cross-peak mentioned earlier. For the other end of the molecule, there was a clear VIII1→3VII correlation; simultaneously, the VIII1→4VII interaction also induced an n.O.e., in accord with previous findings concerning O-3-linked galactose residues[7,8,31,33,34]. This latter property enabled us to discriminate between the III1→4II and the V1→4IV connectivities, whereas the III1→3II and the V1→3IV connectivities reflecting the same sequence are indistinguishable, being represented by overlapped cross-peaks. Further, GlcNAc-VI H-1 exhibited two interresidue n.O.e. cross-peaks at δ 3.59 and 3.80. The former of these chemical shifts is common to Gal-IV H-5,6, but O-5 cannot be the site of a glycosyl residue. Although the latter value was attributed to protons at two sites (H-4,6′) potential for a glycosyl residue, H-4 could be ruled out for the following reason. According to

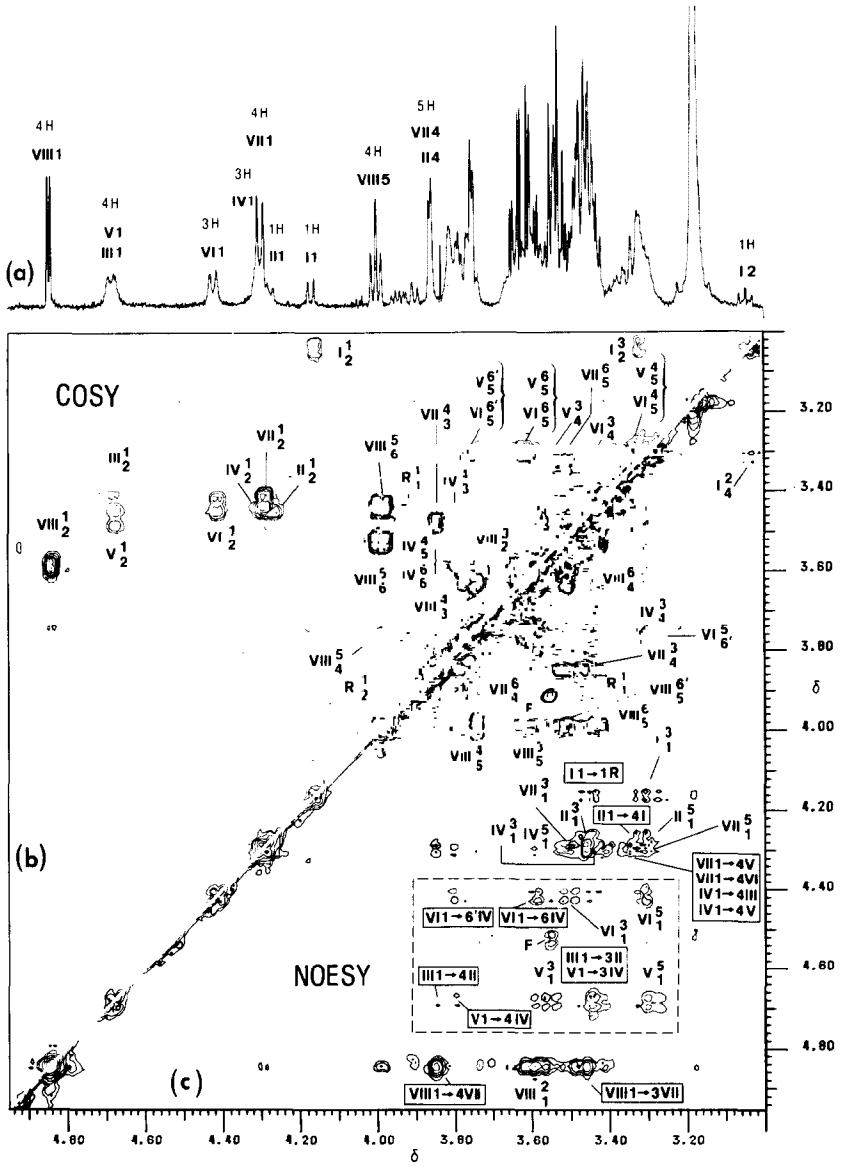


Fig. 8. Part of 500-MHz, ^{1}H-n.m.r. spectra of a 13mM solution of the ceramide eicosasaccharide (BI_{rab}-3): (a) The resolution-enhanced 1D spectrum. (b) Contour plot of the COSY spectrum. Recycle delay was 1.9 s and 96 transients were collected for each t_1. Saturation of HDO with two power levels was applied. (c) NOESY spectrum recorded by use of the 90-t_1-90-t_m-90 pulse sequence with HDO presaturation. Recycle delay was 2 s; mixing delay t_m was varied randomly by ±7% of its average value of 0.3 s to suppress remaining scalar coupling effects. Number of scans per each t_1 was 80. F: folded resonances. The labelling for intraresidue n.O.e. is the same as for COSY, whereas solid-line framed labels refer to interresidue n.O.e. and indicate glycosylation site and sequence. Cross peaks referring to anomeric protons of GlcNAc-III, -V, and -VI were markedly weaker and had to be reproduced from a lower level of the 2D matrix (area within the dashed-line frame). Other details as in Fig. 6.

TABLE III

MOLAR RATIOS OF SUGAR COMPONENTS IN BI_{rab}-3 AND BI_{rab}-4[a]

Residue	*Proton*	*BI_{rab}-3*		*BI_{rab}-4*	
		Calc.	*Found*	*Calc.*	*Found*
I	H-1	1	1.0[b]	1	1.0[b]
I	H-2	1	0.9	1	1.1
R[c]	H-5	1	1.1	1	1.1
VIII	H-1	4	3.8	5	4.8
II + IV + VII	H-1	8	8.1	10	10.6
VI	H-1	3	3.1	4	4.1
III + V	H-1	4	4.1	5	4.8

[a]As obtained from signal integrals in 1D ^{1}H-n.m.r. spectra. [b]Default value. [c]For numbering, see Table II, footnote *c*.

