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Sequence determination of a non-sulfated glycosaminoglycan-like polysaccharide from melanin-free ink of the squid *Ommastrephes bartrami* by negative-ion electrospray tandem mass spectrometry and NMR spectroscopy

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Abstract A non-sulfated polysaccharide was isolated from the ink sac of squid *Ommastrephes bartrami* after removal of the melanin granules. The carbohydrate sequence of this polysaccharide was assigned by negative-ion electrospray tandem mass spectrometry with collision-induced dissociation of the oligosaccharide fractions produced by partial acid hydrolysis of the polysaccharide. The structural determination was completed by NMR for assignment of anomeric configuration and confirmation of linkage information and it was unambiguously identified as a glycosaminoglycan-like polysaccharide containing a glucuronic acid–fucose (GlcA-Fuc) disaccharide repeat in the main chain and a *N*-acetylgalactosamine (GalNAc) branch at Fuc position 3: $-[3\text{GlcA}\beta1\text{–}4(\text{GalNAc}\alpha1\text{–}3)\text{Fuc}\alpha1]_n-$. Partial hydrolysis of the polysaccharide to obtain several oligosaccharide fractions with different numbers of the repeating unit assisted the assignment. In the negative-ion tandem mass spectrometric analysis, the unique ^{0,2}A type fragmentation was important to establish the presence of a 4-linked fucose in the main polysaccharide chain and a GalNAc branch at the Fuc position –3 of the disaccharide repeat.

Keywords Oligosaccharide · Sequence determination · Squid ink polysaccharide · Electrospray mass spectrometry · NMR

Abbreviations

ES-MS	electrospray mass spectrometry
CID	collision-induced dissociation
COSY	correlation spectroscopy
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear single quantum coherence
TFA	trifluoroacetic acid
PMP	1-phenyl-3-methyl-5-pyrazolone
GAG	glycosaminoglycan
SIP	squid ink polysaccharide
SIO	squid ink oligosaccharide
Fuc	Fucose
Ara	arabinose
Glc	glucose
Man	mannose
Gal	galactose
GalN	galactosamine
GlcN	glucosamine
GlcA	glucuronic acid
GalA	galacturonic acid
GalNAc	*N*-acetylgalactosamine

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Introduction

There has been rising interest in recent years in the structure and function of the diverse oligosaccharides that decorate glycolipids, glycoproteins and proteoglycans due to increased awareness of the biological roles of the oligosaccharide chains, *e.g.* signaling, cellular adhesion and protein modification. The use of carbohydrate molecules in pharmaceutical applications is also growing. An example of this is heparin and its low molecular weight fragments that is used as an anticoagulant agent. It has been recently found that marine organisms are a rich source of diverse oligosaccharide sequences with valuable pharmaceutical and biomedical potential [1]. Many exotic polysaccharides with unique carbohydrate sequences have been identified in marine plants and animals that differ from those of mammalian origin [2–4]. As these often are of polyanionic nature, containing hexuronic acids and/or sulfates, similar to mammalian glycosaminoglycans (GAGs), they pose a difficult challenge for structural characterization and functional studies.

A sulfated GAG-like polysaccharide has been identified in squid ink and has been reported to have antibacterial [5–7], anti-tumor [8, 9], and anti-retroviral activities [10]. Several active components, including a tyrosinase and an angiotensin-converting-enzyme inhibitor have been identified [11–14]. Squid belongs to the invertebrate *Mollusca* and, like other cephalopods, relies on ejection of dark ink for defense. The ink consists of a suspension of melanin granules in a viscous and colorless medium. A peptidoglycan from the ink sac of *Illex argentinus* has been reported to have anti-tumor activity and the carbohydrate moiety is a sulfated GAG with sulfate content ranging from 8% to 15%, and a trisaccharide repeating unit comprising glucuronic acid (GlcA), *N*-acetylgalactosamine (GalNAc) and fucose (Fuc) [15, 16]. The GAG-like chain was initially reported to have a linear sequence by NMR analysis using various two-dimensional techniques, glycosidase and periodate oxidation treatment, together with mass spectrometric and HPLC identification of the reaction products as pyridinylamino (PA) derivatives [15]. However, in a later tandem mass spectrometric study of the trisaccharide fraction as a PA derivative, the same group found an intense fragment ion for GalNAc-Fuc-PA in the positive ion spectrum and a weak ion for glucuronic acid–fucose (GlcA-Fuc)-PA in the negative ion spectrum. It was concluded that the sequence of the trisaccharide repeat is branched [16].

Mass spectrometry (MS) has become a primary technique in carbohydrate structural analysis in the last two decades [17]. The rapid advancement in electrospray (ES) ionization and matrix-assisted laser desorption/ionization has allowed MS to be used not only to measure the molecular mass for profiling but also to provide sequence, branching pattern, and partial linkage information of oligosaccharides [18–31]. Negative-ion electrospray mass spectrometry (ES-MS) with collision-induced dissociation tandem mass spectrometry (ES-CID-MS/MS) has been successfully used for sequence determination of various types of carbohydrate molecules [19–22]. It has been demonstrated that high sensitivity detection (low picomole to high femtomole) can be achieved without the need of derivatization even for neutral oligosaccharides [23, 24], eliminating the possibility of potential structural modification of labile groups (*e.g. O*-acetylation) during chemical treatment. The presence of several characteristic fragmentations at certain monosaccharide residues with specific linkages under ES-CID-MS/MS conditions can provide important information on sequence, branching pattern and in certain cases on linkages. For instance, a double glycosidic D-type cleavage [23, 24] is unique to 3-linked GlcNAc and Glc residues, whereas ^{0,2}A-type cleavages (nomenclature used to define the cleavage based on that introduced previously [32]), only occur with 4-linked GlcNAc [23], Glc [24] and unsubstituted glucosamine [25]. These fragmentations were used for identification of important structural features on underivatized neutral and sialylated oligosaccharides, including differentiation of type 1 and type 2 chains [23,24] or blood-group types [23, 26–29] (*e.g.* Lewisa or Lewis×determinants). Differential decarboxylation has also been reported for differentiation of sialic acid 2–3/2–6 linkages on sialylated isomeric backbone sequences [27, 30]. ES-CID-MS/MS has also been successfully applied to identification of sulfated oligosaccharides [31].

Although MS is a sensitive method of structural analysis, NMR spectroscopy is also a necessary choice for more complete assignment of carbohydrate structures when sufficient material is available. Using the negative-ion ES-CID-MS/MS strategy eight novel oligosaccharide sequences, including six neutral [26, 27, 29], one sialylated [28] and a glucuronic acid-containing [25, 33] oligosaccharides, have been determined, and the structural assignments completed by NMR analysis.

