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Characterization of a Novel Pyruvylated Carbohydrate Unit Implicated in the Cell Aggregation of the Marine Sponge *Microciona prolifera**

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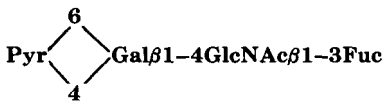
The species-specific Ca^{2+}-dependent reaggregation of dissociated cells of the marine sponge *Microciona prolifera* is mediated by a large extracellular adhesion proteoglycan. The glycans of this molecule are involved in the interactions of the proteoglycan with itself and with the sponge cells. Monoclonal antibodies against the glycans block the aggregation of sponge cells (Misevic, G. N., Finne, J., and Burger, M. M. (1987) *J. Biol. Chem.* 262, 5870–5877). Proteoglycan oligosaccharides were prepared by partial acid hydrolysis of the isolated glycans, and their reactivity with the monoclonal antibodies was monitored after linkage to phospholipid and immunostaining of thin layer chromatograms. One major antibody-reactive oligosaccharide was detected and purified by ion-exchange chromatography and high performance liquid chromatography. ^1H NMR spectroscopy, fast atom bombardment-mass spectrometry, methylation analysis, and sequential chemical and enzymatic degradation studies indicated the structure



for the oligosaccharide. The depyruvylated derivative of the oligosaccharide did not react with the aggregation-blocking antibody, which indicates that the pyruvate acetal is an essential part of the epitope.

For a long time sponges have been used as a simple model of multicellular organisms in the study of the molecular interactions involved in tissue development and organization (1, 2). Reaggregation of dissociated sponge cells occurs in a species-specific manner (1). In the marine sponge *Microciona prolifera* the reaggregation process depends on the presence of a large extracellular adhesion proteoglycan, formerly known as *Microciona* aggregation factor, and a high concentration of Ca^{2+} ions (3, 4). Monosaccharide analyses of the isolated glycans have revealed an unusual composition with the presence of uronic acid, fucose, and other neutral monosaccharides in the same molecule (5, 6). The glycans have been shown to be involved in sponge aggregation (6–8). On the basis of the aggregation activity of the purified glycans in their cross-linked polymeric form, multivalent carbohydrate-carbohydrate interactions of low affinity binding sites have been postulated to form the basis of the cell adhesion (7–9). Carbohydrate-carbohydrate interactions, some requiring Ca^{2+}, have also been suggested to mediate cell interactions in higher animals (10–12).

To reveal the molecular basis of the interaction in sponge aggregation, monoclonal antibodies have been prepared against the purified adhesion proteoglycan (7). Some of the monoclonal antibodies bind to the glycans and specifically block the aggregation activity of the proteoglycan (7). In this report we describe the isolation of an oligosaccharide from a partial acid hydrolysate of the total carbohydrates of the adhesion proteoglycan. This oligosaccharide forms the epitope recognized by a monoclonal aggregation-blocking antibody. The structure is shown to be of a novel type containing a pyruvylated monosaccharide, which is also required for the interaction with the blocking antibody.

EXPERIMENTAL PROCEDURES

Materials—*N*-Acetylneuraminyl α2–3 lactose (3′-sialyllactose) was purified from bovine colostrum as described previously (13). Synthetic 4,6-pyruvylated aminopentyl galactoside (*R*-configuration) was provided by Dr. T. Ziegler, University of Stuttgart, Germany. GlcUAβ1–3GalN (chondrosine), which was *N*-acetylated by standard procedures, Galβ1–3GlcNAcβ1–3Galβ1–4Glc (lacto-*N*-tetraose), and 1,2-dipalmitoyl *sn*-glycero-3-phosphatidylethanolamine (dipalmitoyl phosphatidylethanolamine) were from Sigma. NaB^3H_4 (500 mCi/mmol) was from Amersham International (United Kingdom). Poly(isobutyl methacrylate) (M_r 300,000) was supplied by Aldrich, 4-chloro-1-naphtol by Merck (Darmstadt, Germany), and dextran T 70 by Pharmacia (Uppsala, Sweden). ^2H_2O (99.8 and 99.96 atom % ^2H) was purchased from MSD Isotopes (Montreal, Canada).

Live specimens of *M. prolifera* were collected by the Supply Department of the Marine Biological Laboratory, Woods Hole, MA.

The monoclonal antibody clones Block 1 and C-16 (7) were purified from culture supernatants by affinity chromatography on either protein A- or protein G-Sepharose columns (Pharmacia) according to the manufacturer's recommendations. Horseradish peroxidase-coupled rabbit anti-mouse antibody was received from Dakopatts (Glostrup, Denmark). Enzymes were used as supplied: β-galactosidase from *Charonia lampas* (Seikagaku Kogyo, Tokyo, Japan) and from *Escherichia coli*, α-galactosidase from jack bean (both from Boehringer,

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Mannheim, Germany) and β-*N*-acetylhexosaminidase from jack bean (Sigma).

TLC aluminium Silica Gel 60 sheets and Fractogel TSK DEAE-650 were from Merck. Bio-Gel P-2 (200–400 mesh) and Dowex AG 50W-X8, H^+ form (100–200 mesh) were obtained from Bio-Rad. The CarboPac PA1 HPLC[1] columns and the anionic micromembrane suppressor cartridge were from Dionex (Sunnyvale, CA), and the Dextro-Pac cartridge was obtained from Waters (Milford, MA).

Purification of Adhesion Proteoglycan Glycans—Isolation of the adhesion proteoglycan and its glycans was carried out essentially as described (7). Partial acid hydrolyses were performed as indicated in the figure legends in conical Teflon sealed glass vials. The samples were neutralized and evaporated under vacuum and either linked to lipid for analysis or purified further by anion-exchange chromatography.

Synthesis and Immunochemical Detection of Neoglycolipids—Aliquots of dried oligosaccharides were linked to 2.5 mg/ml dipalmitoyl phosphatidylethanolamine in chloroform/methanol (1:1, v/v) as described (14) with the modification of an overnight incubation at 60 °C. Neoglycolipids were separated on aluminium Silica Gel 60 sheets in chloroform/methanol/water (44:36:8, v/v), dried, and the origins of the TLC sheets covered occasionally by immersion in 0.1% by volume of poly(isobutyl methacrylate) in hexane/dichloromethane (1:1, v/v). Blocking took place in 1% bovine serum albumin in phosphate-buffered saline (8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 131 mM NaCl, 2.1 mM KCl, pH 7.4) at room temperature for 1 h. For immunodetection (15) the sheets were decorated with 2 μg/ml monoclonal antibody in the blocking solution at room temperature for 1 h followed by washing with three changes of phosphate-buffered saline before incubation with the second antibody, a 1:1,000 diluted horseradish peroxidase-coupled rabbit anti-mouse antibody at room temperature for 1 h. After three washes in phosphate-buffered saline the sheets were developed with 0.017% 4-chloro-1-naphtol, 0.006% H_2O_2 in phosphate-buffered saline.

Column Chromatography—Anion-exchange chromatography was performed by loading 20 mg of total oligosaccharide hydrolysate on a 20-ml column of Fractogel TSK DEAE-650 equilibrated in water. Unbound material was eluted with water before applying a linear gradient of pyridine-acetic acid buffer, pH 5.0, from 0 to 100 mM in 200 ml, followed by a final step with 500 mM pyridine-acetic acid buffer. The gradient was measured by absorbance at 260 nm of 1:500 diluted eluent. Gel filtration was achieved on a Bio-Gel P-2 column (1.6 × 130 cm) equilibrated in 10 mM pyridine-acetic acid buffer, pH 5.0, at a flow rate of 18 ml/h. Fraction size was about 3 ml and measured for each single run. Standardization was achieved with dextran T 70 hydrolyzed in 100 mM HCl at 100 °C for 4 h (16) and measured by the anthrone reaction (17).

