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Analysis of monosaccharides, fatty constituents and rare *O*-acetylated sialic acids from gonads of the starfish *Asterias rubens*

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Abstract

A previous study (Bergwerff et al., Biochimie 74 (1992) 25–37) reported that sialic acids present in *Asterias rubens* gonads were essentially composed of 8-methyl-*N*-glycolylneuraminic acid (Neu5Gc8Me), a large part of it being acetylated in position 9. Using GC/MS of heptafluorobutyrate derivatives (Zanetta et al., Glycobiology 11 (2001) 663–676) on the chloroform/methanol soluble and insoluble fractions, we showed that most sialic acids were found in the latter and demonstrated that all sialic acids were derived from *N*-glycolylneuraminic acid, most of them being 8-methylated, but that the majority were also acetylated in position 4 or 7 (or both positions). GC/MS analyses of the constituents liberated using acid-catalysed methanolysis verified that major glycoprotein-bound glycans were N-linked and of the gluco-oligomannosidic type. Major fatty acids were poly-unsaturated (especially C20:4) and long-chain bases were C22:1 phytosphingosine and C22:2 6-hydroxysphingenine. Major monosaccharides found in the chloroform/methanol extract (quinovose and fucose) were derived from steroidal saponins.

Keywords: Asterias; *N*-acetylneuraminic acid; *N*-glycolylneuraminic acid; *N*-glycolyl-8-methyl-neuraminic acid; *O*-acetylated sialic acids; N-linked glycans; Unsaturated lipids

1. Introduction

In recent years, increasing interest was drawn to the diversity of sialic acids, since these monosaccharides could play important biological roles (for reviews see Refs. [1–4]). For example, 9-*O*-acetyl *N*-acetylneuraminic acid is a specific ligand for the agglutinin of *influenza* virus C [5], whereas 4-*O*-acetylated *N*-acetylneuraminic acid is a specific ligand for the agglutinin of murine hepatitis S virus [6]. Based on previous work [1,2], sialic acids present an extreme diversity and more than 40 different compounds were identified differing in the presence in position 5 of an amino group (neuraminic acid derivatives) or an hydroxyl group (3-deoxy-D-glycero-D-galacto-nonulosonic acid (Kdn)), different acylations of the NH_2 group at position 5 (acetyl, glycolyl) and various substituents of the different hydroxyl groups (acetyl, lactyl, methyl, sulphate, phosphate, etc.). Complex monosaccharides structurally identical with sialic acids were identified in bacteria (legionaminic acid and derivatives), which may represent important epitopes [7,8]. Sialic acids show sometimes a clear species specificity. For example, *N*-glycolylneuraminic acid (Neu5Gc) (but not Kdn) is absent from man, although it was found in all other mammalian species so far analysed. The starfish *Asterias rubens* presents the

Abbreviations: aag, alkyl-acyl-glycerol; C22:1phyt, 1,3,4-trihydroxy-2-amino-docosene; C22:2sphe6oh, 1,3,6-trihydroxy-2-amino-4,x-ene-docosene; CM, chloroform methanol-soluble; CMI, chloroform methanol-insoluble; EI, electron impact; FAME, fatty acid methyl-ester; HFB, heptafluorobutyrate; HFBAA, heptafluorobutyric acid anhydride; GC, gas chromatography; Kdn, 3-deoxy-D-glycero-D-galacto-nonulosonic acid; LCB, long-chain base; MS, mass spectrometry; the nomenclature used for sialic acids is according to Schauer and Kamerling (1997): Neu5Ac = *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Gc8Me, 8-methyl-*N*-glycolylneuraminic acid; Neu4Ac5Gc8Me, 4-*O*-acetyl-8-methyl-*N*-glycolylneuraminic acid; Neu7Ac5Gc8Me, 7-*O*-acetyl-8-methyl-*N*-glycolylneuraminic acid; Neu4,7$Ac_2$5Gc8Me, 4,7-di-*O*-acetyl-8-methyl-*N*-glycolylneuraminic acid; Qui, quinovose (6-deoxyglucose).