In the present study, we extend the negative-ion ES-CID-MS/MS strategy to a polysaccharide and determine the sequence of a non-sulfated GAG-like polysaccharide which was isolated from the melanin-free ink of squid *Ommastrephes bartrami*. The overall strategy includes partial depolymerization to obtain oligosaccharide fractions with different numbers of repeating unit and their sequence determination (including branching pattern and partial linkage) by negative-ion ES-CID-MS/MS to derive the overall sequence of the squid ink polysaccharide (SIP). The structural analysis was finally completed by NMR spectroscopy for assignment of anomeric configuration and confirmation of linkage information.

Materials and methods

Materials

The ink of squid *Ommastrephes bartrami* was obtained from Zhou-Shan Fishery Company (Zhejiang, China) and stored at −40°C before use. Gel-filtration medium Bio-Gel P-2 was from BioRad (Richmond, CA, USA), HW40-C and TSK G4000PWXL columns from TOSOH BIOSEP (Tokyo, Japan), and Sephacryl S-300 from Amersham Biosciences (Uppsala, Sweden). DEAE ion-exchange gel was from Whatman (Brentford, England). D-mannose (Man) and L-fucose (Fuc), papain and cystein (Cys) were purchased from Fluka (Seelze, Germany), while L-arabinose (Ara), D-galactose (Gal), D-galactosamine (GalN), D-glucosamine (GlcN), D-glucuronic acid (GlcA), D-galacturonic acid (GalA) and disaccharide lactose from Sigma (St. Louis, Missouri, USA). The derivatization reagent 1-phenyl-3-methyl-5-pyrazolone (PMP) was from Sinopharm Chemical Reagent (Shanghai, China).

Isolation and purification of non-sulfated squid ink polysaccharide

Fresh ink (ca. 1,000 g) taken directly from 10 ink sacs of squid *Ommastrephes bartrami* was acidified to pH 4–5 with 0.1 M HCl and the solution was allowed to stand for 24 h at 4°C to participate melanin. Melanin was removed after centrifugation at 5,000 g for 1 h. Melanin-free ink was then digested with 2 volumes of 1% (w/v) papain in Tris–HCl buffer (50 mM, pH 6.8; containing 5 mM Cys and 5 mM EDTA) at 60°C for 24 h. Digestion was repeated twice to ensure the cleavage of the protein/peptide moiety. Crude melanin-free SIP was obtained after precipitation with 4 volumes of ethanol. The crude SIP was purified by sequential gel filtration and anion-exchange chromatography on a Sephacryl S-300 column (1.6×100 cm) and a DEAE ion-exchange column (2.6×30 cm), respectively. Carbohydrate was detected by phenol/sulfuric assay [34].

The purity of the isolated SIP was determined by cellulose-acetate membrane electrophoresis, which was carried out in 0.1 M HCl (pH 1.2) at 3 mA for 30 min as described [35]. Membrane was stained with 0.2% Alcian blue in 0.1% acetic acid. The molecular weight of SIP was determined by liquid chromatography on an Agilent 1100 system (Palo Alto, CA, USA) with a TSK G4000PWXL column (TOSOH BIOSEP) by elution with 0.2 M NaCl.

Chemical composition determination

Monosaccharide compositions were determination by a PMP-HPLC method [36]. In brief, poly- or oligosaccharides (typically 1 mg) were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 110°C under nitrogen for 8 h with lactose added as internal standard. The monosaccharide hydrolysate was dried under vacuum, and then derivatized with 450 μL PMP solution (0.5 M, in methanol) and 450 μL of 0.3 M NaOH at 70°C for 30 min [36]. The reaction was stopped by neutralization with 450 μL of 0.3 M HCl and extraction with chloroform (1 mL, three times). HPLC analyses were performed on an Agilent ZORBAX Eclipse XDB-C18 column (5 μm, 4.6×150 mm,) at 25°C with detection at UV 250 nm. The mobile phase was 0.05 M KH_2PO_4 (pH 6.9) with 15% (solvent A) and 40% (solvent B) acetonitrile in water. A gradient of B from 8% to 19% in 25 min was used.

Sulfate content was determined by $BaCl_2$/gelatin method [37] and ion-chromatography [38], and protein by the Lowry method [39].

Periodate oxidation of SIP

Periodate oxidation was carried out with 0.05 M aqueous $NaIO_4$ solution at 4°C for 4 days. The reaction was stopped by dialysis against distilled water for 24 h and the retained reaction solution was lyophilized. Periodate oxidation products were then hydrolyzed and the compositions were determined by monosaccharide composition analysis using the HPLC-PMP method as described above.

Preparation of SIP oligosaccharides

Oligosaccharides from SIP were obtained by partial depolymerization of the polysaccharide with 0.05M H_2SO_4 at 80°C for 16 h. The oligosaccharide mixture was fractionated by gel filtration on a HW40-C column (2.6×120 cm) eluted with 0.2M NH_4HCO_3. Each fraction was desalted by a Bio-Gel P-2 column (1.6×100 cm). Carbohydrate content was monitored by phenol/sulfuric method [35].

A disaccharide fraction was prepared from the polysaccharide using 0.1 M TFA at 100°C for 3 h and purified on a HW-40C column (2.6×120 cm).

Negative-ion ES-MS and CID MS/MS

Negative-ion ES-MS and CID MS/MS were carried out on a Waters Ultima mass spectrometer (Manchester, UK) with a Q-TOF configuration. Nitrogen was used as desolvation and nebulizer gas at a flow rate of 250 L/h and 150 L/h, respectively. Source temperature was 80°C, and the desolvation temperature 150°C. A cone voltage of 40 V was used for both negative- and positive-ion detection and the capillary voltage was maintained at 3 kV. Product-ion spectra were obtained from CID using argon as the collision

gas at a pressure of 0.17 MPa. The collision energy was adjusted between 10 and 36 V for optimal fragmentation. A scan rate of 1.0 s/scan was used for both ES-MS and CID MS/MS experiments, and the acquired spectra were summed for presentation. Oligosaccharides were dissolved in acetonitrile/water (1:1, v/v), typically at a concentration of 20 pmol/μL, of which 5 μL was loop-injected. Solvent (acetonitrile/1 mM ammonium bicarbonate, 1:1, v/v) was delivered by a Harvard syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 10 μL/min.

NMR spectroscopy and infrared spectroscopy

For NMR analysis, squid ink polysaccharide (50 mg) and oligosaccharides (10 mg) were co-evaporated with D_2O (99.8%) twice by lyophilization before final dissolution in 500 μL high-quality D_2O (99.96%), containing 0.1 μL acetone. ^{1}H-NMR experiments were carried out at 600 MHz and ^{13}C-NMR at 150 MHz. Spectra were recorded at 25°C for the oligosaccharides and 60°C for the polysaccharide. The temperatures were chosen in order to place the HDO signals with minimal disturbance to carbohydrate protons. The observed ^{1}H chemical shifts were reported relative to internal acetone (2.23 ppm). Correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and heteronuclear single quantum coherence (HMQC) experiments were also carried out at 25°C.