High performance anion-exchange chromatography was carried out on a Dionex System (series 4500i) (18). Separation was achieved either on an analytical (4 × 250-mm) or semipreparative scale (9 × 250-mm) column of Dionex CarboPac PA1 pellicular anion-exchange resin. The columns were eluted as indicated in the figure legends. Flow rates were 1 ml/min. For detection 200 mM NaOH was added postcolumn at a flow rate of 1 ml/min, and the following electrode potentials were applied: E_1 = 0.05 V (t_1 = 360 ms), E_2 = 0.8 V (t_2 = 120 ms), E_3 = −0.6 V (t_3 = 420 ms) with a response time of 3 s. Postcolumn addition and detection were omitted during preparative runs. Instead, the column eluent was desalted by on-line passage through an anionic micromembrane suppressor cartridge continuously regenerated by 12.5 mM H_2SO_4 at a flow rate of approximately 4 ml/min. Collected eluents were immediately neutralized by the addition of 1 M pyridine.

Chemical Treatments—Reduction was achieved in 0.2 M $NaBH_4$, 1 mM NaOH for 2 h at room temperature, and periodate oxidation was done in 50 mM $NaIO_4$ in 50 mM sodium acetate buffer, pH 4.4, at 4 °C for 24 h. Depyruvylation was achieved by incubation in 10 mM HCl at 100 °C for 6 h (19). Methanolysis was carried out as described (20) except for a higher temperature (80 °C), and the methyl glycosides were analyzed on a Waters Dextro-Pac column (8 × 100 mm) (21).

Radioactive Labeling of Reducing Sugars—Up to 100 nmol of purified oligosaccharides or reference sugars were labeled by reduction with 2 mCi of NaB^3H_4 in 10 mM NaOH overnight at room temperature, neutralized with 1 M acetic acid, and desalted by passing through a 200-μl Dowex AG 50W-X8 column followed by repeated evaporations with methanol.

Enzymatic Digestions—Equal amounts (about 1 nmol of oligosaccharide) of ^3H-labeled oligosaccharides were digested with 1 milliunit each of β-galactosidase from *C. lampas* in 50 mM sodium citrate buffer, pH 3.4, β-galactosidase from *E. coli* in 50 mM potassium phosphate-citrate buffer, pH 6.5, α-galactosidase from jack bean in 50 mM potassium phosphate-citrate buffer, pH 6.0, at 37 °C overnight. The specificity of the β-galactosidases was confirmed by digestion of ^3H-labeled lactose and lacto-*N*-tetraose under identical conditions, which showed cleavage of the β1–4 but not the β1–3 linkage, in accordance with published data for the *E. coli* enzyme (22). Ten milliunits of β-*N*-acetylhexosaminidase from jack bean was used in 50 mM sodium phosphate-citrate buffer, pH 5.0, at 37 °C. Complete digestion with the hexosaminidase was achieved by incubation for 110 h with 8 aliquots of 1 milliunit each of enzyme added in intervals of 12 h. Controls were incubated for the same periods in buffer only.

Purification of the Depyruvylated Oligosaccharide—The purified oligosaccharide was depyruvylated by hydrolysis in 10 mM HCl at 100 °C for 1 h, and the resulting hydolysate was passed over a 1-ml Fractogel TSK DEAE-650 column equilibrated and eluted with water, followed by 50 mM pyridine-acetic acid buffer, pH 5.0. Aliquots of each fraction were labeled by reduction with 0.5 mCi of NaB^3H_4 and analyzed on the Bio-Gel P-2 column (1.6 × 130 cm). Equal amounts of the purified peaks were linked to lipid and tested for immunoreactivity as described above. Monosaccharide analysis from the purified peaks was performed as described above.

^1H NMR Spectroscopy—Prior to ^1H NMR spectroscopic analysis the desalted sample was exchanged twice in 99.8% ^2H_2O and finally dissolved in 450 μl of 99.96% ^2H_2O. ^1H NMR spectra were recorded at 500 MHz on a Bruker AMX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University). Chemical shifts (ppm) are expressed by reference to internal acetone (δ = 2.225). One-dimensional spectra and two-dimensional homonuclear Hartmann-Hahn (HOHAHA; Ref. 23) spectra were recorded essentially as described (24).

Fast Atom Bombardment-Mass Spectrometry (FAB-MS)—FAB-mass spectra were obtained in either the positive or negative ion mode using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (Bijvoet Center) using either 10 kV (positive ion mode) or −10 kV (negative ion mode) accelerating voltage. The FAB gun was operated at an emission current of 10 mA with xenon as the bombarding gas. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used and recorded and processed on a Hewlett Packard HP9000 series data system using the JEOL Complement software. Tandem mass (MS-MS) spectra were obtained on the same instrument, using helium as the collision gas at a pressure sufficient to reduce the parent ion to one-third of its original intensity. Samples were dissolved in water (underivatized samples) or methanol (permethylated samples), and 1 μl of the sample solution was loaded into the thioglycerol matrix. Permethylated samples were prepared by the method of Ciucanu and Kerek (25).

Linkage Analysis—Partially methylated alditol acetates were prepared as described (26) and analyzed by gas chromatography-mass spectrometry on a JEOL JMS-AX505W mass spectrometer fitted with a Hewlett Packard 5890 gas chromatograph (Bijvoet Center) using an on-column injector and helium as the carrier gas at a flow rate of 2 ml/min. The monosaccharide derivatives were separated on a CP-Sil 5CB column (0.32 mm × 25 m, Chrompack) using the following temperature program: 60 °C for 2 min, a gradient of 40 °C/min to 140 °C, holding for 2 min at 140 °C, then a gradient of 4 °C/min to 230 °C, and finally holding the temperature at 230 °C for 10 min. Mass spectra were obtained under conditions of electron impact and were recorded using linear scanning from *m/z* 50–450 at an accelerating voltage of 3 kV.

Absolute Configuration of Fucose—The absolute configuration of the fucose residue was determined by gas chromatography-mass spectrometry of its trimethylsilylated (−) 2-butyl glycoside as described (27, 28).

RESULTS

Preparation and Immunological Detection of Oligosaccharides—Preliminary experiments showed that the glycans from the adhesion proteoglycan, although having some similarity with glycosaminoglycans in their composition, could not be

[1] The abbreviations used are: HPLC, high performance liquid chromatography; Fuc, L-fucose; Hex, hexose; Pyr, pyruvate; HOHAHA, homonuclear Hartmann-Hahn; FAB, fast atom bombardment; MS, mass spectrometry; G_{M3}, *N*-acetylneuraminylgalactoceramide.

cleaved by commercially available glycosaminoglycan-digesting enzymes (data not shown). Chemical degradation by de-*N*-acetylation and deamination did not produce oligosaccharides that would bind to the monoclonal antibodies. Therefore, partial acid hydrolysis was used for the fragmentation of the glycans. Purified glycans, prepared by extensive Pronase digestion, were subjected to partial acid hydrolysis, and the resulting oligosaccharides were linked to dipalmitoyl phosphatidylethanolamine for immunochemical analysis (14). After separation as neoglycolipids on TLC plates, one oligosaccharide component was identified as reacting the strongest with the monoclonal antibody Block 1, which inhibits the aggregation of sponge cells (Fig. 1, *lane 4*). Free oligosaccharides did not remain on the plate (Fig. 1, *lane 3*), and unrelated control lipid-linked saccharides were unreactive (Fig. 1, *lanes 1* and *2*). The control monoclonal antibody C-16, which does not inhibit aggregation but binds to the glycans of the adhesion proteoglycan, did not bind to any of the lipid-linked oligosaccharides (results not shown).

Optimization of the Conditions of Partial Acid Hydrolysis—The production of the oligosaccharide reactive with the Block 1 antibody was dependent on the duration of the partial acid hydrolysis in hydrochloric (not shown) or acetic acid (Fig. 2). Some of the larger, uncleaved oligosaccharides that were not lipid-linked because of their poor solubility in the solvents used for linking did not migrate on TLC. They were kept in place by plastic decoration and could therefore be detected at the site of sample application. This allowed us to follow the disappearance of the reactivity from the large polymers and the concomitant appearance of the main oligosaccharide recognized by antibody Block 1.

Purification of the Reactive Oligosaccharide—The oligosaccharides liberated by partial acid hydrolysis of the glycans were fractionated on a Fractogel TSK DEAE anion-exchange column, and the hexose-positive peaks were pooled and tested for immunoreactivity (Fig. 3). Most of the antibody Block 1 reactivity was bound by the column and eluted at approximately 20 mM pyridine-acetic acid, which indicated that the active oligosaccharides were negatively charged.