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peculiarity of producing 8-*O*-methyl-5-glycolylneuraminic acid (Neu5Gc8Me) as the major compound [9,10], thus providing a good model for studying the sialate 8-methyltransferase [11]. Using a new method, which allows the determination of the sialic acid diversity without prior purification of the monosaccharides [12], we demonstrate that the major sialic acids of the gonad of *A. rubens* are mono-*O*-acetylated derivatives (in position 4 and 7) of Neu5Gc8Me. Therefore, this material also constitutes a good model for studying the sialate-*O*-acetyltransferases involved in their biosynthesis.

2. Materials and methods

2.1. Materials

A. rubens (2–3 years old) was collected in the North-Friesian part of the North Sea in early spring. The gonads were dissected, lyophilised and stored at –80 °C. The powder was extracted using chloroform/methanol (CM) mixtures [13] and the CM soluble (CM) and insoluble (CMI) fractions were separated by centrifugation (4000 rpm at room temperature). The two samples were evaporated using a rotary evaporator (at room temperature) and the remaining water was eliminated by lyophilisation. Samples were shipped to Villeneuve d'Ascq as dried material.

2.2. Analyses of the sialic acid diversity

The samples were weighted (about 1 mg of each) in reaction vials [14] and submitted first to a mild acid hydrolysis (1 ml, 2 M acetic acid during 105 min at 80 °C) and dried using a rotary evaporator. The samples were suspended adding 500-µl anhydrous methanol and 500 µl of a diazomethane solution in diethyl-ether [12]. The samples were left overnight in the closed vials. Before analysis, the samples were dried under a stream of nitrogen and supplemented with 400 µl dried acetonitrile and 50 µl of heptafluorobutyric acid anhydride (HFBAA; Fluka, 99% purity). After cooling at room temperature, the samples were evaporated under a stream of nitrogen and taken up in 400 µl of dried acetonitrile. 1–2 µl were injected onto the needle of the Ross injector of the GC/MS apparatus.

For alkaline treatment of sialic acids, the dried material obtained after mild acid hydrolysis (see above) was suspended in 500 µl of 0.1 M ammonia and incubated for 15 min at room temperature, neutralised with 0.1 M formic acid and evaporated to dryness at room temperature in a rotary evaporator. The dried residue was methyl-esterified and derivatised with HFBAA as above.

For sialic acid purification, 300 mg of the CMI fraction was submitted to mild acid hydrolysis under the conditions described above. After centrifugation, the pellet was submitted again to mild acid hydrolysis. Resorcinol staining [15] indicated that the supernatant obtained after this second hydrolysis contained less than 5% of the sialic acids liberated during the first step. The combined supernatants were evaporated to dryness with a rotary evaporator and passed through a DE52 column (Whatman; 20 × 1 cm) equilibrated in water. The column was washed with 40 ml water and eluted successively using 25 ml 0.1 M KCl, 0.4 M KCl and 1 M KCl in water and the eluates were evaporated to dryness. All sialic acids were found in the 0.4 M KCl fraction. An aliquot was analysed for sialic acids after direct methyl-esterification with diazomethane followed by acylation with HFBAA.

2.3. Monosaccharide analyses

Once the analyses of sialic acids were performed, the samples were dried under a stream of nitrogen and supplemented with 1 ml of methanolysis reagent (0.5 M gaseous HCl dissolved in anhydrous methanol [16]) and heated for 20 h at 80 °C. After drying under a stream of nitrogen, the samples were supplemented with dried acetonitrile and HFBAA as above and heated for 15 min at 150 °C. Before analysis the samples were evaporated under nitrogen and taken up in 400-µl dried acetonitrile. Again 1–2 µl were injected on the needle of the Ross injector.