An infrared spectrum of the polysaccharide (×0.5 mg) was taken on a Perkin-Elmer instrument as KBr pellets at room temperature.

Results and discussion

Isolation of non-sulfated glycosaminoglycan-like polysaccharide from squid ink

From 10 ink sacs of *Ommastrephes bartrami* about 15 g (yield: 1.5%) of crude melanin-free polysaccharide was obtained following centrifugation, papain digestion and ethanol precipitation. Crude SIP was then fractionated by Sephacryl S-300 gel chromatography (Fig. 1a) and the major fraction indicated by the solid bar was pooled for further purification by DEAE anion-exchange chromatography (Fig. 1b). The purity of the SIP was determined by cellulose-acetate membrane electrophoresis, which showed a single band (lane 2, Fig. 1c) with a slower migration than chondroitin sulfate A and B (lanes 3 and 4, respectively, Fig. 1c). The molecular mass of SIP was determined to be 48 kD by gel filtration chromatography on a TSK G4000PWXL column (data not shown).

Chemical compositions of SIP

Monosaccharide composition analysis was carried out by HPLC following acid hydrolysis and derivatization with PMP [34]. Three monosaccharides, Fuc, GlcA and GalN in a molar ratio of 1:1:1, were found to be the main components, whereas other monosaccharide residues (*e.g.*

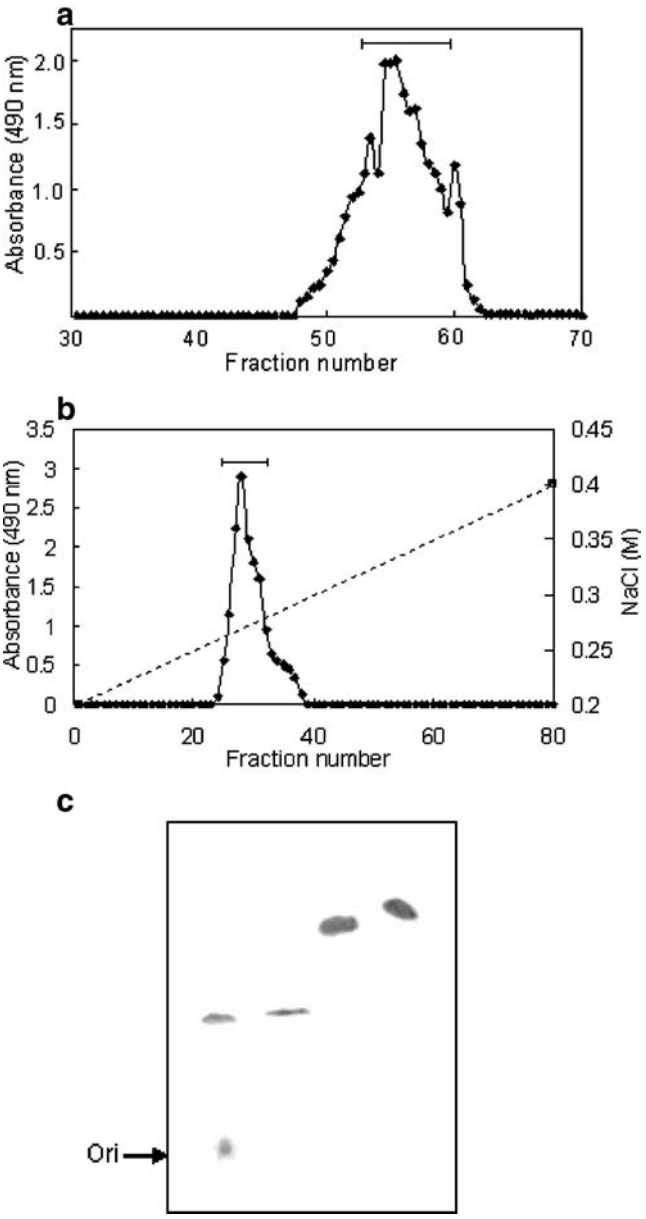

Fig. 1 Isolation of a non-sulfated glycosaminoglycan-like polysaccharide from squid ink. The crude SIP was fractionated by gel filtration chromatography of on a Sephacryl S-300 column (**a**) and the collected fraction was further purified by ion-exchange chromatography on a DEAE ion-exchange column (**b**). *Solid bars* indicate the fractions collected. The purity of the purified SIP was analyzed by cellulose-acetate membrane electrophoresis (**c**); *lane 1*: crude SIP, *lane 2*: purified SIG, *lane 3*: chondroitin sulfate A, and *lane 4*: chondroitin sulfate B

Table 1 Monosaccharide compositions and protein contents of SIP and its oligosaccharide fractions

Fractions	Molar ratio of monosaccharides								Protein contents (%)
	Fuc	GlcN	GlcA	GalA	GalN	Gal	Ara	Man	
SIP	1.00	0.14	0.96	0.03	1.01	0.03	0.03	0.08	3.81
OF-I	1.00	0.36	0.98	0.05	1.02	0.12	0.06	0.29	–
OF-II	1.00	0.41	0.96	0.05	0.99	0.12	–	0.26	–
OF-III	1.00	0.02	0.95	0.04	0.94	0.02	–	0.07	–
OF-IV	1.00	–	0.99	–	1.02	–	–	–	–
OF-V	1.00	0.10	–	0.02	3.72	0.12	–	–	–

Monosaccharide content was in molar ratio relative to Fuc and protein content in percentage mass. Dashes: not detected (value<0.01).

GalA, GlcN, Gal, Ara and Man) were found in very minor concentrations (Table 1). A small amount of protein was also detected.

In the purified SIP, sulfate was not detected by either the $BaCl_2$/gelatin or ion-chromatography method. The absence of sulfate is different from the GAGs isolated from *Illex argentinus* (11) in which sulfate content of 8–15% were detected. The lack of sulfate in SIP was further confirmed by IR spectroscopy with the absence of the expected peaks at 860 cm^{-1} (C–O–S) and 1,240 cm^{-1} (S=O) (data not shown).