The antibody-reactive oligosaccharide fraction was subjected to chromatography on a Dionex semipreparative PA1 anion-exchange column using an anionic micromembrane suppressor for on-line desalting during the purification of oligosaccharides. The purity of individual fractions was monitored by rerunning aliquots on an analytical column (Fig. 4, *A* and *B*). Analysis of the immunoreactivity of the individual oligosaccharides revealed one component recognized by antibody Block 1 (Fig. 4*C*), and all other peaks were negative. This oligosaccharide also ran as a single peak on the same PA1 anion-exchange column at high pH used to ionize hydroxyl groups (18), which suggested that it consisted of a single component (data not shown).

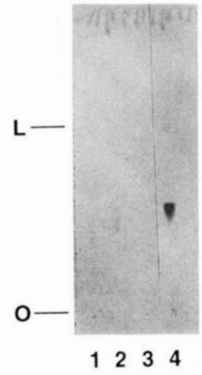


FIG. 1. **Immunodetection of adhesion proteoglycan-derived oligosaccharides as neoglycolipids.** Oligosaccharides linked to dipalmitoyl phosphatidylethanolamine (*lanes 1*, *2*, and *4*) or free reference oligosaccharides (*lane 3*) were separated by TLC and detected by immunostaining with monoclonal antibody Block 1. *Lane 1*, lactose (25 μg); *lane 2*, GlcUAβ1–3GalNAc (25 μg); *lanes 3* and *4*, partial acid hydrolysate (1 M HCl, 80 °C, 1 h) of total adhesion proteoglycan oligosaccharides (50 μg). The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

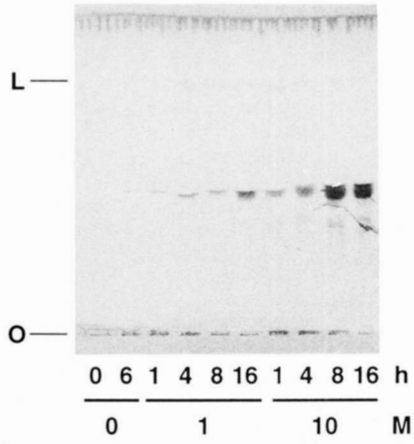


FIG. 2. **Partial acid hydrolysis of the adhesion proteoglycan glycans.** The adhesion proteoglycan glycans were hydrolyzed in acetic acid at 80 °C at the concentrations and for the times indicated and were then linked to dipalmitoyl phosphatidylethanolamine and analyzed by TLC and immunostaining with monoclonal antibody Block 1. The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

Structural Characterization of the Oligosaccharide—The purified oligosaccharide, labeled by reduction with NaB^3H_4, eluted from a Bio-Gel P-2 gel filtration column at a position equivalent to 7.5 glucose units, which corresponds roughly to the elution position of the charged trisaccharide 3′-sialyllactose (Fig. 5). Compositional analysis after methanolysis indicated the presence of equimolar proportions of *N*-acetylglucosamine and fucose, but no other monosaccharide component was found (Table I). Reduction of the oligosaccharide before methanolysis resulted in the disappearance of the fucose peaks, which suggested that fucose is at the reducing terminus (fucitol is not detected in the HPLC monosaccharide analysis system). The sugar composition did not identify the nature of the negative charge in the oligosaccharide. Although glucuronic acid and sulfate were previously detected as components of the sponge glycans (5, 6, 8), glucuronic acid was not found by monosaccharide analysis in the purified compound. Solvolysis, carried out under conditions reported to desulfate carbohydrates (29), did not change the elution volume of the oligosaccharide on gel filtration (data not shown).

^1H NMR Spectroscopy—The one-dimensional ^1H NMR spectrum of the oligosaccharide (not shown) revealed four major signals in the region for anomeric protons (5.5 ppm > δ > 4.3 ppm; (30)). On the basis of their lower intensities, and patterns in the two-dimensional HOHAHA spectrum (Fig. 6), the signals at 5.230 ppm ($^3J_{1,2}$ = 4 Hz) and 4.567 ppm ($^3J_{1,2}$ = 8 Hz) could be assigned to the α- and β-anomeric forms, respectively, of the reducing Fuc. The corresponding CH_3 resonances occurred at 1.205 ppm (Fucα) and 1.245 ppm (Fucβ). The β-anomeric signal ($^3J_{1,2}$ = 8 Hz) at 4.70 ppm was attributed to the GlcNAc residue because the two-dimensional HOHAHA experiment allows for magnetization transfer throughout the monosaccharide spin system (Fig. 6). The GlcNAc H-1 signal was distorted, possibly because of virtual couplings and/or the effect of the adjacent reducing Fuc

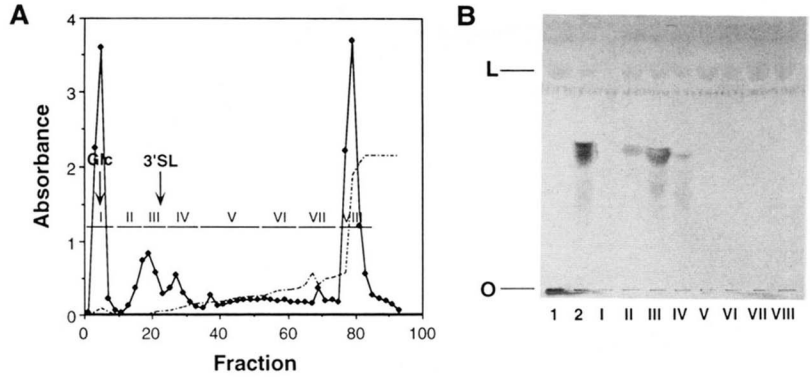


FIG. 3. **Fractionation of the adhesion proteoglycan-derived oligosaccharides by ion-exchange chromatography.** *Panel A*, adhesion proteoglycan-derived oligosaccharides (20 mg, hydrolyzed in 10 M acetic acid at 80 °C for 8 h) were fractionated on a column of Fractogel TSK DEAE. Elution was achieved by washing with water followed by a linear gradient of pyridine-acetic acid buffer, pH 5.0, from 0 to 0.1 M, and final elution with 0.5 M pyridine-acetic acid buffer. The gradient was measured at 260 nm (– · – · –). The hexose content was determined by the anthrone reaction at 620 nm (♦——♦). The elution positions of the reference compounds glucose (*Glc*) and 3′-sialyl lactose (*3′SL*) are indicated. Fractions I–VIII were pooled as indicated. *Panel B*, analysis of the oligosaccharides linked to dipalmitoyl phosphatidylethanolamine by TLC and immunodetection with monoclonal antibody Block 1. *Lane 1*, nonhydrolyzed glycans; *lane 2*, unfractionated partial acid hydrolysate; *lanes I–VIII* correspond to pools I–VIII as indicated in *panel A*. The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

