Carbohydrate Research, 228 (1992) 289–297
Elsevier Science Publishers B.V., Amsterdam

Further definition of the size of the blood group-i antigenic determinant using a chemically synthesised octasaccharide of poly-*N*-acetyllactosamine type*

Ten Feizi, Elizabeth F. Hounsell,
Glycoconjugates Section, Clinical Research Centre, Watford Road, Harrow, Middlesex, HA1 3UJ (United Kingdom)
Jocelyne Alais, Alain Veyrières†, and Serge David
Institut de Chimie Moléculaire d'Orsay, U.R.A. du C.N.R.S. D. 0462, Université Paris-Sud, Bât. 420, F-91405 Orsay (France)

(Received June 26th, 1991; accepted in revised form January 23rd, 1992)

ABSTRACT

In earlier studies, the minimum structure which inhibited the binding of anti-i to an i-active glycoprotein was the linear trisaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-D-Gal. There was an increasing hierarchy of inhibitory activities in the linear tetrasaccharide, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-GlcNAc, its methyl β-glycoside, and in the methyl β-glycoside of the hexasaccharide. The linear octasaccharide methyl β-glycoside in this series is approximately only half as active as the hexasaccharide methyl β-glycoside. Analyses by high resolution ^{1}H-n.m.r. of these two oligosaccharides indicated that they have similar conformations in solution, and there is no evidence for the occurrence of inter-molecular interactions which might partially hinder the binding of anti-i to the octasaccharide methyl β-glycoside. These results are consistent with the size of the i antigen being in the region of a hexasaccharide. It is proposed that the methyl aglycon group of the hexasaccharide methyl β-glycoside confers an above normal activity by presenting a hydrophobic area for additional contact in the vicinity of the antibody-combining site.

INTRODUCTION

The i and I antigens are carbohydrate determinants of human erythrocytes recognised by monoclonal autoantibodies in sera of patients with an autoimmune haemolytic disorder, cold agglutinin syndrome[1]. The expression of the i antigen, which predominates on the erythrocytes of the foetus and neonate, diminishes during the first year of life, while expression of the I antigen increases. These antigens are not confined to human erythrocytes but are expressed in a variety of human and animal cells, and their levels change in embryonic development, cell differentiation, and oncogenesis[1,2].

By haemagglutination inhibition and quantitative precipitation using mucin-type

* T.F., E.F.H., J.A., and A.V. congratulate Professor S. David on the occasion of his 70th birthday and thank him for the stimulus he has given for the collaborations between our two laboratories.
† Present address: Laboratoire de Chimie des Glucides, T74-E6, Université Pierre et Marie Curie, 4 Place Jussieu, F-75252 Paris, France.

0008-6215/92/$ 05.00

glycoproteins derived from human milk and ovarian cyst fluids and other sources, it was established[3,4] that the antigenic determinants recognized by anti-i and anti-I are expressed on oligosaccharides that serve as backbone structures for the major blood group antigens. Inhibition of binding experiments using oligosaccharides derived from ovarian cyst mucins[4,5] and glycosphingolipids derived from erythrocytes[6,7] established that i and I antigens are expressed on linear and branched oligosaccharides, respectively, consisting of the repeated disaccharide unit, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3 or 6) (poly-*N*-acetyllactosamine), of the type detected by biochemical approaches on erythrocytes[8–12].