Preparation and fractionation of SIP oligosaccharide fragments

Oligosaccharide fragments were prepared by partial depolymerization of SIP with mild acid hydrolysis. The extent of hydrolysis was assessed by high performance TLC of aliquots of reaction mixtures obtained under various conditions. Optimized conditions with H_2SO_4 (0.05 M) at 80°C for 16 h were chosen for large scale preparation. The hydrolysate was fractionated by gel filtration chromatography on a HW40-C column and five squid ink oligosaccharide fractions (SIO-I to SIO-V, Fig. 2) were collected. Monosaccharide composition analysis (Table 1) showed that the fractions SIO-I to SIO-IV each contained primarily the same monosaccharides (Fuc, GlcA and GalN) in the same molar ratio (1:1:1), indicating a repeating trisaccharide unit in the polysaccharide chain. SIO-V was eluted at the included volume (V_t, Fig. 2) contained mainly the monosaccharide GalN (~80%) together with some Fuc (~20%).

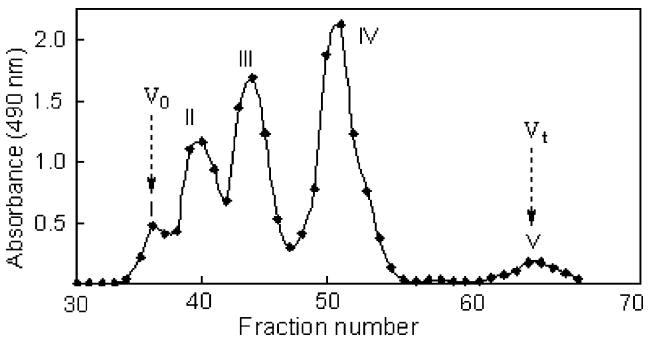

Fig. 2 Fractionation of SIP oligosaccharides by gel filtration chromatography. Oligosaccharide fragments were obtained by partial depolymerization of SIP with 0.05 M H_2SO_4, fractionation on a HW40-C column, elution by 0.2 M NH_4HCO_3 at a flow rate of 15 mL/h, and detected by phenol/sulfuric assay off-line

As SIO-I was collected at the void volume and contained multiple components, it was not subjected to further structural analysis. The major fractions SIO-II, III and IV were shown to contain nona-, hexa- and trisaccharides, respectively, with the formula of $(GlcA.Fuc.GalNAc)_n$ (n= 1–3), by negative-ion ES-MS (Table 2). Some very minor components (<5%) with one GalNAc less were also detected by ES-MS in these fractions and these minor components were isolated by further fractionation on a Bio-Gel P-2 column. The minor subfractions SIO-IIa and SIO-IIIa thus obtained from SIO-II and SIO-III were the octasaccharide $GlcA_3.Fuc_3.GalNAc_2$ and pentasaccharide $GlcA_2.Fuc_2.GalNAc_1$, respectively, by ES-MS analysis (Table 2). The loss of a GalNAc was likely to be caused by the acid hydrolysis procedure used for depolymerization and the cleaved GalNAc was eluted in the fraction SIO-V.

Sequence determination of SIP oligosaccharides by negative-ion ES-CID-MS/MS

Product-ion spectra were acquired with tri-, hexa- and nonasaccharide fractions to determine their sequences. The di- and pentasaccharide fractions lacking a GalNAc were also used to assist the assignment. The unequivocal sequences of individual oligosaccharides obtained were used to derive the overall sequence of the polysaccharide.

Trisaccharide SIO-IV The product-ion spectrum of SIO-IV in the negative-ion mode gave limited information with only a C_1 ion at *m/z* 193 (Fig. 3a), indicating a GlcA at the non-reducing terminus. The lack of a C_2 ion was highly

Table 2 Negative-ion ES-MS of SIP oligosaccharide fractions

Fractions	Observed ions			Assignments	
	$[M-H]^-$	$[M-2H]^{2-}$	$[M-3H]^{3-}$	Mol masses	Compositions
OF II		796.0 (80)	530.3 (100)	1,593.5	$GlcA_3.Fuc_3.GalNAc_3$
OF IIa		694.4 (22)	462.6 (5)	1,390.4	$GlcA_3.Fuc_3.GalNAc_2$
OF III	1,067.4 (100)	533.2 (65)		1,068.3	$GlcA_2.Fuc_2.GalNAc_2$
OF IIIa	864.4 (18)	431.7 (40)		865.3	$GlcA_2.Fuc_2.GalNAc_1$
OF IV	542.2 (100)			543.2	$GlcA_1.Fuc_1.GalNAc_1$
OF Iva	339.1			340.1	$GlcA_1.Fuc_1$

indicative of a branched sequence. The positive spectrum of MH^+ (Fig. 3b) was also acquired and this produced Y_1 and Z_1 ions at *m/z* 350 and *m/z* 368, respectively, confirming the presence of a non-reducing terminal GlcA and a disaccharide Fuc.GalNAc at the reducing end. A fragment ion at *m/z* 204 indicated a terminal GalNAc but there was no direct evidence whether the GalNAc was in a linear or a branched sequence.