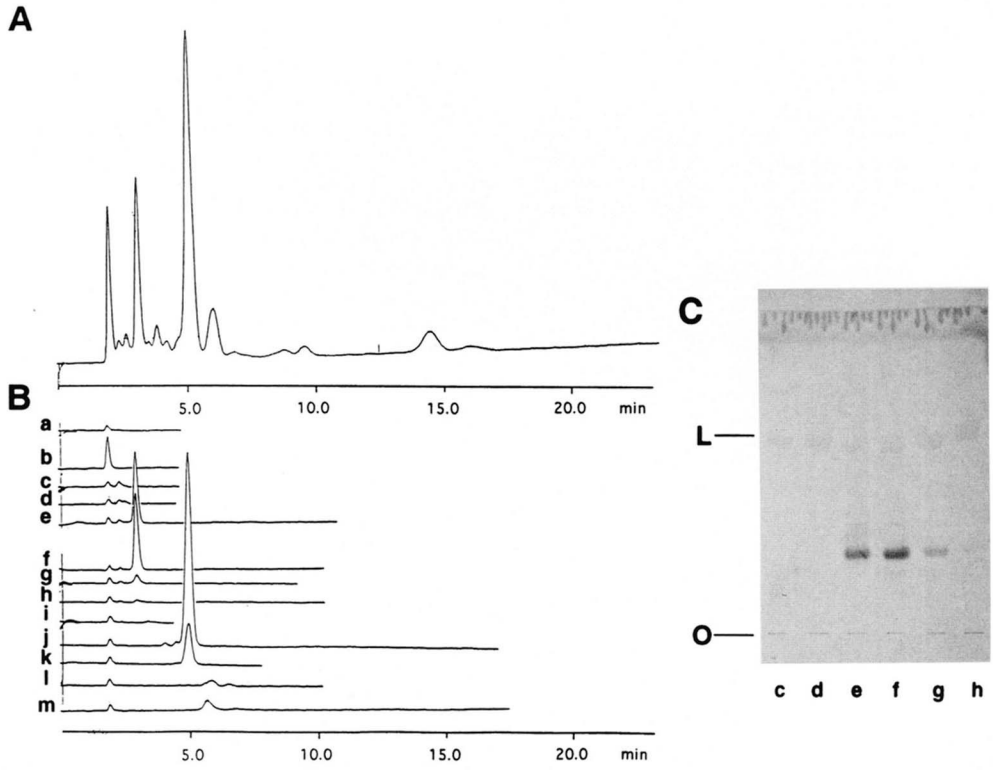


FIG. 4. **Purification of adhesion proteoglycan-derived oligosaccharides by HPLC.** Pool III oligosaccharides (Fig. 3) were separated on a semipreparative PA1 column and fractions collected after on-line desalting. *Panel A*, an aliquot of pool III oligosaccharides run on a PA1 analytical column eluted isocratically with 80 mM sodium acetate, 10 mM NaOH, detector sensitivity 300 nA. *Panel B*, aliquots of individual fractions (*a–m*), separated on the semipreparative PA1 column, rechromatographed using the analytical column under the same conditions as in *panel A*. *Panel C*, analysis of the oligosaccharides from *panel B* linked to dipalmitoyl phosphatidylethanolamine by TLC and immunodetection with monoclonal antibody Block 1. The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

residue. The GlcNAc NAc methyl protons resonated at 2.051 ppm. The NMR spectrum of the oligosaccharide revealed an additional β-anomeric ($^3J_{1,2}$ = 8 Hz) monosaccharide residue (H-1, 4.503 ppm), which showed a Gal-like spin system in the two-dimensional HOHAHA spectrum (Fig. 6). In Gal the small $^3J_{4,5}$ coupling constant usually prevents observable magnetization transfer between H-4 and H-5 in a HOHAHA experiment. Finally, a singlet at 1.461 ppm suggested the presence of a pyruvate group in the oligosaccharide (31). The chiral C-2 of the pyruvate moiety has the *R*-configuration. The ^{1}H chemical shift value of the acetal methyl group in the pyruvylated trisaccharide (1.461 ppm) is close to that observed in the synthetic 4,6-pyruvylated aminopentyl galactoside containing a pyruvylated acetal in *R*-configuration (1.455 ppm), whereas the value between the *R*- and *S*-isomers of 4,6-*O*-(1-carboxyethylidene)-β-D-galactopyranoside differs by 0.14 ppm (32).

FAB-MS—The underivatized sample was examined using both positive and negative ion mode FAB-MS. In the positive ion mode (data not shown) pseudo-molecular ions were ob-

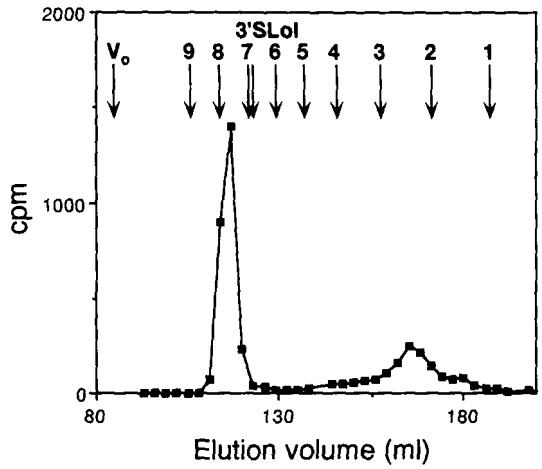


FIG. 5. **Gel filtration of the isolated oligosaccharide.** The purified oligosaccharide was reduced with NaB^3H_4 and subjected to gel filtration on a Bio-Gel P-2 column (1.6 × 130 cm) equilibrated in 10 mM pyridine-acetic acid buffer, pH 5.0. V_0, void volume; *1–9*, elution volumes of glucose monomer to nonamer; *3'SLol*, elution volume of ^3H-labeled 3'-sialyllactitol.

TABLE I

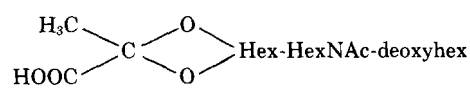
Monosaccharide composition of the native and chemically modified oligosaccharide

The monosaccharide compositions of the purified native oligosaccharide, the oligosaccharide reduced with $NaBH_4$, the oligosaccharide treated with mild acid, and the oligosaccharide treated with periodate followed by mild acid were determined after methanolysis by HPLC as their methyl glycosides. The compositions are expressed per mol of *N*-acetylglucosamine.

Constituent	Native	$NaBH_4$	Mild acid	Periodate
		mol/mol		
Gal	≤0.2[a]	≤0.1[a]	1.0	≤0.3[a]
Man	0.0	0.0	0.0	0.0
GlcNAc	1.0	1.0	1.0	1.0
Fuc	1.1	0.0	1.2	0.0
GlcUA	0.0	0.0	0.0	0.0

[a] Maximum value. Because of interfering peaks, the possible presence of low amounts of galactose could not be excluded.

served at *m/z* 600 ($M+H^+$), *m/z* 622 ($M+Na^+$), and *m/z* 644 ($M-H+2Na^+$). In the negative ion mode an $M-H^-$ pseudomolecular ion was observed at *m/z* 598. These ions indicate the presence of a deoxy$Hex_1Hex_1HexNAc_1$ trisaccharide bearing an additional substituent with a mass that is consistent with an acetal-linked pyruvate group, in which the pyruvate substitutes two of the carbohydrate hydroxyl groups. This conclusion was further supported by the results of positive ion mode FAB-MS and MS-MS analyses of the permethylated product. The FAB-mass spectrum of the permethylated derivative (data not shown) contained a major pseudomolecular ion at *m/z* 726 corresponding to $M+H^+$ for acetal-linked pyruvylated deoxy$Hex_1Hex_1HexNAc_1$. The mass increase on permethylation corresponds to the incorporation of nine methyl groups as expected for the reducing trisaccharide, in which two OH groups are bound to a pyruvate that carries a carboxyl group which becomes methyl esterified. Two structurally informative fragment ions were observed at *m/z* 522 (corresponding to an A^+-type fragment ion with the structure $Pyr_1Hex_1HexNAc^+$) and *m/z* 488 (formed from the *m/z* 520 ion by β-elimination of the substituent (CH_3O–) on C-3 of the charged HexNAc residue (33)). These ions confirm that the deoxyHex residue represents the reducing terminus of the trisaccharide and demonstrate that the HexNAc is internal and not substituted at C-3, which means that the pyruvyl group must be attached in acetal linkage to a nonreducing terminal Hex residue. This conclusion was formally proven by the results of the MS-MS analysis of the ion at *m/z* 726 (Fig. 7). This spectrum also contains the ions at *m/z* 520 and 488 and also an additional important pair of ions at *m/z* 246 and 274. Both arise by double cleavage of the A^+-type ion at *m/z* 520; *m/z* 246 arises by β-cleavage (34) of Pyr-Hex from the charged HexNAc residue, demonstrating that the Pyr-Hex moiety is terminal, whereas *m/z* 274 is related and arises by cross-ring cleavage of the nonreducing terminal Hex residue to yield an ion containing C-1 and the ring oxygen of the nonreducing terminal residue (34). The presence of β-cleavage ions in collision-induced dissociation spectra of permethylated carbohydrates is frequently accompanied by these "+28" ions.[2] These data demonstrate the following partial structure



STRUCTURE 1

for the oligosaccharide.