all experience with glycosylation-induced shifts[7,8,31,33–35], one would expect that H-4 for a O-4-linked residue would exhibit a low-field shifted resonance when compared with H-4 of the nonglycosylated at O-4 Gal-II and Gal-VII but, in fact, the opposite was true (δ 3.80 *vs.* 3.86). Hence, the VI1→6IV partial sequence seems to be well established and the problem of the "vicious circle" of the Gal-IV H-4,5,6,6′ resonances mentioned above can be viewed as resolved.

After the aforementioned partial sequences had been established, it remained to be shown, in which order the β-D-galactosyl residues are linked to the 2-acetamido-2-deoxy-D-glucosyl residues. That the first branching Gal-IV had to be bound to the head GlcNAc-III unit is obvious and does not require any experimental proof. In this sense, it is meaningless that the corresponding interresidue n.O.e. cross-peak was overlapped by those referring to the IV1→4V, VII1→4V, and VIII1→4VI linkages. However, the observation that the last two are also indistinguishable admits of different structural patterns. The essential question is whether each next bifurcation occurs in the (1→3)-, or rather the (1→6)-linked branch. This variation leads to five possible structures for BI_{rab}-3 and fourteen for BI_{rab}-4. We have shown[36] that, in analogy with BI_{rab}-2 (ref. 9), each consecutive bifurcation takes place in the (1→3)-linked branch, yielding a maximally elongated molecule containing a maximum number of (1→6)-linked branches. This branching pattern was established on the basis of the intensity ratio of the H-1 signals of GlcNAc-V and -VI for the degradation products obtained after removal of all α-D-Gal*p*-(1→3)-β-D-Gal*p* fragments. This leaves structures **2** and **3** as the only ones representing BI_{rab}-3 and -4.

After α-D-galactosidase treatment, permethylated glycosphingolipids furnished qualitatively identical, partially methylated alditol acetates. Their molar ratios, however, as determined by g.l.c. were converted to 4.3:2.7:0.8:7.2 (BI_{rab}-3_α) and 4.6:1:3.7:0.8:9.5 (BI_{rab}-4_α), respectively (Table I). This indicated that all four or five terminal α-D-galactopyranosyl groups are linked (1→3) to subterminal

D-galactopyranosyl residues, in agreement with a tetra- or penta-antenary structure. From the former compound, 5 or 6 mol of 3-*O*-substituted galactosyl residues, only 1 mol remained in both substances. The number and type of linkage of the residual 2-acetamido-2-deoxy-D-glucose, branching D-galactose, and D-glucose residues was unchanged in both degraded BI_{rab}-3 and BI_{rab}-4. Consequently, both substances were converted to a ceramide hexadecasaccharide (BI_{rab}-3_{α}) and a ceramide eicosasaccharide (BI_{rab}-4_{α}), respectively.