Chemically synthesised oligosaccharides of poly-*N*-acetyllactosamine-type have been invaluable in estimating the sizes of the antigenic determinants recognized by individual monoclonal anti-i and anti-I antibodies[13–17] since limited amounts of oligosaccharides of this type can be isolated from natural sources. The question of the size of the antigenic determinant(s) recognized by anti-i has been addressed in three sets of experiments with synthetic oligosaccharides. In initial experiments (Experiment I, Table I), the chemically synthesised trisaccharides **1**, **2**, and **3** (refs. 18 and 19) were found not to inhibit binding of five monoclonal anti-i antibodies tested (designated Den, Galli, McC, Tho, and Nic)[13]. In a second series of experiments (Table I) where the chemically synthesised di- and tri-saccharides **4** and **5** (refs. 19 and 20) were tested with three monoclonal anti-i antibodies (Den, McC, and Tho), inhibition was achieved with compound **5**, but the amount of oligosaccharides required was high; 1600 and 800 nmol, respectively, in a reaction volume of 50 μL were required to give 50% inhibition of binding[15]. This indicated that compound **5** is only a part of the i antigenic determinants recognized by these antibodies. In a third series of experiments (Table I), trisaccharide **5**, tetrasaccharide **6**, its methyl β-glycoside (**7**), and the hexasaccharide methyl β-glycoside **8** (refs. 21, 22) were examined[17]. The tetrasaccharide **6** was 7, 35, and > 135 times more active than **5** as an inhibitor of McC, Tho, and Den antibodies, respectively, whereas the tetrasaccharide methyl β-glycoside (**7**) had a two–three-fold greater inhibitory activity in comparison with compound **6**. It was suggested[17] that the superior reactivity of the methyl β-glycoside is due to the stabilised β-D-anomeric configuration of the (formerly reducing) terminal 2-acetamido-2-deoxy-D-glucose residue, contrasting with the α-D-anomeric configuration that is predicted to be favoured[21,23] by the mutarotation equilibrium in aqueous solution of **6**. These observations together with the three–five-fold greater inhibitory activity of the hexasaccharide methyl β-glycoside (**8**), as compared with **7**, indicated that the anti-i combining sites recognise a sequence of at least three β-(1→3)-linked *N*-acetyllactosamine units. In the present study we compare the inhibitory activity of a chemically synthesised octasaccharide methyl β-glycoside (**9**) with that of **8** toward four of the anti-i antibodies (Den, Galli, McC, and Tho) and compare the results with those of high-resolution ^{1}H-n.m.r. spectroscopy which set out to detect any differences in the solution conformations of the two oligosaccharides that have four and three repeated *N*-acetyllactosamine units, respectively.

β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-D-Gal

1

β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)-D-Gal

2

β-D-Galp-(1→3)-β-D-GlcpNAc-(1→6)-D-Gal

3

β-D-Galp-(1→4)-D-GlcNAc

4

β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-D-Gal

5

β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-GlcNAc

6

β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-GlcNAcOMe

7

a *b* *c* *d*
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-GlcpNAc-(1→3)-

e *f*
-β-D-Galp-(1→4)-β-D-GlcpNAcOMe

8

a *b* *c* *d*
β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-

c' *d'* *e* *f*
-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAcOMe

9

β-D-Galp-(1→4)-β-D-GlcpNAcOMe

10

EXPERIMENTAL

Nuclear magnetic resonance spectroscopy. — 1D ^{1}H-n.m.r. spectroscopy was carried out at 600 MHz by use of a Varian instrument equipped with a Sun Microsystems computer. The sample (500 nmol) was lyophilised three times from D_2O (D-4501 from Sigma Chem. Co. Ltd., Poole, Dorset), and the residue taken up in 400 μL of D_2O (D-9764 from Sigma) containing 0.2 μL of acetone (Analar) as internal standard. The spectra were recorded at 22° and the chemical shifts reported relative to the signal of acetone at δ 2.225 from reference sodium 4,4-dimethyl-4-silapentane-1-sulphonate. 2D Correlated spectroscopy (DQCOSY) was performed at 500 MHz by use of a Bruker AM500 instrument equipped with an Aspect 3000 computer. Nuclear Overhauser enhancement (n.O.e.) experiments were carried out as a HOHAHA/ROESY experiment using a Bruker AM400, Aspect 3000 hardware, and a program written by Drs. C.

Bauer and T. Frenkiel of the National Institute for Medical Research, Mill Hill, London, NW7 1AA, United Kingdom.

Antibodies. — The four human anti-i antibodies, Den, Galli, McC, and Tho, investigated have been described previously[13,15,17] and were used as plasma.

Inhibition of antibody binding. — The inhibition of binding of antibodies to a Ii-active glycoprotein extract from foetal meconium[24], coated onto plastic microwells, at a concentration of 30 μg/mL was assayed[25] by use of anti-i Den, Galli, McC, and Tho sera at dilutions of 1:50 000, 1:40 000, 1:50 000, and 1:1000, respectively, and a total reaction volume of 50 μL. Bound antibodies were detected with ^{125}I-labeled rabbit antibodies to human μ chains (Dakopatts a/s, DK-2600 Glostrup, Denmark).