[2] J. Thomas-Oates, unpublished results.

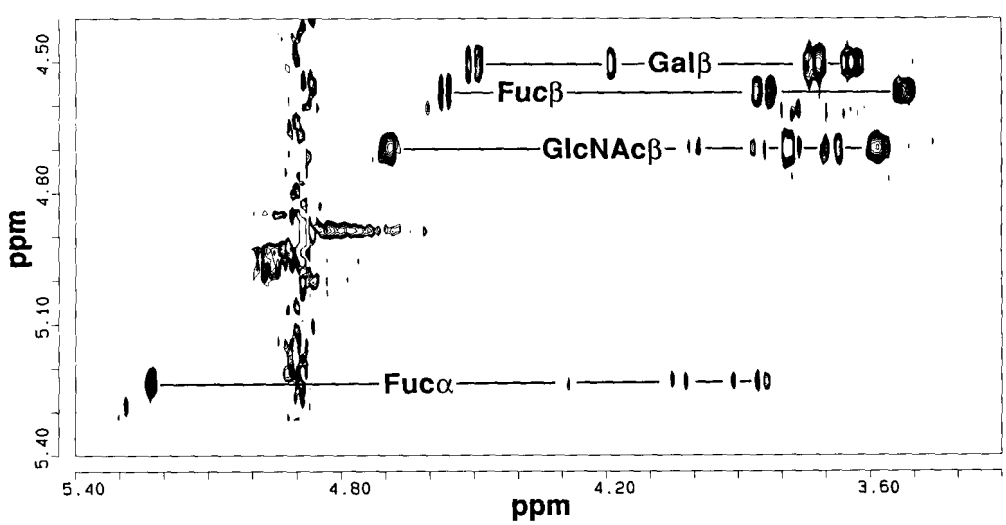


FIG. 6. **Part of the two-dimensional HOHAHA spectrum of the *Microciona* adhesion proteoglycan-derived oligosaccharide.** The spectrum was recorded with a 92-ms mixing time.

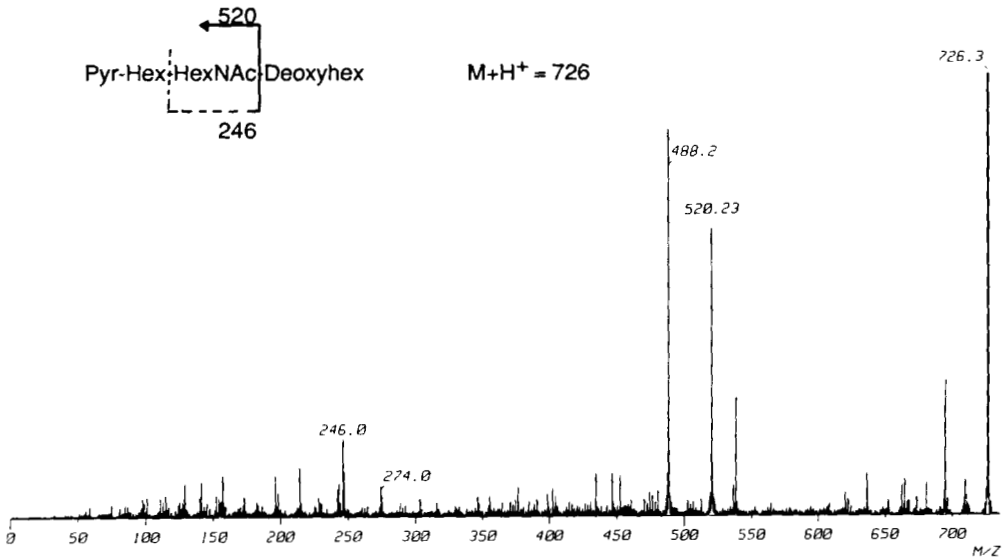


FIG. 7. **FAB-MS-MS spectrum of the permethylated oligosaccharide.** The elimination of methanol from the ion at *m/z* 520 to yield *m/z* 488 shows that C-3 of the HexNAc residue is unsubstituted. The ion at *m/z* 246 is formed by β-cleavage from *m/z* 520, whereas the related ion at *m/z* 274 arises by cross-ring cleavage of the Pyr-Hex residue in *m/z* 520 (see "Results").

Enzymatic and Chemical Degradation—Attempts to digest the native oligosaccharide with α- or β-galactosidases failed, apparently because of the presence of the pyruvate moiety. To remove the pyruvate selectively, the oligosaccharide was subjected to mild acid treatment. After this, sugar analysis of the oligosaccharide revealed the presence of 1 mol of galactose (Table I).

The products of mild acid treatment of the oligosaccharide were separated by gel filtration yielding four major peaks (Fig. 8). Peak I was the uncleaved compound, and IV eluted at the position of fucitol. Peak II appeared after 1 h of hydrolysis, and peak III was created after 2–3 h, which reflects the different stabilities of the glycosidic linkages. Whereas peak I was resistant to galactosidases (Fig. 9*A*), peak II was susceptible to β-galactosidase but not to α-galactosidase (Fig. 9*B*). The product of this digestion coeluted with peak III. The digestion of peak III with β-*N*-acetylhexosaminidase yielded a product coeluting with peak IV (Fig. 9*C*). These data prove the presence of galactose and confirm the following sequence.

Pyr-Galβ-GlcNAcβ-Fuc

STRUCTURE 2

To study the linkage positions the oligosaccharide was oxidized with periodate (Table I). Galactose was degraded, which indicated the presence of vicinal unsubstituted hydroxyl groups and thus suggested that the pyruvate acetal is 4,6-linked to the galactose. *N*-Acetylglucosamine was not oxidized, which indicated substitution of the hydroxyl at C-3 or C-4. This result was also in accordance with the internal position of the *N*-acetylglucosamine in the trisaccharide. Combined with the fact that the β-galactosidases from *C. lampas* and *E. coli* cleave β1–4 linkages but not β1–3 linkages (see "Experimental Procedures"), this finding indicated a β1–4 linkage of galactose to *N*-acetylglucosamine.

Linkage Analysis—Gas chromatography-MS analysis of the partially methylated alditol acetates generated from the oligosaccharide allowed identification of derivatives corresponding to 3-substituted deoxyHex, 4,6-disubstituted Hex, and 4-substituted HexNAc (Fig. 10). The 4,6-disubstituted Hex arises from the substitution of the terminal Hex residue at two positions by acetal-bound pyruvate. Taken together with the other data, the complete structure of the oligosaccharide could thus be determined (Fig. 11).

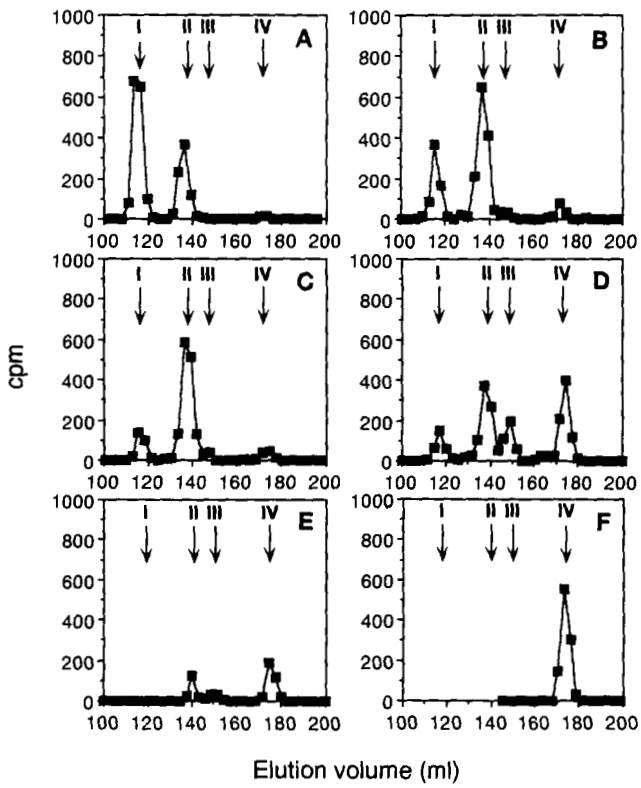


FIG. 8. **Degradation of the isolated oligosaccharide by mild acid.** The sample labeled by reduction with NaB^3H_4 was hydrolyzed in 10 mM HCl at 100 °C for 1 h (*panel A*), 2 h (*panel B*), 3 h (*panel C*), 4 h (*panel D*), and 6 h (*panel E*) and subjected to gel filtration on a Bio-Gel P-2 column eluted with 10 mM pyridine-acetic acid buffer, pH 5.0. For comparison, ^3H-labeled fucitol was run under the same conditions (*F*). The elution volumes of the native oligosaccharide (*I*) and its degradation products (*II–IV*) are indicated by *arrows*.