2.4. GC/MS analyses

For GC/MS analysis, the GC separation was performed on a Carlo Erba GC8000 gas chromatograph equipped with a 25 m × 0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 µm film phase (Chrompack France. Les Ulis. France). The temperature of the Ross injector was 260 °C and the samples were analysed using the following temperature program: 90 °C for 3 min, 90–260 °C at 5 °C/min and 260 °C for 20 min. The column was coupled to a Finnigan Automass II mass spectrometer (mass detection limit 1000) or, for masses larger than 1000, to a Nermag 10-10H mass spectrometer (mass detection limit 2000). The analyses were performed routinely in the electron impact mode (ionisation energy 70 eV; source temperature 150 °C). In order to preserve the filament of the ionisation source, the GC/MS records were performed 5 min after the injection of the sample. The quantitation of the different constituents was performed on the total ion count (TIC) of the MS detector using the Xcalibur software (Finnigan Corp.). For ascertaining the mass of the different derivatives, the MS analyses were also performed in the chemical ionisation mode in the presence of ammonia (ionisation energy 150 eV, source temperature of 100 °C). The detection was performed for positive ions. For quantitative data, the relative molar response factors on the major peaks were those reported elsewhere [14].

3. Results and discussion

3.1. Bulk analysis of CM soluble and insoluble fraction

Although these data were obtained in the second step of the analysis, they were found to be very informative. Indeed,

the methanolysis step cleaved almost all glycosidic bonds and eliminated all *O*-acyl groups and *N*-acyl groups not stabilised by resonance (such as peptide bonds). Therefore, all classical sialic acid groups (*O*-acetyl, *O*-lactyl, *O*-sulfate) of *N*-acetylneuraminic and *N*-glycolylneuraminic acid were transformed (after acylation with HFBAA) into the per*O*(*N*)-HFB derivatives of the *O*-methylglycoside of the methyl-ester of neuraminic acid (Neu). This was also the case for the *O*-acyl derivatives of Kdn (giving the per(*O*)HFB derivative of the *O*-methylglycoside of the methyl-ester of Kdn) and of 8-methylated sialic acids (the *O*-methyl groups being resistant) analysed as the per *O*(*N*)-HFB derivative of the *O*-methylglycoside of the methyl-ester of Neu8Me). Therefore, from these potential initial derivatives of Neu5Ac, Neu5Gc, Kdn and Neu5Ac8Me, the methanolysis step reduced the heterogeneity to compounds derived from Kdn, Neu, and Neu8Me, all of them being characterised by specific retention times and specific mass spectra (with extremely specific reporter ions). Furthermore, since all ester and amide bonds were cleaved, the parent members of the sialic acid families could be directly compared to the levels of other monosaccharides, fatty acids (FAMEs) and/or long-chain bases (LCBs) [14,17]. Therefore, this allowed a global quantification of the parent members of these different families. Sterol derivatives, alkyl-acyl-glycerols could be also quantitatively determined.

As shown in Fig. 1, the GC/MS spectra of the two fractions were essentially different, the CM soluble fraction showing a large amount of lipid constituents, the CM insoluble fraction showing an excess of monosaccharide constituents.

3.2. Bulk analysis of the CMI fraction

The CMI fraction showed Man as the major peak (Fig. 1A). Based on the ratio of Man to the two different series of peaks derived from compounds formed from the second GlcNAc residue (*O*-methyl glycoside of GlcN) and of the first GlcNAc residue involved in the *N*-glycosidic bond (free GlcN, its glycosylamine and its Amadori rearrangement product [18]), it was concluded that the major glycans (0.125 mg/mg protein) corresponded, in a primary approach, to glucosyl-oligomannosidic *N*-glycans (with a mean composition of 8.42 Man and 1.02 Glc residues per two GlcNAc). These data were in agreement with previous studies [19] showing that the majority of the *N*-glycans of *A. rubens* were of the gluco-oligomannosidic type. These experiments also showed the presence of a quantitatively important (but unidentified) compound (1.38 mol per *N*-glycan mole) showing characteristic ions of the family of uronic acids ($m/z = 537$). As shown in Table 1, the levels of Gal and GalNAc relative to the sum of GlcNAc were low (0.08 and 0.014% respectively). The derivative of Neu was detectable but represented a low proportion (4.96%) of the total sialic acids (0.277 mol/mole *N*-glycans) almost entirely recovered as the derivative of Neu8Me (Kdn was absent). The ratio of sialic acids to Gal (2.31/1) was compatible with the observation of others [10] that these sialic