The f.a.b. spectra of permethylated BI_{rab}-3_{α} (Fig. 9a) and BI_{rab}-4_{α} (ref. 38) exhibited characteristic changes both in molecular weight-related ions and sequence ions. In agreement with the removal of four D-galactosyl residues from BI_{rab}-3, the major pseudomolecular ion ($M + Na^+$) was observed at *m/z* 4263 (nominal mass). Sequence ions produced by fission at glycosidic linkages of the 2-acetamido-2-deoxy-D-glucose residues were observed at *m/z* 464, 1362, 2260, and 3158 (see Scheme 3).

Further treatment of both substances by one cycle of Smith degradation led to an almost complete disappearance of terminal D-galactosyl groups and 4-*O*-substituted 2-acetamido-2-deoxy-D-glucosyl residues in both substances (BI_{rab}-$3_{\alpha SD}$ and BI_{rab}-$4_{\alpha SD}$) to give 3.4 or 4.5 mol of terminal 2-acetamido-2-deoxy-D-glucopyranosyl groups. In comparison to the α-D-galactosidase-treated glycolipids BI_{rab}-3_{α} and BI_{rab}-4_{α} the molar ratios of 3,6-di-*O*-substituted galactose, 3-*O*-substituted galactose and 4-*O*-substituted glucose residues remained unchanged, in agreement with a Gal-(1→3)-Gal-(1→4)-GlcNAc trisaccharide sequence of the branches in BI_{rab}-3 as well as BI_{rab}-4. About 15–20% of the former terminal galactosyl groups were not degraded. This corresponds to a relatively too low yield of terminal 2-acetamido-2-deoxyglucosyl groups and a too high yield of 4-*O*-substituted 2-acetamido-2-deoxyglucosyl residues in both substances, when compared with the theoretical yield of the corresponding type and linkage of sugars. These findings were corroborated by the results of f.a.b.m.s. of the permethylated compounds BI_{rab}-$3_{\alpha SD}$ and BI_{rab}-$4_{\alpha SD}$ (ref. 36). The f.a.b. spectrum of BI_{rab}-$3_{\alpha SD}$ is presented in Fig. 9b. The occurrence of several terminal 2-acetamido-2-deoxyglucosyl groups is indicated by the intense ion pair at *m/z* 260 and 228. The ion *m/z* 658 representing the major ceramide residue remained unchanged. An ion pair at *m/z* 464 and 432 clearly showed that a terminal Gal→GlcNAc unit is still present where the terminal galactosyl group escaped the Smith degradation. The f.a.b. spectrum also provided evidence that this disaccharide unit is present at the branch closest to the ceramide residue. The expected sequence ions for the desired molecule carrying only terminal 2-acetamido-2-deoxyglucosyl groups are *m/z* 954, 1648, and 2342 as indicated in Scheme 4A. However, besides *m/z* 2342, another ion 204 mass units higher is to be seen at *m/z* 2546. Since it is only the nonasaccharide ion 2342 that is accompanied by a decasaccharide ion 2546, it could be safely concluded that only the last branch is substituted by one galactosyl group residue (Scheme 4B). Accordingly, the major pseudomolecular ion was observed at *m/z* 3651. In addition, still another compound could be observed; it was represented by the sequence ions at *m/z* 709, 1403, and