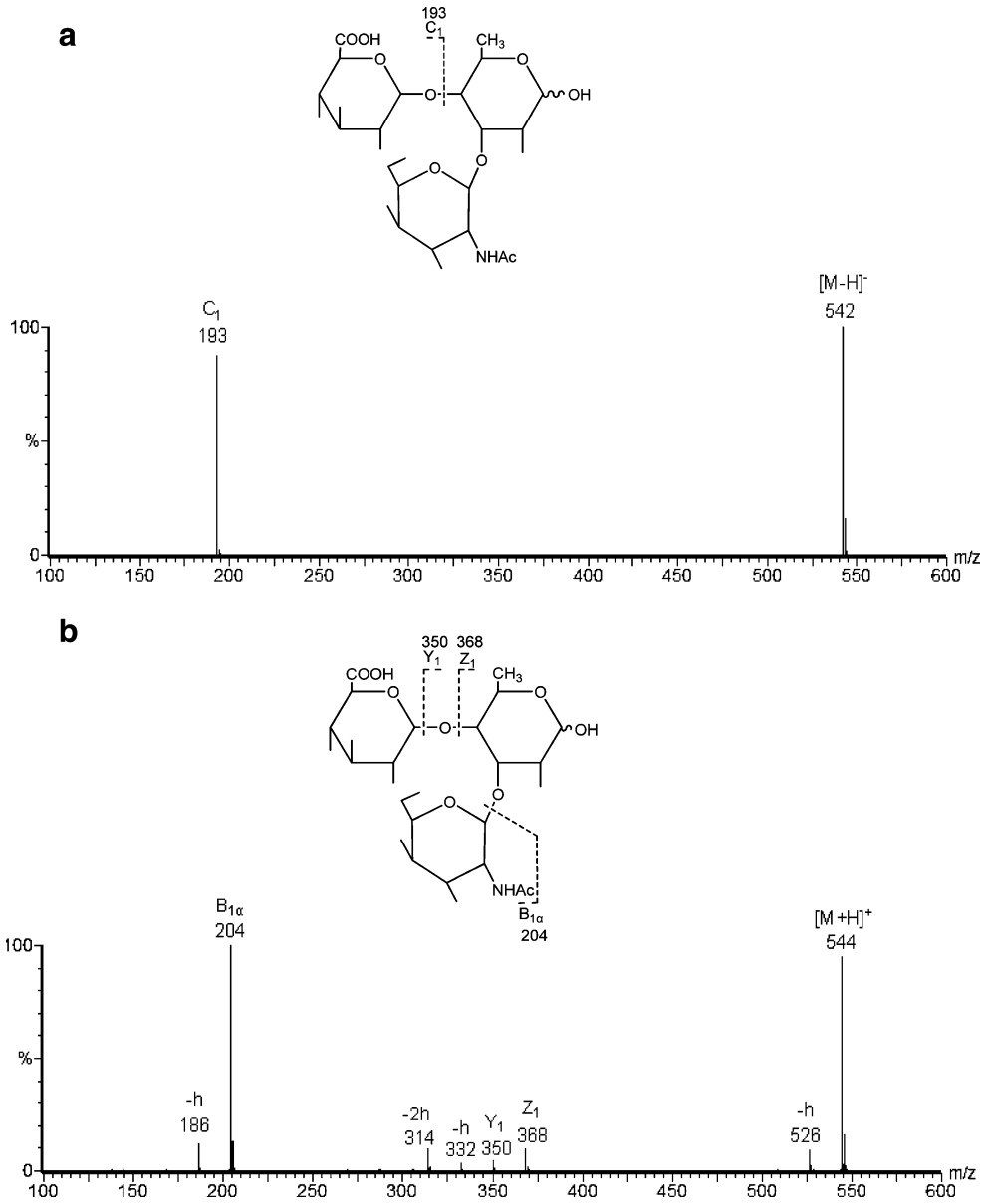

Fig. 3 ESI-CID-MS/MS product-ion spectra of trisaccharide SIO-IV. **a** $[M-H]^-$ and **b** MH^+. Structures are shown to indicate the proposed fragmentation. The nomenclature used to define the cleavage is based on that introduced previously [32]. The ions marked with *h* are fragments produced by dehydration of the major ions

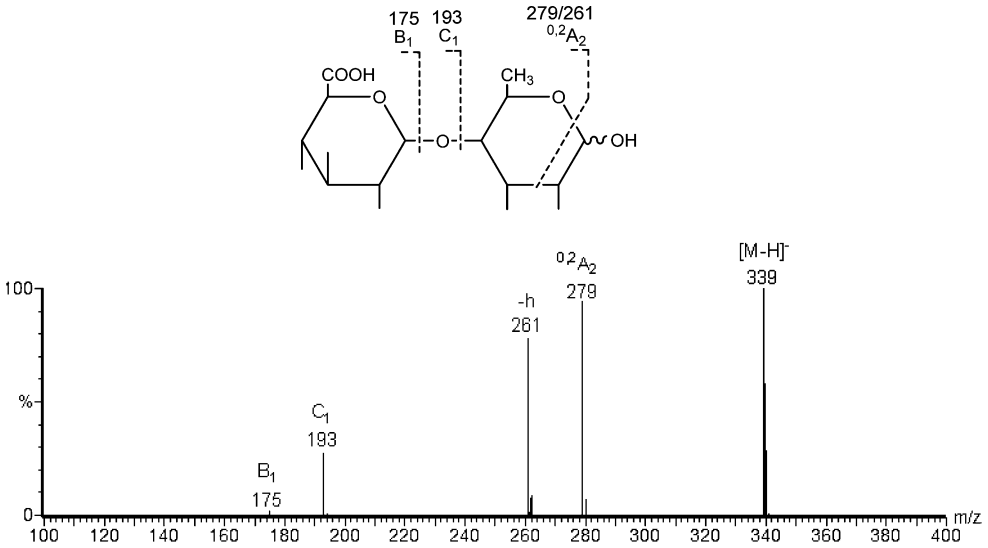

Fig. 4 Negative-ion ESI-CID-MS/MS product-ion spectrum of disaccharide SIO-IVa. Structure is shown to indicate the proposed fragmentation. The nomenclature used to define the cleavage is based on that introduced previously [32]. The ion marked with *h* is a fragment produced by dehydration of the major ion

As only limited sequence information was available with the trisaccharide, a further attempt was made with the disaccharide fraction SIO-IVa containing GlcA and Fuc (Table 2), obtained from acid hydrolysis (0.1 M TFA) of the polysaccharide. In the negative product-ion spectrum of SIO-IVa, in addition to C_1 and B_1 ions (m/z 193 and 175, respectively, Fig. 4) a unique ^{0,2}A ion doublet occurred at *m/z* 279/261. The ^{0,2}A ion doublet is characteristic of a 4-linked residue [23–25], indicating the presence of a -4Fuc and therefore a disaccharide sequence of GlcA1–4Fuc. However, in the negative-ion spectrum of the trisaccharide (Fig. 3a) this ion doublet was absent, suggesting that the 4-linked Fuc was further substituted at the 3-position, preventing such ^{0,2}A fragmentation as previously demonstrated [23, 24]. Taken together, the trisaccharide is deduced to be:

GlcA1–4(GalNAc1–3)Fuc.

Hexasaccharide SIO-III The hexasaccharide SIO-III also gave a simple negative product-ion spectrum (Fig. 5) with exclusively non-reducing terminal ions dominated by C-type fragmentation. The C_1 ion at *m/z* 193 again identified a