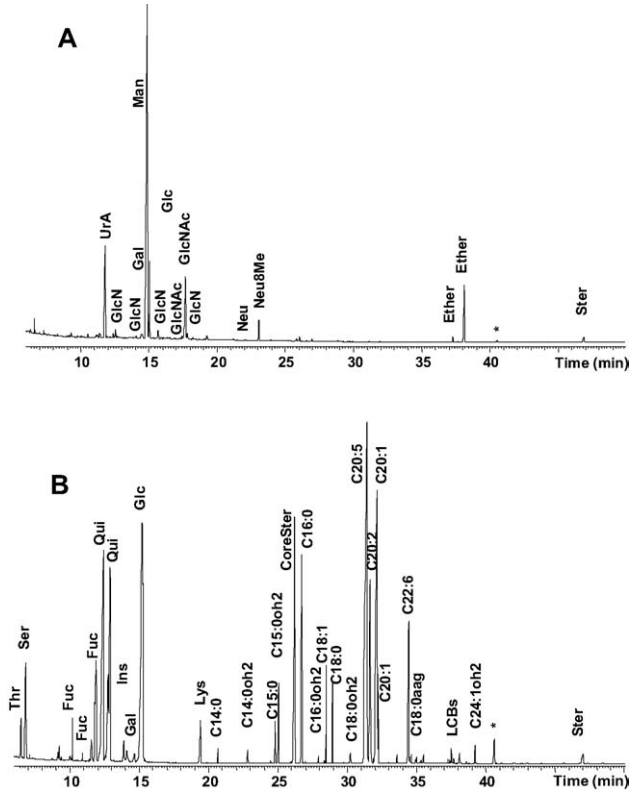

Fig. 1. GC/MS analysis of the HFB derivatives of compounds liberated using acid-catalysed methanolysis from the CMI fraction (A) and from the CM (B) of *A. rubens*.
Note in A, the limited number of molecular entities in contrast with B. C18:0 aag = alkyl-acyl-glycerol with an alcohol chain of 18 carbon atoms. Ster: undefined steroid derivatives (different from cholesterol and cholestanol) having a single alcohol group. Ether: undefined poly-ethers (not cleaved using methanolysis and not derivatised using HFBAA). CoreSter = steroid of saponins.

acids were organised as oligomers (α2,5′-linked, i.e. to the hydroxyl of the glycolyl groups) of 2–3 units. Based on this monosaccharide composition, it was difficult to propose the nature of the glycan structure to which sialic acids were attached.

3.3. Bulk analysis of the CM fraction

The CM fraction showed (except for Glc) a relatively low level of classical monosaccharides involved in the glycosylation of glycosphingolipids (Gal, GalNAc, GlcNAc; Fig. 1B). The major compounds were fatty acids and especially poly-unsaturated compounds with 20 and 22 carbon atoms (Table 1). Hydroxylated fatty acids were very minor compounds and LCBs (minor compounds) were essentially a mono-unsaturated phytosphingenine with 22 carbon atoms and a di-unsaturated 6-hydroxy-sphingenine also with 22 carbon atoms, the chain length being identical to that found in *Asterias amurensis* glycolipids [20,21]. Cholesterol, cholestanol and di-hydrocholestanol were also present but at relatively low levels (Table 1). Major monosaccharides were deoxyhexoses (Qui and Fuc) and Glc. This fraction showed a very low but significant amount of derivatives of the Neu fam-

Table 1
Composition of the CM soluble (CM) and CM insoluble (CMI) fractions of *A. rubens* obtained using GC/MS analysis of HFB derivatives of the methanolysis products

