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Characterization of the specificity of binding of *Moluccella laevis* lectin to glycosphingolipids

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The specificity of *Moluccella laevis* lectin was investigated by analysing its binding to glycosphingolipids separated on thin-layer chromatograms or adsorbed on microtitre wells. The binding activity of the lectin was highest for glycosphingolipids with terminal α-linked *N*-acetylgalactosamine, both in linear structures, as the Forssman glycosphingolipid, GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer, and in branched structures, as glycosphingolipids with the blood group A determinant, GalNAcα3(Fucα2)Galβ. In addition, the lectin bound, though considerably more weakly, to linear glycosphingolipids with terminal α-linked galactose. When considering the use of the *M. laevis* lectin for biochemical and medical purposes this cross-reactivity may be of importance.

Keywords: *Moluccella laevis* lectin; glycosphingolipid; *N*-acetylgalactosamine.

Nomenclature: The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: *Eur J Biochem* (1977) **79**:11–21, *J Biol Chem* (1982) **257**:3347–51, and *J Biol Chem* (1987) **262**:13–18). It is assumed that Gal, Glc, GlcNAc, GalNAc, and NeuAc are of the D-configuration, Fuc of the L-configuration, and all sugars present in the pyranose form.

Introduction

Moluccella laevis seeds contain a lectin which interacts specifically with blood group A, N and Tn determinants, resulting in an 'independent' agglutination of human blood group A, O^{NN} and Tn (O^{MM}) erythrocytes [1, 2]. This is due to pronounced preference of the *M. laevis* lectin (MLL) for α-linked *N*-acetylgalactosamine, as demonstrated by hapten inhibition of haemagglutination [1, 2] and by binding to glycoproteins in an enzyme immunoassay [2]. *N*-Acetylgalactosamine was the best monosaccharide inhibitor of lectin-mediated haemgglutination, 300–500 times more potent than galactose, and the methyl α-glycosides of both *N*-acetylgalactosamine and galactose were better inhibitors than the corresponding methyl β-glycosides. Among the glycoproteins tested, the lectin interacted most strongly with asialo ovine submaxillary mucin, which contains many α-linked *N*-acetylgalactosamine residues bound to serine or threonine.

MLL is a glycoprotein composed of three non-identical subunits: one subunit is a heterodimer of 67 kDa consisting of two S-S-linked polypeptides of 28 and 46 kDa, and the other two subunits have molecular sizes of 42 kDa and 26 kDa, respectively [1, 3]. The latter subunit has been isolated and found to have the same haemagglutinating activity and binding specificity as the native protein [3, 4].

In order to obtain more data on the carbohydrate specificity of MLL, the interaction of the lectin with many glycosphingolipids was investigated, using a chromatogram binding assay and by binding to glycosphingolipids adsorbed in microtitre wells.

Materials and methods

Lectin preparation

MLL was isolated from ground defatted *Moluccella laevis* seeds by affinity chromatography on Sepharose-bound galactose, in the presence of 8 M urea, as described [3]. This preparation, designated U-MLL, was used for the binding studies.

Glycosphingolipid preparations

Total acid and non-acid glycosphingolipid fractions, from the sources given in Table 1, were prepared by extraction with chloroform and methanol, mild alkaline degradation, dialysis, silicic acid and DEAE-cellulose chromatography

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Table 1. Binding of *Moluccella laevis* lectin to glycosphingolipids separated on thin-layer chromatograms.