Absolute Configuration of Fucose—Gas chromatography-MS analysis of the trimethylsilylated (−) 2-butyl glycoside of the fucose residue showed it to have the same gas chromatog-

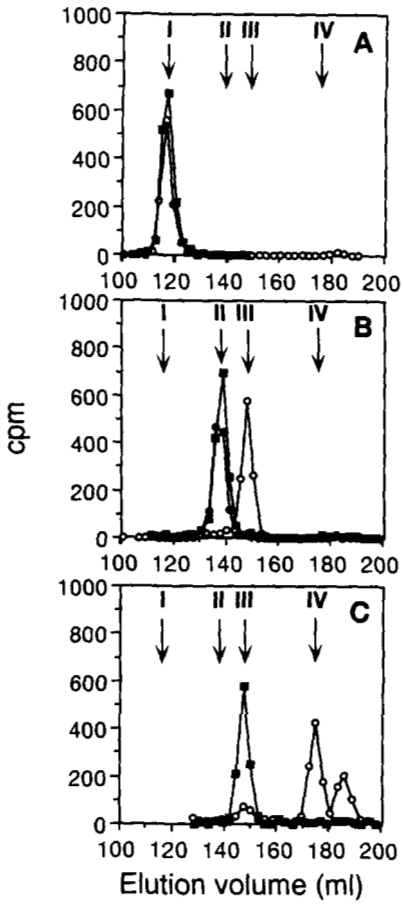


FIG. 9. **Sequential degradation of the isolated oligosaccharide with glycosidases.** *Panel A*, undigested (■) and β-galactosidase-digested oligosaccharide (○). *Panel B*, undigested (■), α-galactosidase (●), and β-galactosidase-digested (○) peak II oligosaccharide from Fig. 8*D*. *Panel C*, undigested (■) and β-hexosaminidase-digested (○) peak III oligosaccharide. The elution volumes of the native oligosaccharide (*I*) and its degradation products (*II–IV*) are indicated by *arrows*. The additional peak which appeared after peak IV during the long incubation seems to be an effect of the hexosaminidase on the fucitol. Incubation of a fucitol standard with the hexosaminidase under identical conditions created the same peak, whereas the peak was not produced without the enzyme (data not shown).

raphy retention pattern as authentic L-fucose (not shown).

Proportion of Pyruvylated Galactose in Sponge Glycans—Because galactose could be detected in the oligosaccharide only after depyruvylation, the sugar composition of the intact undegraded adhesion proteoglycan carbohydrates was also determined with and without depyruvylation to determine the proportion of pyruvylated galactose residues in the molecule. There was a small increase in the ratio of all sugar components to *N*-acetylglucosamine after depyruvylation with mild acid (Table II). The result is most likely caused by a slight instability of *N*-acetylglucosamine under the conditions of mild acid treatment. The increase in galactose was only slightly larger than that of the other sugar components, which indicated that the proportion of pyruvylated galactose in the intact proteoglycan was at most a few percent.

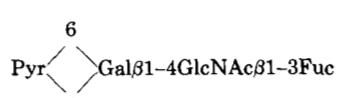
Role of Pyruvate in Antibody Recognition—To assess the importance of the pyruvate for recognition by the antibody, the original and the depyruvylated oligosaccharides were purified after hydrolysis for 1 h on a Fractogel TSK DEAE column. Aliquots of these reducing sugars were labeled by reduction with NaB^3H_4 and their purity checked by gel filtration (Fig. 12*A*) and monosaccharide analysis (data not shown). Equal amounts of the purified compound were linked to lipid, separated on TLC, and tested for immunoreactivity (Fig. 12*B*). Although the original compound containing the pyruvate group reacted with the antibody, the removal of the pyruvate completely abolished recognition by the antibody.

DISCUSSION

Polyvalent interactions of carbohydrates with cellular receptors as well as multivalent carbohydrate-carbohydrate interactions have been postulated to form the basis of cell recognition and aggregation in the marine sponge *M. prolifera* (6–8). A similar molecular mechanism of carbohydrate-carbohydrate interaction has also been suggested to operate during mouse embryonic development between fucose-containing X antigen structures (11, 12) and in the adhesion of tumor cells through binding of G_{M3} ganglioside with other carbohydrate ligands (35, 36). To characterize the molecular basis of the postulated multivalent carbohydrate-based cell aggregation activity in the sponge system, we have used adhesion-blocking monoclonal antibodies against the sponge adhesion proteoglycan (7).

For the purification and analysis of the reactive epitopes of the large proteoglycan adhesion molecule, the total glycan fraction had to be degraded into small oligosaccharide fragments. To allow the immunological analysis of the small highly water-soluble oligosaccharides they were linked by reductive amination to a lipid anchor (14). The method is limited to the study of small oligosaccharides, since structures larger than about a decasaccharide cannot be linked to the lipid (14). It was therefore not possible to compare directly the original material with the purified fragments in the same assay to judge the yield of the active oligosaccharide during the partial acid hydrolysis. Hydrolysis conditions were chosen to create a maximum amount of oligosaccharide recognized by the antibody. Some epitope structures might have been destroyed by this procedure, and others could have escaped detection because of conformational factors in the lipid-linked form on the TLC plate. Nevertheless, immunostaining of neoglycolipids on TLC plates revealed one major band reacting with the antibody Block 1, which may thus represent the major epitope of the Block 1 antibody in the intact adhesion proteoglycan.

The binding of the active oligosaccharide to an anion-exchange column indicated the presence of a negative charge in the molecule, and the oligosaccharide was resistant toward the action of glycosidases. However, neither glucuronic acid nor sulfate, previously known acidic components of the adhesion proteoglycan, was detected. By the combination of NMR and FAB-MS the charged group was identified as an acetal-bound pyruvate attached to the nonreducing terminal residue of a trisaccharide. Together with the specific degradation by glycosidases and chemical methods, the structure



STRUCTURE 3

was assigned for the oligosaccharide.

The yield of the oligosaccharide after partial acid degradation and purification was 200–300 nmol/20 mg of adhesion proteoglycan, which corresponds to 200–300 binding sites in each adhesion proteoglycan molecule. This value could be an underestimation caused by the acid lability of the pyruvate group and is also smaller than the 1,100 binding sites formerly