Oligosaccharides. — Chemical synthesis of the linear hexasaccharide methyl β-glycoside (**8**)[22] and of the octasaccharide methyl β-glycoside (**9**)[26,27] have been described previously.

RESULTS AND DISCUSSION

The inhibitory potency of the octasaccharide methyl β-glycoside **9**, toward the four anti-i antibodies, Den, Galli, McC, and Tho, was compared with that of the hexasaccharide methyl β-glycoside **8** (Table I); 25, 28, 34, and 25 nmol of **9** were required

TABLE I

Inhibition of the binding of anti-i antibodies by chemically synthesised linear oligosaccharides of the poly-*N*-acetyllactosamine series

Oligosaccharide	*Antibody (nmol of oligosaccharides giving 50% inhibition)*				
	Den	*Galli*	*McC*	*Tho*	*McDon*
Experiments I[a]					
1	[b]	[b]	[b]	[b]	[b]
2	[b]	[b]	[b]	[b]	[b]
3	[b]	[b]	[b]	[b]	[b]
Experiments II[c]					
4	[b]	[d]	[b]	[b]	[d]
5	[b]	[d]	800	1600	[d]
Experiments III[e]					
5	[b]	[d]	900	1100	[d]
6	105	70	100	29	110
7	41	27	52	9	60
8	9	9	11	2	12
Present experiments					
8	8	13	15	9	[d]
9	25	28	34	25	[d]

[a] Inhibition data taken from ref. 13. [b] Not inhibitory at highest concentration tested. [c] Inhibition data taken from ref. 15. [d] Not tested. [e] Inhibition data taken from ref. 17.

to give 50% inhibition of binding of the four antibodies, respectively. Clearly, with each antibody, compound **9** is approximately only half as active as an inhibitor when compared to compound **8**, which in the present experiments gave 50% inhibition at 8, 13, 15, and 9 nmol, respectively, with the four antibodies.

Three possible explanations were considered for the observed lower inhibitory activity of the octasaccharide methyl β-glycoside **9**, as compared to the hexasaccharide methyl β-glycoside **8** with the anti-i antibodies. First, that the octasaccharide methyl β-glycoside (**9**) might adopt a different, less favourable conformation in solution. There is precedent for the adoption of different conformations of oligosaccharides of different chain-length. For example, in the decasaccharide, $[\alpha\text{-NeuAc-}(2\rightarrow8)]_{10}$, the inner six residues adopt a conformation that differs from that of the shorter oligosaccharides of this series[28,29]. Second, it was considered that the octasaccharide glycoside may undergo intermolecular interactions which render inaccessible a part of the i-antigenic determinant. It is known, for example, that polysaccharide chains in solution occur as intermolecular aggregates in the form of two and three stranded helices[30], and it has been

TABLE II

^{1}H-N.m.r. chemical shifts (δ) of the hexa- (**8**) and octasaccharide methyl β-glycosides (**9**)

Unit of hexasaccharide **8**			*Unit of octasaccharide* **9**		
β-D-Galactose					
a	H-1	4.479	a	H-1	4.480
	H-2	3.538		H-2	3.538
	H-3	3.670		H-3	3.669
	H-4	3.923		H-4	3.924
c	H-1	4.465	c,c′	H-1	4.465
	H-2	3.578		H-2	3.578
	H-4	4.160		H-4	4.161
e	H-1	4.455	e	H-1	4.455
	H-2	3.576		H-2	3.576
	H-4	4.157		H-4	4.157
2-Acetamido-2-deoxy-β-D-glucose					
b	H-1	4.698	b	H-1	4.697
	H-2	3.784		H-2	3.784
	H-5	3.6		H-5	3.6
	H-6	3.952		H-6	3.952
	H-6′	3.85		H-6′	3.85
d	H-1	4.695	d,d′	H-1	4.694
	H-2	3.780		H-2	3.780
	H-6	3.949		H-6	3.949
	H-6′	3.85		H-6′	3.85
f	H-1	4.459	f	H-1	4.458
	H-2	3.74		H-2	3.74
	H-6	3.998		H-6	3.998
	H-6′	3.82		H-6′	3.82
	OMe	3.505		OMe	3.505
	NAc	2.032/2.029 (2:1)		NAc	2.032/2.029 (2:2)

proposed[31] that carbohydrate-to-carbohydrate interactions may occur with certain short oligosaccharides of clustered glycolipids. Third, it may be that some aspect inherent in the molecular geometry of the hexasaccharide methyl glycoside results in a tighter association with the antibody combining-site. ^{1}H-n.m.r. spectroscopy, with