No. Trivial name	*Glycosphingolipid structure*[a]	*Binding*[b]	*Source*	*References*
1. Globotri	Galα4Galβ4GlcβCer	+	Human erythrocytes	[30]
2. Isoglobotri	Galα3Galβ4GlcβCer	(+)	Dog small intestine	[29]
3. A-4	GalNAcα3(Fucα2)Galβ4GlcβCer*	+	Rat small intestine	[28]
4. Forssman	GalNAcα3GalNAcβ3Galα4Galβ4GlcβCer	++	Dog small intestine	[29]
5.	GalNAcα3GalNAcβ3Galα3Galβ4GlcβCer	++	Rat colon carcinoma	[31]
6. P_1	Galα4Galβ4GlcNAcβ3Galβ4GlcβCer	+	Human erythrocytes	[32]
7. B5	Galα3Galβ4GlcNAcβ3Galβ4GlcβCer	(+)	Rabbit erythrocytes	[33]
8.	Galα3Galβ3GlcNAcβ3Galβ4GlcβCer*	+	Monkey intestine[c]	
9.	GalNAcα3Galβ4GlcNAcβ3Galβ4GlcβCer*	+	Human erythrocytes[c]	
10. A6-1	GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4GlcβCer*	+	Rat small intestine	[34]
11. A6-2	GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcβCer	++	Human erythrocytes	[35]
12. A7-1	GalNAcα(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4GlcβCer	++	Human small intestine	[36]
13. A7-2	GalNAcα(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4GlcβCer	++	Dog small intestine	[10]
14. A-12	GlcNAcα3(Fucα2)Galβ3GlcNAcβ3(GalNAcα3(Fucα2)Galβ3-GlcNAcβ6)Galβ3GlcNAcβ3Galβ4GlcβCer*	+	Rat small intestine	[37, 38]
15. A-12	GlcNAcα3(Fucα2)Galβ3GlcNAcβ3(GalNAcα3(Fucα2)Galβ4-GlcNAcβ6)Galβ3GlcNAcβ3Galβ4GlcβCer*	+	Rat small intestine	[37, 38]
16.	Galα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcβCer*	+	Rabbit erythrocytes	[39]
17.	Galα3Galβ4GlcNAcβ3(Galα3Galβ4GlcNAcβ6)Galβ4GlcNAcβ-3Galβ4GlcβCer*	+	Rabbit erythrocytes	[40]
18.	GlcβCer*	–	'Various'	[30]
19.	GalβCer*	–	'Various'	[30]
20. Sulfatide	SO_3-3GalβCer*	–	Human kidney	[41]
21. LacCer	Galβ4GlcβCer*	–	Dog small intestine	[29]
22. TriN	GlcNAcβ3Galβ4GlcβCer	–	Malignant melanoma[d]	
23. GgO3	GalNAcβ4Galβ4GlcβCer	–	Guinea pig erythrocytes	[42, 43]
24. GM3	NeuAcα3Galβ4GlcβCer*	–	Human brain	[44]
25. Globoside	GalNAcβ3Galα4Galβ4GlcβCer	–	Human erythrocytes	[30]
26. Isogloboside	GalNAcβ3Galα3Galβ4GlcβCer	–	Rat colon carcinoma	[45]
27. GgO4	Galβ3GalNAcβ4Galβ4GlcβCer	–	Mouse small intestine	[46]
28. Paragloboside	Galβ4GlcNAcβ3Galβ4GlcβCer*	–	Human erythrocytes[e]	
29. Lactotetra	Galβ3GlcNAcβ3Galβ4GlcβCer	–	Human meconium	[47]
30. GM2	GalNAcβ4(NeuAcα3)Galβ4GlcβCer	–	Human brain	[48, 49]
31. Para-Forssman	GalNAcβ3GalNAcβ3Galα4Galβ4GlcβCer	–	Human erythrocytes	[50]
32. SPG	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcβCer*	–	Human erythrocytes	[51]
33. GM1	Galβ3GalNAcβ4(NeuAcα3)Galβ4GlcβCer*	–	Human brain	[30]
34. H5-2	Fucα2Galβ4GlcNAcβ3Galβ4GlcβCer	–	Human erythrocytes	[52]
35. B6-2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcβCer	–	Human erythrocytes	[52]
36. B6-1	Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4GlcβCer	–	Monkey intestine[f]	
37. B7-1	Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4GlcβCer	–	Monkey intestine[f]	
38. $iGbnLc_6$	GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4GlcβCer	–	Rat colon carcinoma	[45]

[a] The GalNAcα and Galα parts have been underlined.
[b] ++ indicates a significant darkening on the autoradiogram when 0.1 μg of the glycosphingolipid was applied on the thin-layer plate, + indicates that 0.5 μg was required to obtain binding, (+) that 2 μg was required for binding, while – indicates no binding even at 4 μg of glycosphingolipid. For the glycosphingolipids denoted by an * the binding/non-binding was tested in mixtures, i.e. the data are not quantitative.
[c] Glycosphingolipid No. 8 was generated from Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4GlcβCer from monkey intestine (No. 36), and glycosphingolipid No. 9 from GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcβCer from human erythrocytes (No. 11), by incubation in 0.05 M HCl at 80 °C for 2 h.
[d] Karlsson K-A, Samuelsson BE, unpublished.
[e] Prepared by neuraminidase-treatment of NeuAcα3Galβ4GlcNAcβ3Galβ4GlcβCer from human erythrocytes (No. 32).
[f] Strömberg N, Karlsson K-A, unpublished.

as described [5]. The individual glycosphingolipids were in general obtained by acetylation [6] of the total glycosphingolipid fractions, and separation by repeated chromatography on silicic acid columns. The identity of the purified glycosphingolipids was confirmed by mass spectrometry [7], proton NMR spectroscopy [8–11], and degradation studies [12, 13].