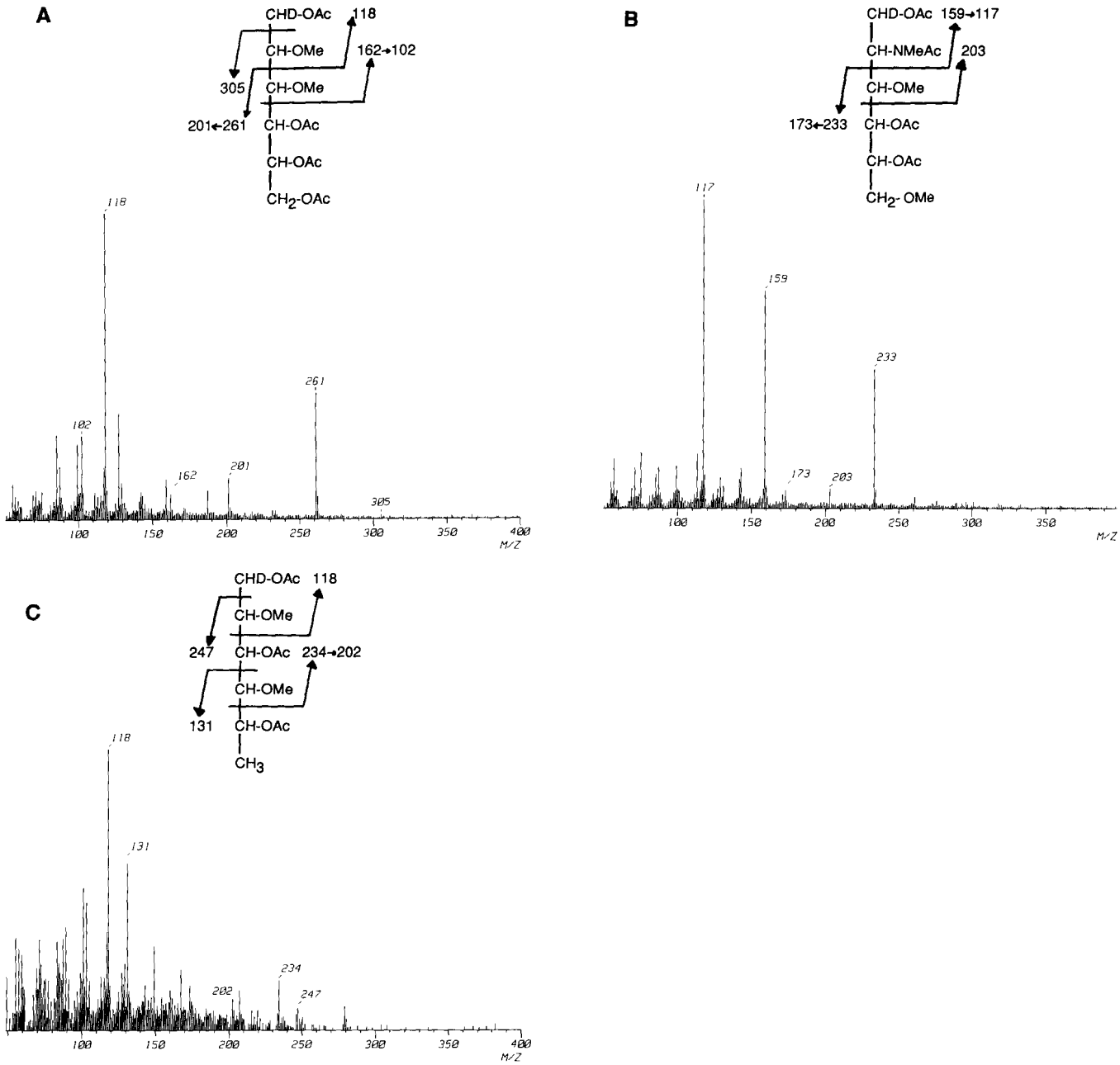


FIG. 10. **Linkage analysis of the isolated oligosaccharide.** Electron impact spectra obtained following gas chromatography of partially methylated alditol acetate derivative of 4,6-disubstituted Hex (*panel A*), 4-substituted HexNAc (*panel B*), and 3-substituted deoxyhex (*panel C*).

determined by antibody binding assays on the intact molecule (7). In spite of the high number of binding sites they represent only a few percent of the mass of the adhesion proteoglycan. This may explain why the presence of pyruvate was not detected previously.

Pyruvate as a part of oligo- or polysaccharide structures has been found previously in bacteria (37–39). Recently, however, this charged group has also been detected in another marine organism, *Aplysia kurodai* (19, 40). The fact that agar also contains pyruvate (41) might indicate a connection between the marine origin and the presence of pyruvate. However, the specific localization of pyruvate in the nerve fibers of *Aplysia* or the adhesion proteoglycan of *Microciona* may indicate more specific functions. It is also possible that the presence of this group has so far been overlooked in higher organisms. In this regard, it may be of significance that human serum amyloid P binds pyruvylated galactose in a Ca^{2+}-dependent manner (41).

The isolated oligosaccharide demonstrates, in addition to the presence of pyruvate, the existence of another unusual feature, substituted fucose. In the sea cucumber difucose branches modify classical glycosaminoglycan structures and render them resistant to glycosaminoglycan-degrading enzymes (42, 43), whereas in the jelly coat of the Mexican axolotl difucosylated branches modify *O*-linked acidic antennary structures (44). In higher animals, substituted fucose has been found in amino acid fucosides isolated from rat kidney cells (45, 46) and normal human urine (47). Most interestingly, substituted and nonsubstituted *O*-linked fucose have been found in several serum factors on their epidermal growth factor-like domains (48–51). So far no function has been assigned to these carbohydrates.

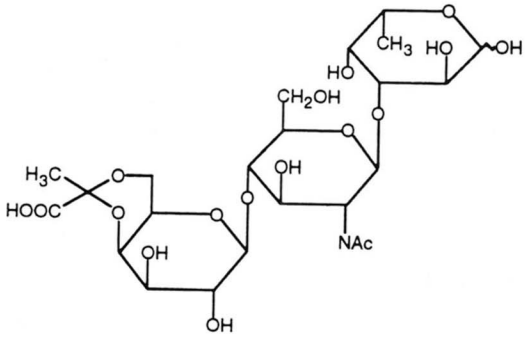


FIG. 11. **Structure of the pyruvylated oligosaccharide.** The complete structure of the oligosaccharide is Pyr-4,6-Galβ1–4GlcNAcβ1–3Fuc. The pyruvate forms a six-membered ring with galactose. The substituent of the chiral acetal carbon in the pyruvate moiety is in the *R*-configuration.

TABLE II

Monosaccharide composition of native and depyruvylated glycans of the adhesion proteoglycan

The monosaccharide compositions of the undegraded glycans in their native form and after depyruvylation with mild acid treatment were determined after methanolysis by HPLC as their methyl glycosides. The compositions (means of five samples) are expressed per mol of *N*-acetylglucosamine.

Constituent	Native	Depyruvylated
	mol/mol	
Gal	1.3	1.6
Man	0.5	0.6
GlcNAc	1.0	1.0
Fuc	1.4	1.6
GlcUA	1.8	2.1

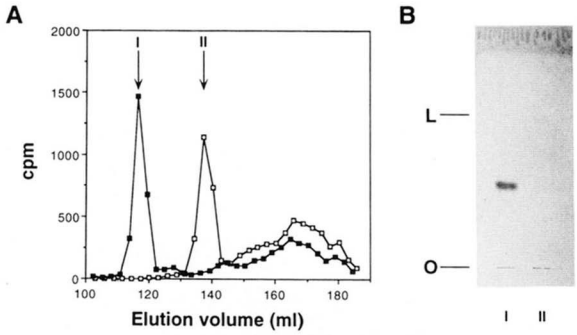


FIG. 12. **Effect of depyruvylation on antibody reactivity of the oligosaccharide.** The oligosaccharide was depyruvylated by treatment with 10 mM HCl at 100 °C for 1 h and purified by passing through a 1-ml DEAE-column. *Panel A*, gel filtration of the native (□) and depyruvylated (■) oligosaccharides after labeling with Na-B^{3}H$_4$on the Bio-Gel P-2 column. *Panel B*, the native (*I*) and depyruvylated (*II*) oligosaccharides were linked to dipalmitoyl phosphatidylethanolamine and analyzed by TLC and immunostaining with monoclonal antibody Block 1. The positions of the origin (*O*) and free lipid (*L*) are indicated on the *left*.

Fucose linkages are more acid-labile than most other glycosidic linkages and represent a preferred cleavage site during partial acid hydrolysis. The finding of fucose at the reducing end of the oligosaccharide and the acid sensitivity of its linkage seem to explain the success in isolating the active oligosaccharide despite the acid sensitivity of the pyruvate acetal. It is not known whether the oligosaccharide exists as a repeating unit, represents a branch on a linear backbone, is an antenna on a classical *N*-linked structure, or is directly linked to the peptide backbone. Characterization of the overall structure of the glycan containing the oligosaccharide would require alternative degradation methods that would conserve the acid-labile fucose linkage.