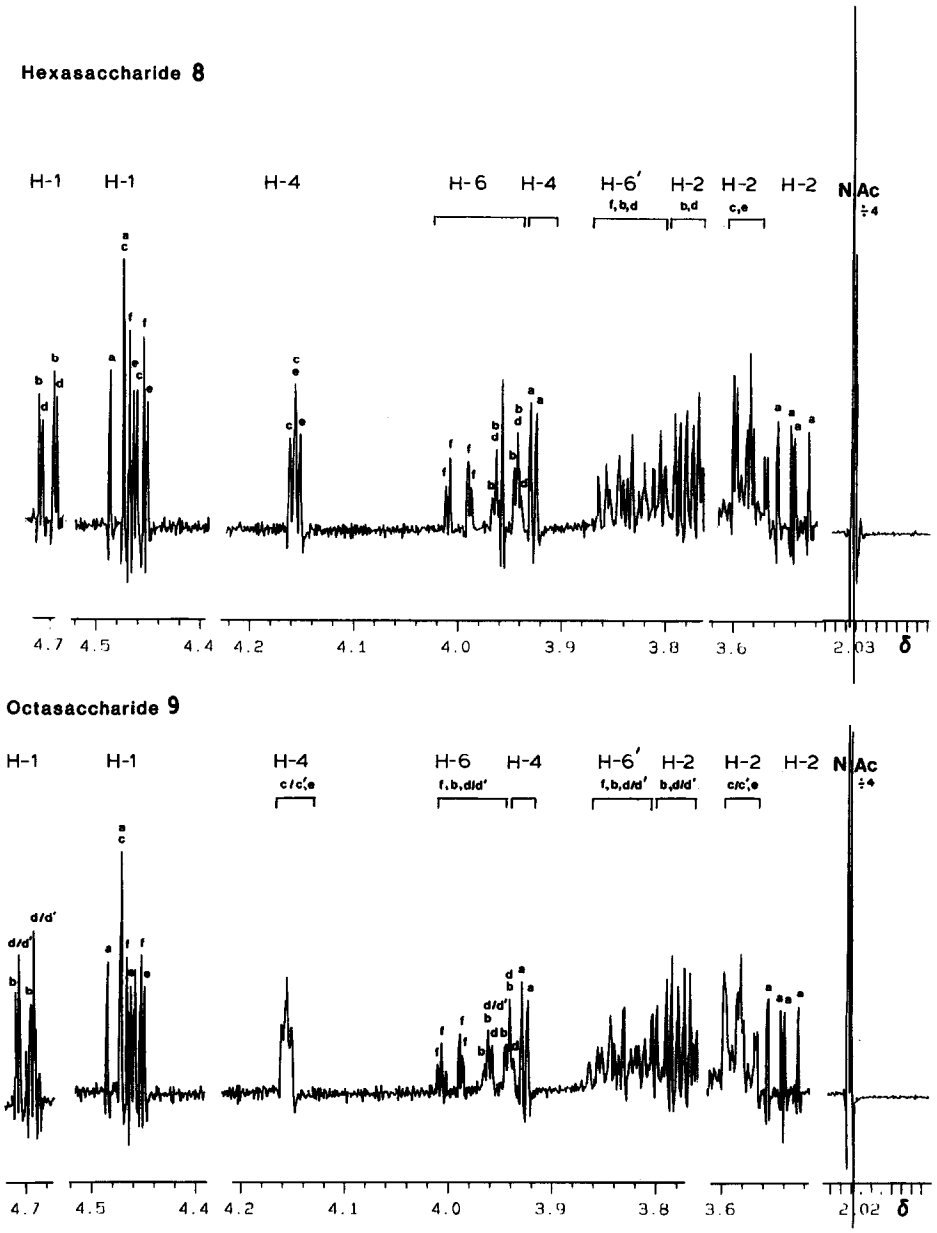

Fig. 1. 600-MHz ^{1}H-N.m.r. spectra of hexasaccharide methyl β-glycoside **8** and octasaccharide methyl β-glycoside **9**.

extensive assignment of chemical shifts by 2D methods, helped to exclude the first two alternatives as follows.