For generation of substances No. 8 and No. 9 in Table 1, 1.1 mg of B6 type 1 glycosphingolipid [Galα3(Fucα2)Galβ3-GlcNAcβ3Galβ4Glcβ1Cer from monkey intestine; No. 36], and 2.3 mg of A6 type 2 glycosphingolipid [GalNAcα3-(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer from human erythrocytes; No. 12], were incubated in 0.05 M HCl at 80 °C for 2 h, followed by partition according to Folch-Pi *et al.* [14]. The lower phase was evaporated and the glycosphingolipids used for chromatogram binding experiments.

Labelling

Goat anti-rabbit antibodies (Dakopatts a/s, Glostrup, Denmark) were ^{125}I-iodinated using the IODO-GEN method [15].

Chromatogram binding assay

The binding of U-MLL to glycosphingolipids separated on thin-layer plates was examined as described elsewhere [16]. Mixtures of glycosphingolipids (40 µg) or pure compounds (1–4 µg) were separated on aluminium-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), using chloroform:methanol:water 60:35:8 (by vol) as solvent. Chemical detection was made by staining with the anisaldehyde reagent [17]. The chromatograms prepared for binding experiments were treated with 0.3% polyisobutylmethacrylate, w/v (Plexigum P28, Röhm, GmbH, Darmstadt, Germany) in hexane:diethylether 1:3, by vol, dried and subsequently soaked in 0.01 M phosphate-buffer/0.14 M NaCl, 5 mM KCl, pH 7.3 (PBS), containing 2% bovine serum albumin, w/v, 0.1% NaN_3, w/v, and 0.1% Tween 20, v/v (Sol. I). After 2 h at room temperature, the plates were covered with U-MLL (diluted to 50–100 µg ml^{-1} in Sol. I). Incubation was for 2 h at room temperature, followed by repeated washings with PBS. The chromatograms were thereafter incubated with rabbit anti-U-MLL serum (diluted 100 times in PBS containing 2% bovine serum albumin, w/v, 0.1% NaN_3, w/v; Sol. II) for 2 h at room temperature, followed by washings with PBS. Final incubation was with ^{125}I-labelled anti-rabbit antibodies (diluted in Sol. II to $2–4 \times 10^6$ cpm ml^{-1}) for 2 h at room temperature, followed by washings with PBS. The plates were exposed to XAR-5 X-ray films (Eastman Kodak, Rochester, NY) for 12–72 h, using an intensifying screen.

Microtitre well assay

Binding to pure glycosphingolipids in microtire wells was performed as described [18]. In brief, serial dilutions of glycosphingolipids (each dilution in triplicate) in methanol were applied into microtitre wells (Cooks M24, Nutacon, Holland), and left overnight at room temperature to let the solvent evaporate. The wells were then blocked with 200 µl Sol. I per well for 2 h at room temperature. Thereafter, 50 µl of U-MLL (50 µg ml^{-1} in Sol. I) was added per well, and incubated for 4 h at room temperature. After six washings with PBS containing 0.1% Tween 20, v/v (Sol. III), 50 µl of rabbit anti-U-MLL serum (diluted 100 times in Sol. II) was added to the wells, followed by incubation for 4 h at room temperature. After washings with Sol. III, 50 µl of ^{125}I-labelled goat anti-rabbit antibodies (diluted in Sol. II to 2×10^6 cpm ml^{-1}) was applied per well, and incubated for 12 h at room temperature. The wells were thereafter washed six times with Sol. III; they were then cut out and the radioactivity counted in a gamma counter.

Molecular modelling

Minimum energy conformations of selected glycosphingolipids were calculated within the Biograf molecular modelling program (Molecular Simulations Inc., Waltham, MA) using the Dreiding-II force field [19] on a Silicon Graphics 4D/25TG workstation. Charges were generated using the charge equilibration method [20], and a distance dependent dielectric constant of 3.5 was used for the Coulomb interactions. In addition, a special hydrogen bonding term was used in which D_{hb} was set at 4 kcal mol^{-1} [19].