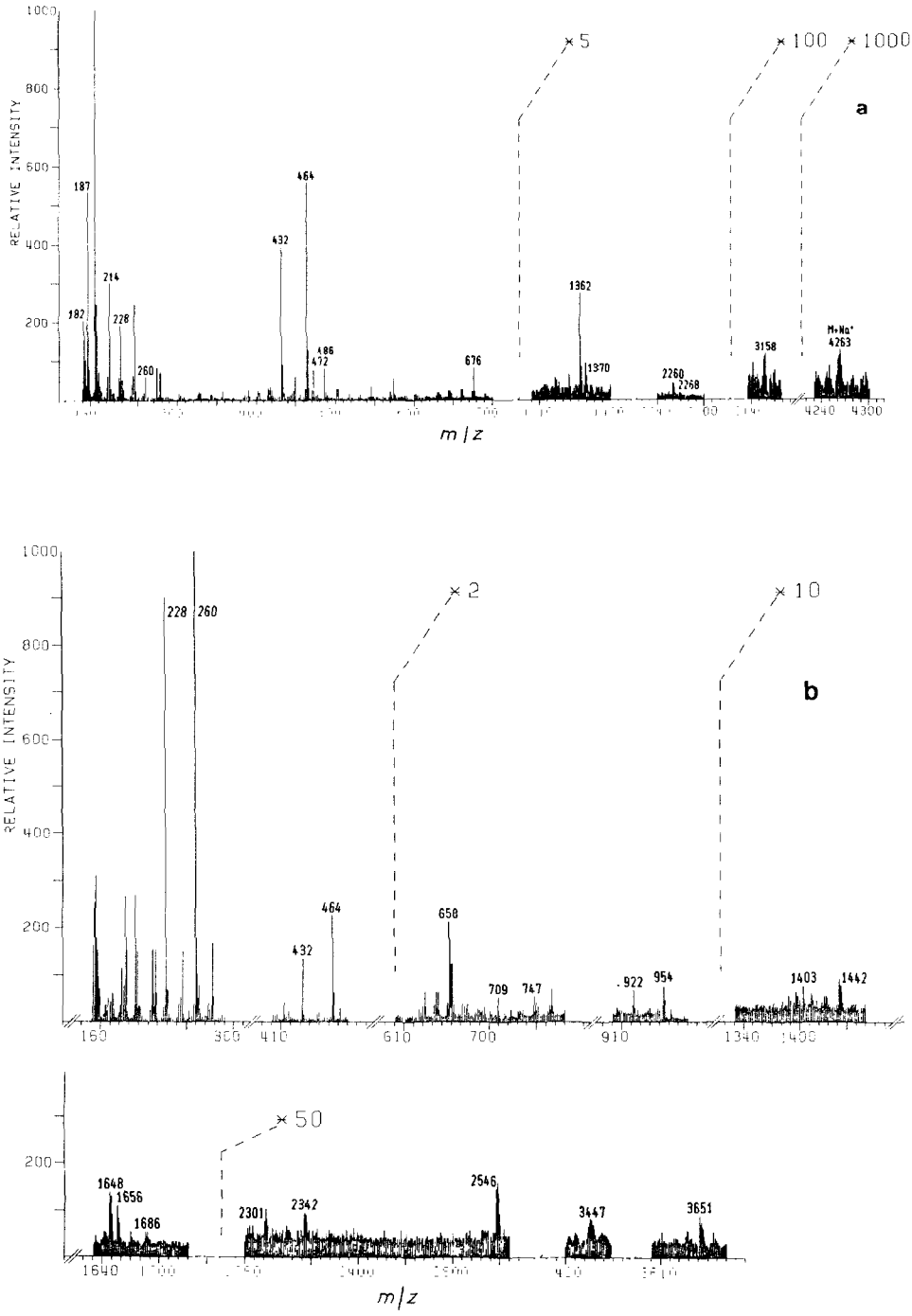
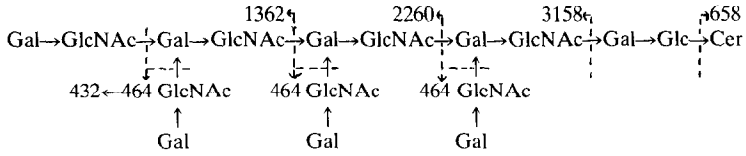
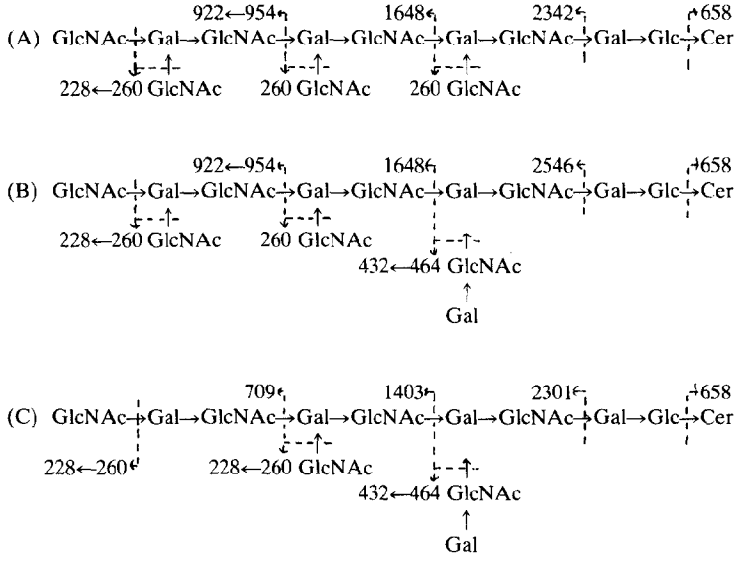