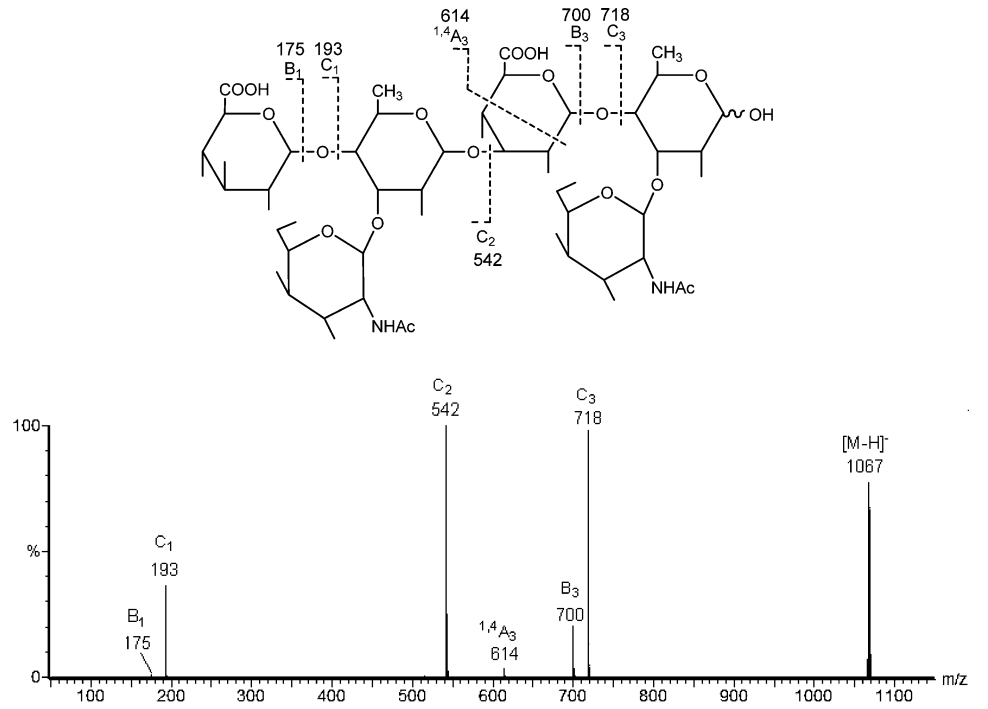

Fig. 5 Negative-ion ES-CID-MS/MS product-ion spectrum of hexasaccharide SIO-III. Structure is shown to indicate the proposed fragmentation. The nomenclature used to define the cleavage is based on that introduced previously [32]

non-reducing terminal GlcA, whereas the mass difference of 349 Da between C_1 and C_2 (*m/z* 542) clearly indicated a GalNAc branch at the Fuc position (203+146 Da). The ion C_3 at m/z 718 was indicative of a GlcA extending the chain further at the Fuc. A gap of a 349 Da between C_3 and $[M–H]^-$ again suggested a GalNAc branch at the reducing terminal Fuc. A weak $^{1,4}A_3$ ion (*m/z* 614) was also observed and considered to derive from the cross-ring 1,4-cleavage of the internal GlcA. As cross-ring double cleavages do not normally take place at a linkage position this strongly suggested that the GlcA was 3-substituted (see the structure shown in Fig. 5 for fragmentation).

The assignment of the hexasaccharide was corroborated by the product-ion spectrum (Fig. 6) of the pentasaccharide fraction SIO-IIIa, which has a GalNAc less than the hexasaccharide (Table 2). As the loss of a GalNAc can take place at either the internal or the reducing terminal Fuc positions, two possible pentasaccharides, SIO-IIIa and SIO-IIIa′, were obtained (see structures in Fig. 6 for illustration). The product-ion spectrum clearly indicated the presence of this mixture. Apart from a similar fragmentation to the hexasaccharide, additional $^{0,2}A$ fragmentation occurred at the mono-4-substituted Fuc, *e.g.* the ion doublet $^{0,2}A_2'$ at *m/z* 279/261 from the pentasaccharide SIO-IIIa′ and $^{0,2}A_4$ at *m/z* 804/786 from SIO-IIIa (Fig. 6). The $^{1,4}A_3$ fragmentation similarly gave two ions at *m/z* 614 and *m/z* 411 for SIO-IIIa and SIO-IIIa′, respectively. The assignment of the 3-substitution of the GlcA was confirmed by periodate oxidation treatment of SIP. The oxidation products were analyzed by PMP-HPLC and an intact GlcA was detected (data not shown). This demonstrated a 3-linked GlcA which was not affected by the periodate treatment as there was no vicinal diol present.

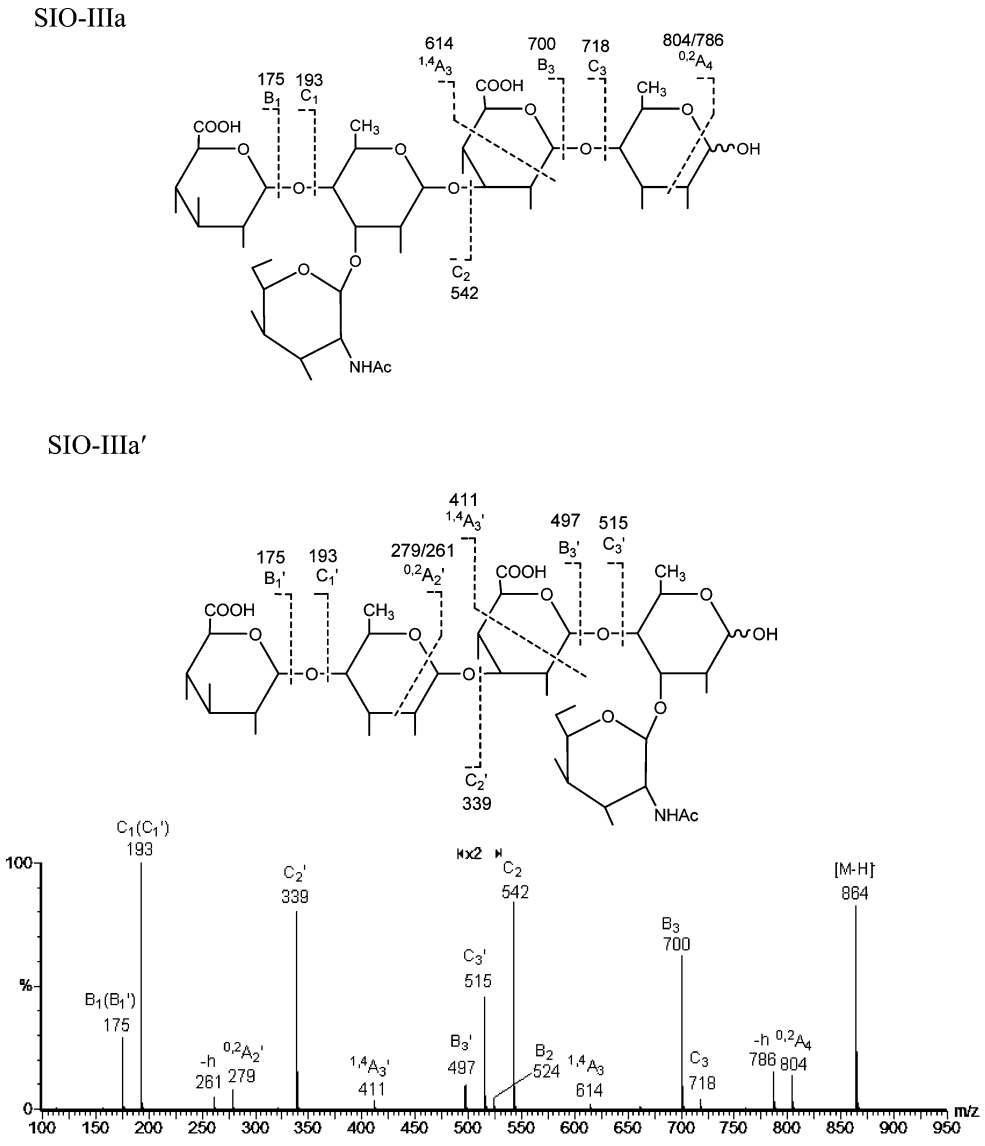

Fig. 6 Negative-ion ES-CID-MS/MS product-ion spectrum of pentasaccharide fraction SIO-IIIa. Structures are shown to indicate the proposed fragmentation. The nomenclature used to define the cleavage is based on that introduced previously [32]. The ions marked with *h* are fragments produced by dehydration of the major ions