Results

Chromatogram binding assay

The binding of U-MLL to glycosphingolipids separated on thin-layer chromatograms (Fig. 1) was investigated using a broad panel of reference compounds (summarized in Table 1). The binding activity of the lectin was approximately the same for all glycosphingolipids having a terminal α3-linked *N*-acetylgalactosamine, as the Forssman glycosphingolipid (GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer; No. 4 in Table 1, and lane 3 in Fig. 1), the Forssman analogue from rat colon carcinoma (GalNAcα3GalNAcβ3Galα3Galβ4Glcβ1Cer; No. 5), and all glycosphingolipids with the blood group A determinant [GalNAcα3(Fucα2)Galβ; Nos. 3 and 10–15 in Table 1, and lane 8 in Fig. 1]. The minimal detection level for the Forssman glycosphingolipid was 50–100 ng and for the A7 type 2 glycosphingolipid 5–10 ng. In contrast, glycosphingolipids with terminal non-reducing β-linked *N*-acetylgalactosamine (Nos. 23, 25, 26, 30, 31 and 38) were not bound by the lectin.

In addition to binding to terminal α-linked *N*-acetylgalactosamine, U-MLL also bound to glycosphingolipids with terminal α-linked galactose. Of these glycosphingolipids, those with terminal α4-linked galactose, such as globotriaosylceramide (Galα4Galβ4Glcβ1Cer; No. 1 in Table 1 and lane 1 in Fig. 1) and the P_1 glycosphingolipid (Galα4Galβ4GlcNAcβ3Galβ4Glcβ1Cer; No. 6, and lane 4), had a higher binding activity than the glycosphingolipids with terminal α3-linked galactose, such as isoglobotriaosylceramide (Galα3Galβ4Glcβ1Cer; No. 2), the B5 glycosphingolipid (Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer; No. 7, and lane 5) and the Galα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer glycosphingolipid (No. 8) produced by defucosylation of the B6 type 1 glycosphingolipid (No. 35).

The higher binding activity of U-MLL for glycosphingolipids with terminal α4-linked galactose was reflected in a detection level of 0.5 µg for the P_1 glycosphingolipid, compared with 2 µg for the B5 glycosphingolipid.

The B6 type 1 and 2 glycosphingolipids (Galα3(Fucα2)-Galβ3GlcNAcβ3Galβ4Glcβ1Cer; No. 35, and Galα3(Fucα2)-Galβ4GlcNAcβ3Galβ4Glcβ1Cer; No. 34) having a fucose in α2-linkage to the penultimate galactose, were consistently negative in the binding assay. Also, the binding was