	CM		CM		CMI
AA		FAMEs		AA	
Val	0.04	C12:0	0.22	Leu	0.021
Thr	1.13	C14:0	0.38	Pro	0.013
Ser	5.24	C15:0	0.21	Lys	0.070
Leu	0.12	C14:0oh2	0.39	Tyr	0.006
Pro	0.406	C16:1	0.07	spermine	0.005
Asp	0.08	C15:0oh2	0.98		
Lys	2.68	C16:0	3.331	Sugars	
		C16:0oh2	0.86	UrA	1.380
Sugars		C17:0	0.04	GlcN	0.986
Fuc	12.35	C18:1	4.26	Gal	0.162
Qui	24.43	C18:0	2.93	Man	8.425
Ins	1.04	C18:0oh2	0. 56	Glc	1.020
Ins*	0.59	C20:4	35.56	GalNAc	0.027
Gal	1.00	C20:2	10.10	GlcNAc	1.000
Glc	34.67	C20:1	18.65	Neu	0.013
GalNAc	0.02	C20:1	2.72	Neu8Me	0.264
GlcNAc	0.01	C20:0	0.02	unk-506	0.022
Neu	0.0004	C21:2	0.03	unk-791	0.123
Neu8Me	0.02	C22:6	6.11	unk-611	0.009
		C22:4	0.21	disacch	0.050
		C22:2	0.15		
sug/FAMEs	1.081	C24:0	0.08	FAMEs	
LCBs/FAMEs	0.021	C24:1oh2	0.55	C16:0oh2	0.004
Chol/FAMEs	0.17				
sug/LCBs	52.63	LCBs		LCBs	
Neu/LCBs	0.11	C22:1phyt	1.15	C21:1phyt	0.031
		C22:2Sphe6oh	0.82	211-510	0.789
Qui/CoreSter	2.46	CoreSter	16.96		
Fuc/CoreSter	1.24	Ster	1.09	Ster	0.242

For CM, the data were expressed relative to Gal. For CMI data were expressed relatively to GlcNAc. Ins* = inositol different from meso-inositol; GlcN = residues from GlcNAc involved in the *N*-glycosidic bond [18]. Qui = quinovose. Unk = unknown compounds with specific fragment ions. C18:0oh2 = 2-hydroxy-octadecanoate; C22:1phyt = mono-unsaturated phytosphingosine (four different compounds were present); C22:2Sphe6oh = C22 6-hydroxysphingenine with an additional desaturation (two different compounds were present). Ster = sterol derivatives (six different compounds were present in the CM fraction). Note the presence of free amino acids in both fractions. CoreSter = steroid of saponins.

ily, including also Neu8Me as the major compound (Neu represented 1.59% of the total sialic acids in this fraction). Again Kdn was absent. The ratio between total Neu and total LCBs indicated that gangliosides (actually found in this material; [22,23] were extremely minor compounds (1.57%), the majority of the LCBs being likely associated with sphingomyelins present as major constituents of the CM soluble fraction (using HPTLC chromatography; not shown). The question was asked on the origin of the major monosaccharides found in this fraction (Qui, Glc and Fuc). One possibility was that these compounds were actually free in the gonads. Indeed, the gonad material was lyophilised as soon as recovered and the CM extract was performed directly on it, in such a way that free monosaccharides could be solubilised in this fraction. Another possibility was that at least part of these compounds, were associated with a hydrophobic core molecule. The careful examination of the GC/MS chromatogram revealed an abundant constituent (relative retention time to linear C18:0 FAME of 0.906) with a strong molecular ion at $m/z = 620$ and a very intense secondary ion at $m/z = 605$ (see Fig. 3 for its mass spectrum). This was a characteristic of compounds having polycyclic structures (such as the HFB derivative of progesterone). Therefore, based on this molecular mass, it was tentatively concluded that this compound (termed CoreSter in the following) was a steroid with 28 carbon atoms, a single hydroxyl group (1 HFB residue), two keto groups and two double bonds (NMR studies should give its correct structure). Similar compounds were actually isolated from *A. rubens* [24,25]. Quantitative analysis indicated a ratio Qui/CoreSter of 2.46, a ratio compatible with the reported structures containing two or three Qui residues per molecules. Based on these previous studies, the high level of Fuc could be also explained as components of saponins. In contrast, the high level of Glc (also detected in saponins [24,25] could not be explained as a unique constituent of these compounds. Part of it belonged to glusosylceramides and lactosylceramide [21,22], but HPTLC analysis (not shown) indicated that the majority of Glc was present as a free monosaccharide. A puzzling question was the presence in the CM fraction of amino acids (especially Thr, Ser and Lys) present in proportions not representative of amino acids in biological fluids. The possibility could not be excluded that these amino acids were not free compounds but associated to other molecules as esters or amides (no evidence was provided on this point in the literature).