Fig. 9. Simplified f.a.b. mass spectra of permethylated ceramide hexadecasaccharide BI_{rab}-3_{α} (a) and the ceramide dodecasaccharide fraction BI_{rab}-$3_{\alpha SD}$ (b) obtained after additional Smith degradation. Mass numbers indicated are nominal masses: compare Schemes 3(a) and 4A–C(b).



Scheme 3. Fragmentation diagram of permethylated, α-D-galactosidase-treated BI_{rab}-3_{α}; $C_{203}H_{364}N_8ONa_{83}$, mol. wt. 4268.14; nominal mass 4263.



Scheme 4. Fragmentation diagrams of permethylated, α-D-galactosidase-treated and Smith-degraded BI_{rab}-$3_{\alpha SD}$: (A) $C_{167}H_{300}N_8NaO_6$, mol. wt. 3451.24, nominal mass 3447; (B) $C_{176}H_{316}N_8O_{68}Na$, mol. wt. 3655.47, nominal mass 3651; and (C) $C_{165}H_{297}N_8NaO_{63}$, mol. wt. 3410.00, nominal mass 3406.

2301, where one of the terminal 2-acetamido-2-deoxyglucosyl group residues is also lost (Fig. 9b and Scheme 4C). The ions present at 8 mass units above the major sequence ions were due to sodium salts of compounds that were undermethylated.

Blood-group serological activities. — As measured by the haemagglutination inhibition assay, ~6–8 μg of each BI_{rab}-1, -2, -3, and -4 completely inhibited the agglutination of human B erythrocytes by 50 μL of 4 haemagglutinating units of human anti-B (Table IV). In comparison to the very small amounts of human erythrocyte B-blood-group active glycosphingolipids necessary for an inhibition of B-specific haemagglutination, these amounts are relatively big. However, the weak activity identifies the B-like blood-group character of almost all rabbit erythrocyte glycolipids isolated thus far.

When a typical human monoclonal, cold-reacting IgM κ-type auto-anti-I was

TABLE IV

HAEMAGGLUTINATION INHIBITION TESTS OF GLYCOSPHINGOLIPIDS FROM RABBIT ERYTHROCYTE MEMBRANES

Glycolipid[a]	*Haemagglutination*[b]	
	Anti-B	*Anti-I*
BI_{rab}-1[c]	6.2	18
BI_{rab}-1_{α}[c]		2.1
BI_{rab}-2[c]	8.1	5.4
BI_{rab}-2_{α}[c]		0.9
BI_{rab}-3	6.2	6.3
BI_{rab}-3_{α}		0.4
BI_{rab}-4	8.1	0.6
BI_{rab}-4_{α}		0.7

[a]Abbreviations of glycolipids, see text. [b]Numbers are the minimum amount of glycolipids which completely inhibit specific haemagglutination by 4 haemagglutinating units of each human anti-B and auto-anti-I (Wal) against B- and O_{adult}-erythrocytes, respectively. Anti-I assays were performed at 0°. [c]See ref. 10.

used in the same haemagglutination inhibition test at 0° against human O_{adult} erythrocytes, BI_{rab} showed almost no inhibition of haemagglutination[10]. In contrast, BI_{rab}-2 and -3 exhibited weak (5–6 μg/50 μL of 4 haemagglutinating units antiserum) and BI_{rab}-4 strong (0.6 μg/50 μL) inhibitory activities (Table IV). Strong inhibition of I-specific haemagglutination was also achieved by all α-D-galactosidase-degraded derivatives having at least two (1→3,6)-galactosyl branching points (0.4–0.9 μg/50 μL). Moderate inhibition was accomplished by the single-branched partial degradation product BI_{rab}-1_{α}. For reasons of blood-group B-like character and I-activity of the corresponding rabbit red-cell glycolipids published elsewhere[10] and in this paper, they are shortly designated as "BI_{rab}" substances.