The antibody reactivity seems to depend critically on the presence of the pyruvate acetal in the oligosaccharide, since the removal of this group completely abolished recognition by the antibody. A similar observation has been made for antibodies reacting with pyruvylated glycolipids of nerve fibers of *Aplysia* (19, 40). However, the binding site for the antibody is larger than the terminal pyruvylated galactose residue because this substituted sugar coupled to an aminohexane spacer was not able to inhibit binding of the antibody to the sponge oligosaccharide in TLC overlay experiments.[3] Although the monoclonal antibody blocks aggregation of sponge cells, it is not yet known what is the functional role of the pyruvate moiety. In view of the requirement for Ca^{2+} in the aggregation it could be postulated that the charged pyruvate group is involved in binding the calcium ion. The identification of the antibody recognition site on the sponge glycans and its structural characterization will now facilitate the definition of the molecular mechanisms mediating the species-specific cell aggregation activity of sponge cells.

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REFERENCES

1. Wilson, H. V. (1907) *J. Exp. Zool.* **5,** 245–258
2. Galtsoff, P. S. (1929) *Biol. Bull.* **57,** 250–260
3. Humphreys, T. (1963) *Dev. Biol.* **8,** 27–47
4. Jumblatt, J. E., Schlup, V., and Burger, M. M. (1980) *Biochemistry* **19,** 1038–1042
5. Henkart, P., Humphreys, S., and Humphreys, T. (1973) *Biochemistry* **12,** 3045–3050
6. Misevic, G. N., and Burger, M. M. (1986) *J. Biol. Chem.* **261,** 2853–2859
7. Misevic, G. N., Finne, J., and Burger, M. M. (1987) *J. Biol. Chem.* **262,** 5870–5877
8. Misevic, G. N., and Burger, M. M. (1990) *J. Biol. Chem.* **265,** 20577–20584
9. Varner, J. A., Burger, M. M., and Kaufman, J. F. (1988) *J. Biol. Chem.* **263,** 8498–8508
10. Fransson, L.-Å., Carlstedt, I., Cöster, L., and Malmström, A. (1983) *J. Biol. Chem.* **258,** 14342–14345
11. Eggens, I., Fenderson, B. A., Toyokuni, T., and Hakomori, S.-i. (1989) *Biochem. Biophys. Res. Commun.* **158,** 913–920
12. Eggens, I., Fenderson, B., Toyokuni, T., Dean, B., Stroud, M., and Hakomori, S. (1989) *J. Biol. Chem.* **264,** 9476–9484
13. Parkkinen, J., and Finne, J. (1987) *Methods Enzymol.* **138,** 289–300
14. Stoll, M. S., Mizuochi, T., Childs, R. A., and Feizi, T. (1988) *Biochem. J.* **256,** 661–664
15. Magnani, J. L. (1985) *Anal. Biochem.* **150,** 13–17
16. Yamashita, K., Mizuochi, T., and Kobata, A. (1982) *Methods Enzymol.* **83,** 105–126
17. Nikkilä, E. A., and Pesola, R. (1960) *Scand. J. Clin. Lab. Invest.* **12,** 209–220
18. Lee, Y. C. (1990) *Anal. Biochem.* **189,** 151–162
19. Araki, S., Abe, S., Ando, S., Kon, K., Fujiwara, N., and Satake, M. (1989) *J. Biol. Chem.* **264,** 19922–19927
20. Chaplin, M. F. (1982) *Anal. Biochem.* **123,** 336–341
21. Lampio, A., and Finne, J. (1991) *Anal. Biochem.* **197,** 132–136
22. Hedbys, L., Johansson, E., Mosbach, K., Larsson, P. O., Gunnarsson, A., Svensson, S., and Lönn, H. (1989) *Glycoconj. J.* **6,** 161–168
23. Bax, A., and Davis, D. G. (1985) *J. Magn. Reson.* **65,** 355–360
24. Hård, K., Van Zadelhoff, G., Moonen, P., Kamerling, J. P., and Vliegenthart, J. F. G. (1992) *Eur. J. Biochem.* **209,** 895–915
25. Ciucanu, I., and Kerek, F. (1984) *Carbohydr. Res.* **131,** 209–217
26. de Lederkremer, R. M., Lima, C., Ramirez, M. I., Ferguson, M. A. J., Homans, S. W., and Thomas-Oates, J. (1991) *J. Biol. Chem.* **266,** 23670–23675
27. Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. (1978) *Carbohydr. Res.* **62,** 349–357
28. Gerwig, G. J., Kamerling, J. P., and Vliegenthart, J. F. G. (1979) *Carbohydr. Res.* **77,** 1–7
29. Nagasawa, K., Inoue, Y., and Kamata, T. (1977) *Carbohydr. Res.* **58,** 47–55
30. Vliegenthart, J. F. G., Dorland, L., and Van Halbeek, H. (1983) *Adv. Carbohydr. Chem. Biochem.* **41,** 209–374
31. Garegg, P. J., Jansson, P.-E., Lindberg, B., Lindh, F., Lönngren, J., Kvarnström, I., and Nimmich, W. (1980) *Carbohydr. Res.* **78,** 127–132
32. Fontana, J. D., Duarte, J. H., Iacomini, M., and Gorin, P. A. J. (1982) *Carbohydr. Res.* **108,** 221–228
33. Fukuda, M., Dell, A., Oates, J., and Fukuda, M. N. (1984) *J. Biol. Chem.* **259,** 8260–8273
34. Dell, A. (1987) *Adv. Carbohydr. Chem. Biochem.* **45,** 19–72
35. Kojima, N., and Hakomori, S. (1989) *J. Biol. Chem.* **264,** 20159–20162
36. Kojima, N., and Hakomori, S. (1991) *J. Biol. Chem.* **266,** 17552–17558

[3] D. Spillmann, T. Ziegler, and J. Finne, unpublished results.

37. Dudman, W. F., and Lacey, M. J. (1986) *Carbohydr. Res.* **145,** 175–191
38. Kojima, N., Kaya, S., Araki, Y., and Ito, E. (1988) *Eur. J. Biochem.* **174,** 255–260
39. Gil-Serrano, A., Sanchez del Junco, A., Tejero-Mateo, P., Megias, M., and Caviedes, M. A. (1990) *Carbohydr. Res.* **204,** 103–107
40. Watanabe, Y., Abe, S., Araki, S., Kumanishi, T., and Satake, M. (1989) *J. Biochem.* **106,** 972–976
41. Hind, C. R. K., Collins, P. M., Renn, D., Cook, R. B., Caspi, D., Baltz, M. L., and Pepys, M. B. (1984) *J. Exp. Med.* **159,** 1058–1069
42. Vieira, R. P., and Mourao, P. A. S. (1988) *J. Biol. Chem.* **263,** 18176–18183
43. Kariya, Y., Watabe, S., Hashimoto, K., and Yoshida, K. (1990) *J. Biol. Chem.* **265,** 5081–5085
44. Strecker, G., Wieruszeski, J.-M., Michalski, J.-C., Alonso, C., Leroy, Y., Boilly, B., and Montreuil, J. (1992) *Eur. J. Biochem.* **207,** 995–1002
45. Klinger, M. M., Laine, R. A., and Steiner, S. M. (1981) *J. Biol. Chem.* **256,** 7932–7935
46. Larriba, G. (1978) *FEBS Lett.* **95,** 190–193
47. Hallgren, P., Lundblad, A., and Svensson, S. (1975) *J. Biol. Chem.* **250,** 5312–5314
48. Kentzer, E. L., Buko, A., Menon, G., and Sarin, V. K. (1990) *Biochem. Biophys. Res. Commun.* **171,** 401–406
49. Bjoern, S., Foster, D. C., Thim, L., Wiberg, F. C., Christensen, M., Komiyama, Y., Pedersen, A. H., and Kisiel, W. (1991) *J. Biol. Chem.* **266,** 11051–11057
50. Harris, R. J., Ling, V. T., and Spellman, M. W. (1992) *J. Biol. Chem.* **267,** 5102–5107
51. Nishimura, H., Takao, T., Hase, S., Shimonishi, Y., and Iwanaga, S. (1992) *J. Biol. Chem.* **267,** 17520–17525