3.4. Diversity of the sialic acids in A. rubens

A few studies were concerned with the sialic acid composition of *A. rubens* gonads [9,10]. Based on the initial work of Warren [9], this material was characterised by the abundance of 8-methyl-*N*-glycolyl-neuraminic acid. These data were at the origin of several studies on the biosynthetic pathways of these derivatives including isolation of the enzymes, definition of the substrates and, finally cloning of the genes [10,11,26–28]. Nevertheless, it was necessary, using new techniques, to study extensively the diversity of the sialic acids. The technique used here involved mild acid hydrolysis (in order to liberate sialic acids) followed by centrifugation of the insoluble protein material, concentration of the supernatant and formation of volatile derivatives of sialic acids (diazomethane methyl-esterification and formation of HFB derivatives).

The GC/MS chromatogram of the CMI fraction (Fig. 2A) showed the total absence of Neu5Ac and Neu5Ac derivatives. This conclusion was based on the absence of reporter ions specific for Neu5Ac, Neu, mono- and poly-acetylated or lactylated derivatives of Neu5Ac. All the peaks present on the chromatogram showed a medium intensity ion at $m/z = 227$, characteristic of Neu5Gc derivatives. Unsubstituted Neu5Gc was actually present but as a very minor

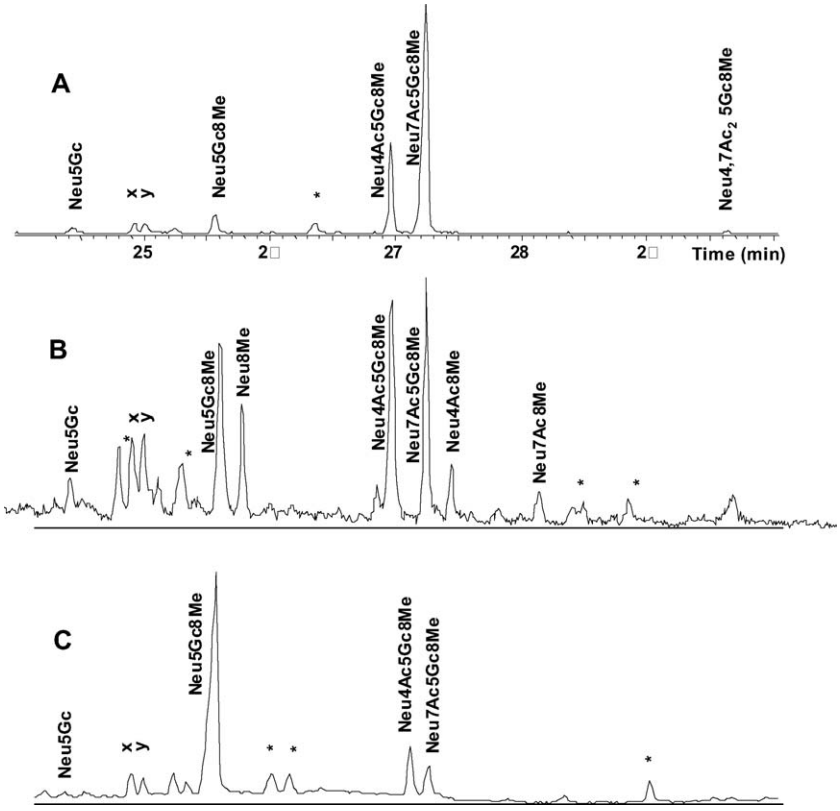

Fig. 2. GC/MS analysis of the diversity of sialic acids present in the CM insoluble fraction of *A. rubens*.
A) Profile of the sialic acids liberated by mild acid hydrolysis without purification. Note in that the major sialic acids were Neu4Ac5Gc8Me and Neu7Ac5Gc8Me. B) Profile obtained after a mild alkaline treatment of the same sample. Note the relative decrease of Neu4Ac5Gc8Me and Neu7Ac5Gc8Me and relative increase of Neu5Gc8Me. Note also that compounds corresponding to de-*N*-acylation were present (Neu8Me, Neu4Ac8Me and Neu7Ac8Me after this treatment. C) Profiles of sialic acids eluted from the DE52 column using 0.4 M KCl. Note that Neu5Gc8Me became the major sialic acid indicating an intense de-*O*-acetylation of the mono-*O*-acetylated sialic acids. *Impurities unrelated to sialic acids (phthalates). **x** and **y** represented the minor peaks being unchanged in A) and B).