The observed chemical shifts for compounds **8** and **9** (Table II) were assigned by comparison of the spectra for the two oligosaccharides with the assumption that the signals that increased in intensity in the 1D spectrum of the octasaccharide **9** relative to that of the hexasaccharide **8** (Fig. 1) arise from the additional disaccharide unit (c′→d′) (Table II) in the octasaccharide **9**. The COSY experiments gave assignments for the H-2–H-4 signals of the Gal, and H-2 and H-6′ of the GlcNAc units. The close similarities of the chemical shifts of the hexa- and octa-saccharides suggested that the two oligosaccharides adopt the same conformations in solution. Neither line-broadening, nor strong n.O.e. effects were apparent in either sample at the concentrations used in the n.m.r. experiments, which were within the range tested in the inhibition assays. This is evidence against the formation of high-molecular-weight aggregates, and suggests the presence of relatively flexible molecules with no strong preference for adopting different conformations in solution.

A structural rather than conformational explanation for the lower inhibitory activity of the octasaccharide methyl β-glycoside **9**, as compared to the hexasaccharide methyl β-glycoside **8**, was therefore sought. In the previous study[17], it was reasoned that the superior activity of the tetrasaccharide methyl β-glycoside **7** as an inhibitor, as compared to that of the free, reducing tetrasaccharide **6**, may reflect the stabilised β-anomeric configuration of the terminal 2-acetamido-2-deoxy-D-glucopyranosyl re-

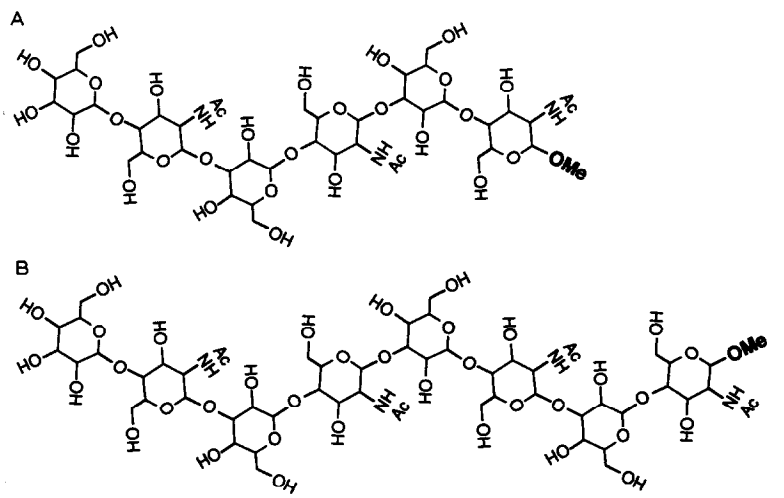

Fig. 2. A representation of the extended chain form of poly-*N*-acetyllactosamine taken from the X-ray crystallographic data for keratan sulphate (Brookhaven files deposited by S. Arnott *et al.*[32] and adapted to show a comparison of the relative orientation of monosaccharide residues and methyl aglycon group in the hexa- (**8**) (A) and octa-saccharide (**9**) (B) methyl β-glycosides. The position of the hydrophobic methyl aglycon group of the hexasaccharide **8** corresponds to the position of a D-galactosyl residue in the octasaccharide **9**. It is proposed that, in the hexasaccharide **8**, the methyl group forms a favourable contact in close proximity to the antibody combining site whereas the methyl aglycon group of the octasaccharide **8** would be in a different orientation and at a distance from the site.