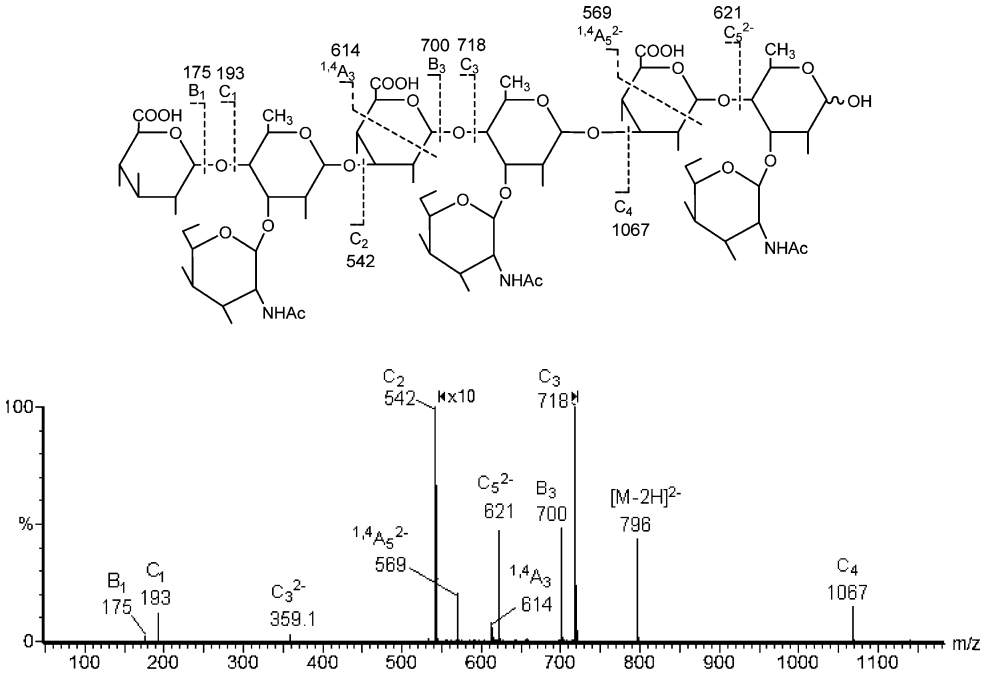

Fig. 7 Negative-ion ES-CID-MS/MS product-ion spectrum of nonasaccharide SIO-II using doubly charged $[M-2H]^{2-}$ as the precursor. Structures are shown to indicate the proposed fragmentation. The nomenclature used to define the cleavage is based on that introduced previously [32]. The ions marked with *h* are fragments produced by dehydration of the major ions

Therefore, the hexasaccharide can be concluded to have the following sequence:

GlcA–4(GalNAc1–3)Fuc–3GlcA–4(GalNAc1–3)Fuc.

Nonasaccharide SIO-II As the content of the acidic residue GlcA increased in the nonasaccharide, the intensity of the singly charged ion $[M-H]^-$ was decreased considerably and this made product-ion scanning difficult. However, the doubly charged ion $[M-H]^{2-}$ can also be used as the precursor to obtain similar fragmentation for sequence determination. As shown in Fig. 7, the expected full set of C-type ions were all present, although some were doubly charged, *e.g.* C_1 (*m/z* 193), C_2 (*m/z* 542), C_3 (*m/z* 718), C_4 (*m/z* 1,067) and C_5 (*m/z* 621.4, doubly charged). Two $^{1,4}A$

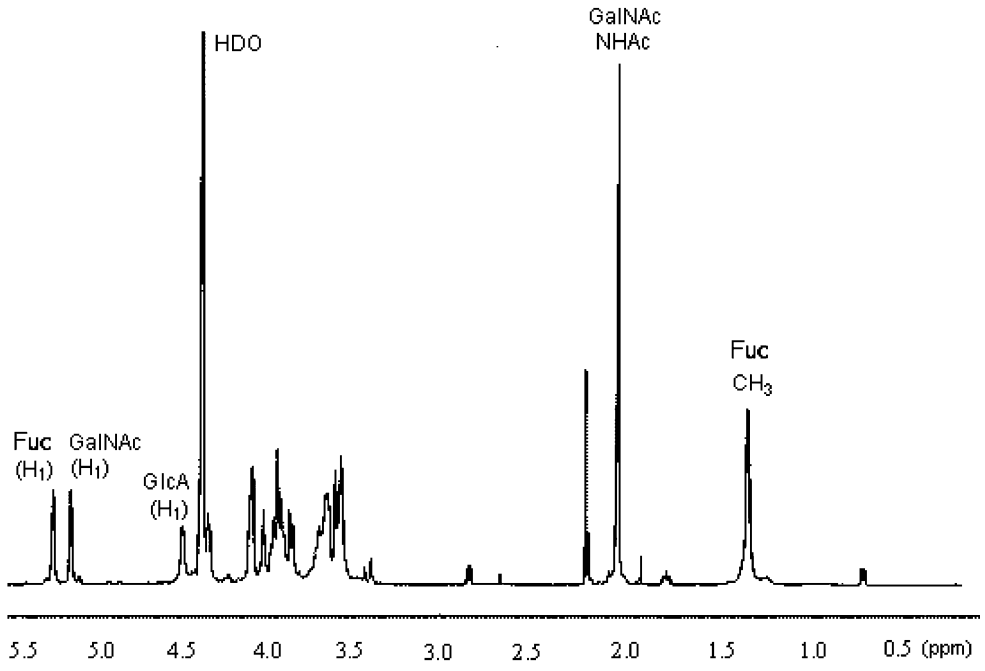

Fig. 8 Six hundred megahertz ^{1}H-NMR spectrum of SIP. Polysaccharide SIP (50 mg) was co-evaporated with D_2O twice by lyophilization before final dissolution in 500 μL high-quality D_2O (99.96%), containing 0.1 μL acetone. ^{1}H-NMR spectrum was recorded 60°C. ^{1}H chemical shifts were reported relative to internal acetone (2.23 ppm)