compound (1.81% of total sialic acids). The fine GC/MS analysis of the chromatogram indicated that none of the *O*-acetyl and *O*-lactyl-derivatives of Neu5Gc already described elsewhere [12] were detectable. The comparisons of the levels of Neu (during the methanolysis; 4.96%) and of Neu5Gc recovered after acid hydrolysis (less than 2%) suggested that not yet identified Neu5Gc derivatives were actually present. In fact, two closely migrating peaks (**x** and **y**) of low intensity were detected on the chromatogram showing the ion at *m/z* = 227, representing 2.82 and 1.80% of the total sialic acids. The sum of the areas of Neu5Gc and these two peaks gave a proportion of Neu5Gc compatible with the data obtained after acid-catalysed methanolysis (see above). Therefore, it was suggested that these two peaks corresponded to new forms of Neu5Gc. These peaks showed an intense ion at *m/z* = 192, the nature remaining undetermined.

All the other peaks (except phthalates) on the GC/MS chromatogram (Fig. 2) showed the very intense and typical ion at *m/z* = 271, corresponding to the homolytic cleavage of the bond between a HFB-derivatised $C_{(9)}$ and a *O*-methylated $C_{(8)}$ from the rest of the molecule ($[C_3F_7CO–CH_2–CH–O\text{-}CH_3)^{+\cdot}]$) and characteristic of Neu5Gc8Me derivatives having a free hydroxyl group on the $C_{(9)}$ carbon atom. Among these different compounds, only little Neu5Gc8Me was present (5.67%). The major compounds were Neu4Ac5Gc8Me and Neu7Ac5Gc8Me (Table 2; 19.92 and 67.18%, respectively). A trace of Neu4,7$Ac_2$5Gc8Me was also identified (0.8%). These compounds presented very characteristic fragmentation pattern elicited in Fig. 3. Although the fragmentation mass spectra of these two compounds were very similar, they could be identified by the presence or not of medium intensity ions at *m/z* = 534 and 380 for Neu4Ac5Gc8Me and Neu7Ac5Gc8Me, respectively. These ions corresponded to the substituted $C_{(9)}$–$C_{(5)}$ carbon atoms (with the elimination of the glycolyl group and with 2 double bonds). These ions differed by a loss of mass of 154, the difference between a HFB and acetate groups, indicating (with the presence of the ion at *m/z* = 271 in both spectra) that the $C_{(9)}$ position was occupied by a HFB derivative and consequently a free hydroxyl group in this position and the presence or not of a HFB group on the $C_{(7)}$ position.

In order to verify these findings, the samples were submitted to a partial alkaline hydrolysis, followed by the reformation of the volatile derivatives. The GC/MS analysis (Fig. 2B) indicated a reduced amount of the Neu4,7$Ac_2$5Gc8Me and a decrease of Neu4Ac5Gc8Me and Neu7Ac5Gc8Me with the concomitant increase of Neu5Gc8Me (Table 2). Other peaks with the intense ion at *m/z* = 271 were identified as *N*-HFB