sidue, and the superior activity of the hexasaccharide methyl β-glycoside **8** compared to that of **7** was consistent with areas within the additional disaccharide unit being recognised by the anti-i combining-sites. The present finding that the hexasaccharide methyl β-glycoside **8** is twice as potent as the octasaccharide methyl β-glycoside **9** as an inhibitor of the four anti-i antibodies indicated that the methyl aglycon group of **8** confers a greater inhibitory activity than does the additional inner disaccharide unit in compound **9**, which represents the natural sequence in linear poly-*N*-acetyllactosamine chains (Fig. 2). We suggest, therefore, that the size of the i antigenic determinant recognized by these antibodies is close to that of a hexasaccharide, and that the methyl aglycon group presents a hydrophobic area for additional contact with a hydrophobic area in the vicinity of the antibody combining-site. Such superior inhibitory activity conferred by a methyl β-glycoside aglycon group was also observed[17] in studies with anti-I-Ma for which the natural recognition structures are β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Gal or -D-GalNAc (refs. 4, 5, 7) and in which the key sequence is β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-OCH_2-, -CH_2 being C-6 of the internal D-galactosyl or 2-acetamido-2-deoxy-D-galactosyl residue[14,16]. The chemically synthesised methyl β-glycoside **10** was approximately twice as active as **2** as an inhibitor of the binding of anti-I-Ma (ref. 17). Since longer oligosaccharides with extended sequences, for example, 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→3)]-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc, were not available for comparison, it is not known whether the inhibitory activity of **10** is indeed superior, as it appears to be in the case of **8** relative to **9** with the anti-i antibodies.

A referee has suggested an alternative explanation, namely that the anti-i antibodies may bind along oligosaccharide **9** in two ways, one of these having a lower affinity, whereas oligosaccharide **8** may be bound in one way only, with a high affinity. However, the anti-i antibodies are not necessarily all of groove-type, as discussed below.

Advances in chemical synthesis are facilitating considerably the range of oligosaccharides that can be synthesised[33,34]. Further synthetic work to produce oligosaccharides, such as the penta- and hepta-saccharide methyl β-glycosides terminating with a methyl β-D-galactopyranosyl residue, as well as oligosaccharide analogues having 2-acetamido-2-deoxy-β-D-glucopyranosyl nonreducing end-groups should help to further delineate the i antigenic determinants recognised by the individual antibodies, and also to extend preliminary observations[35] made with immobilized oligosaccharides (neoglycolipids), which suggested that the combining site of anti-i Den is of a cavity-type, *i.e.*, requires the D-galactose residue of the *N*-acetyllactosamine unit to be terminal, and that of anti-i Tho is a groove-type, *i.e.*, is able to accommodate the i sequence in the presence of a 2-acetamido-2-deoxy-β-D-glucopyranosyl residue linked to O-3 of a D-galactose unit at the nonreducing end. Such detailed information is highly desirable for these monoclonal human autoantibodies, anti-i and anti-I, since they are currently unrivalled as reagents that can provide information on the branching patterns of oligosaccharides of poly-*N*-acetyllactosamine type on isolated glycoproteins, on whole cells and tissues[36].

ACKNOWLEDGMENTS

The authors thank Dr. Jean Picard for assistance with the immunochemical assays and Drs. J. Feeney, T. Frenkiel, and C. Bauer of the MRC Biomedical NMR Centre for their support with the n.m.r. experiments.