DISCUSSION

Structural information concerning the identity of the constituent sugar residues and groups, their number and anomeric configuration, the sites of glycosyl linkage, and the sequence was obtained from combined g.l.c.–m.s., and e.i.- and f.a.b.-m.s., and from 1D and 2D COSY, RCT, and n.O.e. ^{1}H-n.m.r. spectra in the *ab initio* manner. These results and the information concerning the gross branching patterns of the oligosaccharide chains[9,36] led to unique structures of **2** for BI_{rab}-3 and **3** for BI_{rab}-4.

Evidence for the homogeneity of these substances was based on analytical silica gel h.p.t.l.c. in different solvent systems and on the combined physicochemical data obtained by f.a.b.m.s. and n.m.r. spectroscopy. Therefore, this paper provides, for the first time, experimental evidence for the purification and complete structural elucidation of oligoglycosylceramides containing up to 25 sugars.

These substances obviously belong to a series of glycolipids occurring in rabbit erythrocytes[7–10,37,38], which, beginning with five sugar units, have an α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc trisaccharide as a common characteristic feature of all nonreducing terminal groups. Therefore, all glycolipids of this series reveal B-like blood-group activity. With increasing complexity, the substances each are enlarged by another five-sugar unit furnishing a ceramide deca- (BI_{rab}-1) (ref. 2), pentadeca- (BI_{rab}-2) (refs. 8–10), eicosa- (BI_{rab}-3), and pentacosasaccharide (BI_{rab}-4). Beginning with the second substance of this series (BI_{rab}-1) (ref. 7), a 3,6-galactosyl branching point is introduced, yielding two trisaccharide antennae as described above. Proceeding to higher oligomers, a new branching point is added each time, thus furnishing an additional trisaccharide branch. Thus, the number of the antennae (a) or of the branching points (b) can be calculated from the number of sugar units (n) by the formulae a = n/5 and b = (n/5) − 1. Common to all these substances, is a lacto-*N-neo*-tetraosylceramide core. An exception to this rule is represented by the ceramide heptasaccharide BI_{rab} (refs. 9,10), in which only one typical terminal trisaccharide is linked β-D-(1→3)-glycosidically to the core tetrasaccharide. Other glycosphingolipid fractions of rabbit erythrocytes previously described are obviously still more complex and comprise ~30 sugar units[39]. Although their purification and structural evaluation has not yet been accomplished, it is rather probable that they also fulfill the rules of the series.

The blood-group serological I-activity of all members of the series having 10 or more than 10 sugars has to be referred to the branching regions[11–14,16,17]. Thus, I-activity increases with the number of branches (Table IV). With decreasing complexity, this I-activity is dependent on the nature of the terminal region of the branches; BI_{rab}-1, -2, and -3 display their complete I-activities only after removal of their terminal α-D-galactosyl groups. I-Activities increase in proportion to the number of branching points. In BI_{rab}-4, however, the I-activity obviously is independent from the terminal groups of the *N*-acetyllactosamine residues linked β-(1→6) to the branching D-galactose units (Table IV).

The question arises as to the possible biological function of these substances which occur on the surface of rabbit erythrocytes in large quantities. Although this question cannot yet satisfactorily be answered, it is obvious, from recent experiments, that specific D-galactoconjugates from rabbit erythroid surface may serve as high density recognition sites in the complex set of cellular interactions during differentiation and maturation[40]. In addition, intact α-D-galactosyl groups are an essential prerequisite for the viability and normal life span of erythrocytes in blood circulation of the rabbit[41]. Apart from their occurrence in rabbits, glycoconjugates bearing terminal α-D-Gal*p*-(1→3) groups have been identified as characteristic markers of several murine malignant tumors[42–44]. Correspondingly, an α-D-(1→3)-galactosyltransferase could be isolated from membranous fractions of Ehrlich ascites cells having a preference for *N*-acetyllactosamine as acceptor structure[45].

In consequence, the blood-group B-like glycosphingolipids isolated in high yield, as well as their degradation products, may serve as model substances for

further studies not only of the I/i blood-group antigen complex, but also of the cellular differentiation and malignant transformation of tissues from rabbit or murine origin.

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