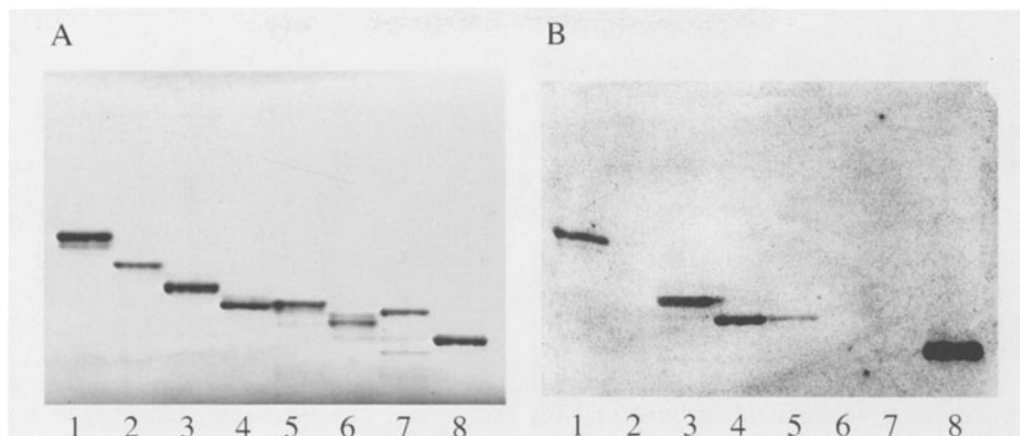

Figure 1. Thin-layer chromatogram stained with anisaldehyde (A) and autoradiogram obtained by overlay with *Moluccella laevis* lectin (B). The glycosphingolipids were separated on aluminium-backed silica gel 60 HPTLC plates (Merck), using chloroform: methanol: water 60: 35: 8, by vol, as solvent system, and further treated as described in Materials and methods. Autoradiography was performed for 48 h. The lanes contained the following glycosphingolipids: (1) globotriaosylceramide from human erythrocytes, 4 μg; (2) globotetraosylceramide from human erythrocytes, 4 μg; (3) Forssman glycosphingolipid from dog small intestine, 4 μg; (4) P_1 glycosphingolipid from human erythrocytes, 4 μg; (5) B5 glycosphingolipid from rabbit erythrocytes, 4 μg; (6) B6 type 2 glycosphingolipid from human erythrocytes, 4 μg; (7) para-Forssman glycosphingolipid from human erythrocytes; 4 μg; (8) A7 type 2 glycosphingolipid from human erythrocytes, 4 μg.

abolished when terminal α-linked galactose was elongated by GalNAcβ in 3-position, as in globotetraosylceramide (GalNAcβ3Galα4Galβ4Glcβ1Cer; No. 25), isoglobotetraosylceramide (GalNAcβ3Galα3Galβ4Glcβ1Cer; No. 26) and GalNAcβ3Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (No. 38).

Microtitre well assay

The relative binding activities for selected glycosphingolipids that bound the lectin were further examined by the microtitre well assay (Fig. 2). In accordance with the results described above, the binding activity of U-MLL was highest for glycosphingolipids with terminal α3-linked *N*-acetylgalactosamine. The binding to the Forssman glycosphingolipid was half maximal at a concentration of 100 ng per well and to the A7 type 2 glycosphingolipid at 300 ng per well.

The compounds with terminal α-linked galactose gave a very weak reaction in this assay. The maximal binding value of the P_1 glycosphingolipid was only 1/5 that of the Forssman glycosphingolipid. Again the glycosphingolipids with terminal α4-linked galactose (globotriaosylceramide and the P_1 glycosphingolipid) had the highest binding activity within this group.

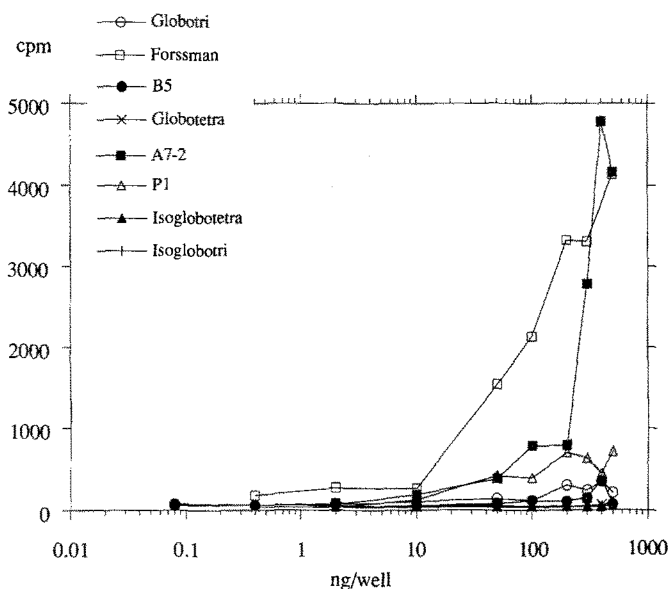

Figure 2. Binding curves obtained by binding of *Moluccella laevis* lectin to serial dilutions of glycosphingolipids adsorbed to microtitre wells. The assay was done as described in Materials and methods.

Discussion

The binding specificity of the lectin from *Moluccella laevis* has previously been characterized by hapten inhibition of haemagglutination and binding to glycoproteins in an enzyme immunoassay, demonstrating a high preference for α-linked *N*-acetylgalactosamine [1, 2]. In the present study a chromatogram binding assay and a microtitre well assay were used to characterize the specificity of the lectin for glycosphingolipids, using a lectin preparation isolated by affinity chromatography in the presence of 8 M urea [3].