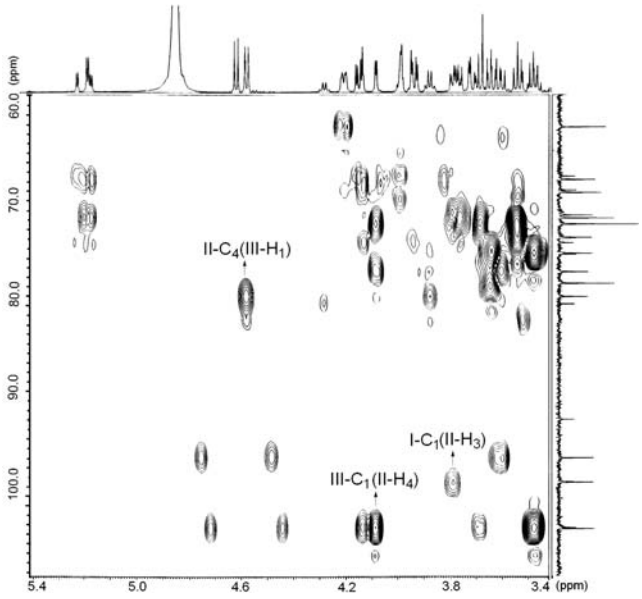

Fig. 9 2D-NMR HMBC spectrum of trisaccharide SIO-IV. Oligosaccharide SIO-IV (10 mg) was co-evaporated with D_2O twice by lyophilization before final dissolution in 500 μL high-quality D_2O, containing 0.1 μL acetone. HMBC experiment was carried out at 25°C and the ^1H chemical shifts were reported relative to internal acetone (2.23 ppm)

ions ($^{1,4}A_3$ at *m/z* 614, and doubly charged $^{1,4}A_5$ at *m/z* 569.4) were also present. Altogether, this clearly indicated the nonasaccharide SIO-II to be:

GlcA–4(GalNAc1–3)Fuc–3GlcA–4
(GalNAc1–3)Fuc–3GlcA–4(GalNAc1–3)Fuc.

Completion of sequence assignment of SIP by NMR

The mass spectrometric assignment of the sequence of SIP was verified and completed by ^1H- and ^{13}C-NMR. The ^1H-NMR spectrum of SIP is shown in Fig. 8 and three narrow doublet peaks were observed at 5.27 ppm (d, *J*=3.42), 5.18 ppm (d, *J*=3.64) and 4.52 ppm (d, *J*=6.06), which were attributed to the anomeric protons of Fuc(α), GalNAc (α) and GlcA(β), respectively, similar to those reported previously for an oligosaccharide [15]. The signals at 1.29 ppm (d, *J*=3.50) and 2.03 ppm (s) were from the methyl group of Fuc and *N*-acetyl group of GalNAc, respectively.

The ^1H-NMR spectrum of SIO-IV (Table 3) was very similar to that of the polysaccharide. However, the peak at 5.27 ppm for the internal Fuc(α) in the polysaccharide chain were absent (Fig. 8) and two new signals appeared. These were the reducing terminal Fuc with α (5.17 ppm, *J*=3.6) and β (4.59 ppm, *J*=7.2) anomeric configurations indicating the Fuc residue at the reducing terminus of the trisaccharide repeat. Assignments of ^1H and ^{13}C chemical-shifts of these residues (Table 3) were made from HMBC (Fig. 9), HMQC and DQF-COSY (not shown). The ^{13}C-NMR spectrum of the trisaccharide ISO-IV (Table 3) gave clear information on the substitution of the reducing terminal Fuc. The upfield shift of C_3 and C_4 signals of Fuc, compared to those of the monosaccharide, indicated substitutions at both 3- and 4-positions (Table 3). The presence of the correlations in the HMBC spectrum (Fig. 9), together with DQF-COSY and HMQC spectra (not shown), unambiguously identified GalNAcα and GlcAβ linked to the C-3 and C-4 positions of the Fuc, respectively. Therefore, the complete sequence for the trisaccharide repeat is:

$-[3\text{GlcA}\beta1\text{–}4(\text{GalNAc}\alpha1\text{–}3)\text{Fuc}\alpha1]_n-$.

Conclusions

A non-sulfated GAG-like polysaccharide was isolated from the melanin-free ink of squid *Ommastrephes bartrami*. The sequence was established initially by negative-ion ES-CID-MS/MS of the oligosaccharide fractions containing various numbers of the trisaccharide repeat. The full structure was completed by NMR analysis for assignment of the anomeric configurations and verification of linkages. It was unambiguously identified as a non-sulfated GAG-like polysaccharide containing a disaccharide GlcA-Fuc repeat in the main chain and a GalNAc branch at the Fuc position: –[3GlcAβ1–4(GalNAcα1–3)Fucα1]$_n$–. Partial hydrolysis of the polysaccharide to obtain several oligosaccharide fractions with

Table 3 ^1H and ^{13}C chemical shifts, H_1, H_2 coupling constants from NMR spectra of SIO-IV

Residue	H_1 (C_1)	H_2 (C_2)	H_3 (C_3)	H_4 (C_4)	H_5 (C_5)	H_6 (C_6)	CH_3	C=O
I GalNAc	5.15, *J*=3.8 (101.87)	4.13 (52.37)	3.91 (70.08)	4.12 (71.46)	4.19 (74.13)	3.73 (64.57)	2.01 (24.82)	(177.49)
II Fuc(α)	5.17, *J*=3.6 (95.25)	3.57 (74.77)	3.75 (79.77)	4.05 (82.40)	3.84 (73.84)	1.35 (18.21)		
II Fuc(β)	4.59, *J*=7.2 (99.3)			4.13 (83.16)	4.22 (69.75)	1.31		
III GlcA	4.55, *J*=7.6 (106.73)	3.45 (76.23)	3.52 (77.93)	3.62 (74.77)	3.65 (81.01)	(177.25)		

Chemical shifts in ppm and coupling constants (*J*) in Hz.

different numbers of the repeating unit assisted the assignment. In the negative-ion tandem mass spectrometric analysis the unique ^{0,2}A type fragmentation was important to establish a 4-linked Fuc in the polysaccharide main chain and a monosaccharide GalNAc branch on this fucose.

Similar sequence has not been found in other sources, but it is interesting to note that polysaccharides isolated from sea cucumber also contains GlcA, GalNAc and Fuc in the monosaccharide composition. However, the sequence is different from SIP. The backbone is composed of repeating disaccharide unit of GlcA-GalNAc with fucose as the branch at GlcA position, a chondroitin sulfate-like backbone sequence [40, 41], rather than a GlcA-Fuc disaccharide repeat backbone with GalNAc as the branch in the case of SIP.

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