REFERENCES

1 T. Feizi, *Immunol. Commun.*, 10 (1981) 127–156.
2 T. Feizi, *Nature (London)*, 314 (1985) 53–57.
3 T. Feizi, E. A. Kabat, G. Vicari, B. Anderson, and W. L. Marsh, *J. Exp. Med.*, 133 (1971) 39–52.
4 T. Feizi, E. A. Kabat, G. Vicari, B. Anderson, and W. L. March, *J. Immunol.*, 106 (1971) 1578–1592.
5 L. Rovis, E. A. Kabat, M. E. A. Pereira, and T. Feizi, *Biochemistry*, 12 (1973) 5355–5360.
6 H. Niemann, K. Watanabe, S. Hakomori, R. A. Childs, and T. Feizi, *Biochem. Biophys. Res. Commun.*, 81 (1978) 1286–1293.
7 T. Feizi, R. A. Childs, K. Watanabe, and S. Hakomori, *J. Exp. Med.*, 149 (1979) 975–980.
8 J. Kościèlak, H. Miller-Podraza, R. Krauze, and A. Piasek, *Eur. J. Biochem.*, 71 (1976) 9–18.
9 J. Jarnefelt, J. Rush, Y. T. Li, and R. A. Laine, *J. Biol. Chem.*, 253 (1978) 8006–8009.
10 J. Finne, T. Krusius, H. Rauvala, R. Kekomaki, and G. Myllyla, *FEBS Lett.*, 89 (1978) 111–115.
11 M. N. Fukuda, M. Fukuda, and S. Hakomori, *J. Biol. Chem.*, 254 (1979) 5458–5465.
12 P. Hanfland, H. Egge, U. Dabrowski, S. Kuhn, D. Roelcke, and J. Dabrowski, *Biochemistry*, 20 (1981) 5310–5319.
13 T. Feizi, E. Wood, C. Augé, S. David, and A. Veyrières, *Immunochemistry*, 15 (1978) 733–736.
14 E. A. Kabat, J. Liao, and R. U. Lemieux, *Immunochemistry*, 15 (1978) 727–731.
15 E. Wood and T. Feizi, *FEBS Lett.*, 104 (1979) 135–140.
16 E. A. Kabat, J. Liao, M. H. Burzynska, T. C. Wong, H. Thøgersen, and R. U. Lemieux, *Mol. Immunol.*, 18 (1981) 873–881.
17 H. C. Gooi, A. Veyrières, J. Alais, P. Scudder, E. F. Hounsell, and T. Feizi, *Mol. Immunol.*, 21 (1984) 1099–1104.
18 C. Augé and A. Veyrières, *J. Chem. Soc., Perkin Trans. 1*, (1977) 1343–1345.
19 C. Augé, S. David, and A. Veyrières, *Nouv. J. Chim.*, 3 (1979) 491–497.
20 J. Alais and A. Veyrières, *Carbohydr. Res.*, 93 (1981) 164–165.
21 A. Veyrières, *J. Chem. Soc., Perkin Trans. 1*, (1981) 1626–1629.
22 J. Alais and A. Veyrières, *Tetrahedron Lett.*, 24 (1983) 5223–5226.
23 R. Kuhn and A. Gauhe, *Chem. Ber.*, 95 (1962) 518–522.
24 E. Wood, J. Lecomte, R. A. Childs, and T. Feizi, *Mol. Immunol.*, 16 (1979) 813–819.
25 H. C. Gooi, J. K. Picard, E. F. Hounsell, M. Gregoriou, A. R. Rees, and T. Feizi, *Mol. Immunol.*, 22 (1985) 689–693.
26 J. Alais and A. Veyrières, *Tetrahedron Lett.*, 28 (1987) 3345–3348.
27 J. Alais and A. Veyrières, *Carbohydr. Res.*, 207 (1990) 11–31.
28 E. A. Kabat, J. Liao, E. F. Osserman, A. Gamian, F. Michon, and H. J. Jennings, *J. Exp. Med.*, 168 (1988) 699–711.
29 F. Michon, J.-R. Brisson, and H. J. Jennings, *Biochemistry*, 26 (1987) 8399–8405.
30 D. A. Rees, *MTP Int. Rev. Sci., Ser. 1*, 5 (1975) 1–42.
31 I. Eggens, B. Fenderson, T. Toyokuni, B. Dean, M. Stroud, and S. Hakomori, *J. Biol. Chem.*, 264 (1989) 9476–9484.
32 S. Arnott, J. M. Guss, D. W. L. Hukins, I. C. M. Dea, and D. A. Rees, *J. Mol. Biol.*, 88 (1974) 175.
33 R. R. Schmidt, *Angew. Chem., Int. Ed. Engl.*, 25 (1986) 212–235.
34 H. Paulsen, *Angew. Chem., Int. Ed. Engl.*, 29 (1990) 823–839.
35 P. W. Tang, P. Scudder, H. Mehmet, E. F. Hounsell, and T. Feizi, *Eur. J. Biochem.*, 160 (1986) 537–545.
36 T. Feizi and R. A. Childs, *Biochem. J.*, 245 (1987) 1–11.