Binding of MLL to all glycosphingolipids with a terminal non-reducing α-*N*-acetylgalactosamine was obtained. No definite determination of the relative affinity of the lectin for terminal α-*N*-acetylgalactosamine in linear carbohydrate chains, as the Forssman glycosphingolipid, and in branched chains, as the blood group A determinant, was possible since the results from the two assay systems were contra-

dictory in this respect. This was presumably due to differences in the conformation of the glycosphingolipids when surrounded by the plastic on the thin-layer plates, versus being adsorbed to the plastic surface in a basically aqueous milieu in the microtitre wells. It has been demonstrated that the glycosphingolipid-binding specificity of Verotoxin 1 on thin-layer chromatograms is changed in the presence of polyisobutylmethacrylate [21], which may be of relevance in this context.

MLL also bound to linear glycosphingolipids with terminal α-galactose. However, the latter binding activity was much lower, in line with earlier results which showed a substantial contribution of the acetamido group at C2 to the interaction with the lectin.

Among the α-galactose-terminated glycosphingolipids, the ones with the sugar in the α4-linkage had a higher binding activity than glycosphingolipids with galactose in an α3-linkage. Glycosphingolipids with terminal α4-linked *N*-acetylgalactosamine or substituted α-*N*-acetylgalactosamine have not been identified in vertebrates [22], but are found in some insects [23, 24]. It would be of interest to study the interaction of MLL with these compounds, in order to obtain further information about the binding specificity of the lectin.

No indications of direct involvement of internal sugars in the interaction with the lectin were found. Still, while the B5 glycosphingolipid and the defucosylated B6 type 1 glycosphingolipid bound the lectin, no binding to the B6 type 2 or the B6 type 1 glycosphingolipid was observed. Thus, the presence of an α-linked fucose at the C2 position of the pentultimate galactose abolished the binding.

The reason for this is suggested by examination of the calculated minimum energy conformation of the binding-positive A6 type 2 glycosphingolipid. The conformation shown in Fig. 3 is in agreement with previous studies [25–27]. The α2-linked fucose protrudes from the same side as the acetamido group of the *N*-acetylgalactosamine, which is essential for high binding activity. Thus, this fucose in α2-linkage to the penultimate galactose may impart partial sterical hindrance. In cases of high binding activity, e.g. the A6 type 2 glycosphingolipid, the inhibitory effect of the α2-linked fucose is compensated by the high affinity for the acetamido group of the terminal *N*-acetylgalactosamine. However, when the binding activity is lower, as for the glycosphingolipids with terminal α-linked galactose, the inhibitory effect of the α2-linked fucose is seen, since no binding to the B6 type glycosphingolipid was obtained.

The binding of MLL to glycosphingolipids with terminal α-galactose may interfere in its use for detection of terminal α-linked *N*-acetylgalactosamine on tissue sections. For example, in the epithelial cells of rat and dog small intestine, globotriaosylceramide or isoglobotriaosylceramide are found in high concentrations [28, 29]. Thus, binding of MLL to the epithelial cells might be due to interaction of the lectin with these α-galactose-terminated triglycosylceramides, as well as, with α-*N*-acetylgalactosamine-terminated glycoconjugates.

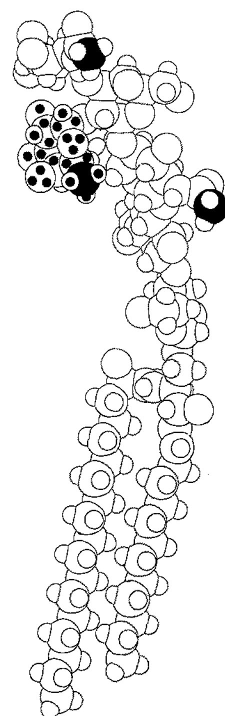

Figure 3. Minimum energy conformation of the *Moluccella laevis* lectin-binding A6 type 2 glycosphingolipid, GalNAcα3(Fucα2)-Galβ4GlcNAcβ3Galβ4Glcβ1Cer [25–27]. The methyl carbons of the acetamido groups are in black, and the fucose residue is dotted. The acetamido group of the terminal *N*-acetylgalactosamine, necessary for high binding activity of the lectin, is exposed on the same side as the fucose. In the absence of this acetamido group, the partial sterical hindrance by the fucose residue was seen, since the lectin did not bind to the B6 type 2 glycosphingolipid, Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer, while the non-fucosylated B5 glycosphingolipid, Galα3Galβ4GlcNAcβ3Galβ4-Glcβ1Cer, bound the lectin, albeit weakly.

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