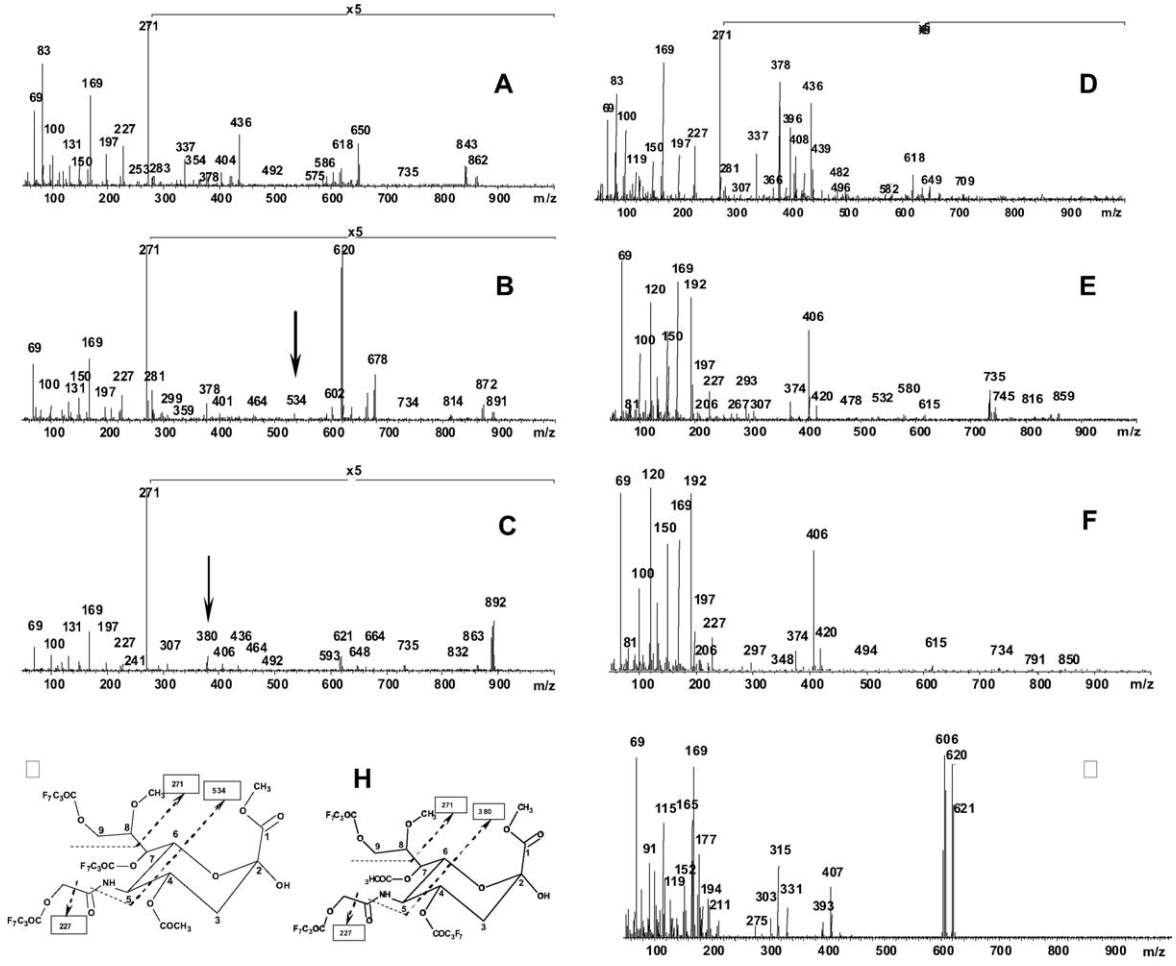

Fig. 3. EI fragmentation mass spectra of sialic acids and of ChloSter present in *A. rubens*.
A) Neu5Gc8Me (*M* = 1137); B) Neu4Ac5Gc8Me (*M* = 983); C) Neu7Ac5Gc8Me (*M* = 983); D); Neu4,7Ac$_2$5Gc8Me (*M* = 829) E) and F) compounds **x** and **y** shown in Fig. 2. The reporter ions for discriminating between Neu4Ac5Gc8Me and Neu7Ac5Gc8Me are indicated by arrows. G) and H) Schematic representation of the generation of reporter ions of Neu4Ac5Gc8Me and Neu7Ac5Gc8Me. I) Fragmentation mass spectrum of CholSter. Note the intensity of the molecular ion and the M-15 ion (*m/z* = 605).

derivatives of the previous sialic acids (completely absent in the original sample), indicating a substantial *N*-de-acylation during the procedure (compounds were recovered as *N*-HFB derivatives). Their retention times were slightly increased relative to the *N*-glycolyl-derivatives. The mass spectra showed, in contrast to the original derivatives, relatively intense ions corresponding to the substituents of the $C_{(7)}$–$C_{(9)}$ carbon atoms. This allowed verifying the position of the acetyl groups in the initial molecules, i.e. Neu4Ac5Gc8Me and Neu7Ac5Gc8Me. These data actually verified the presence of mono-and di-acetylated derivatives of Neu5Gc8Me. In contrast, the two other peaks (**x** and **y**) were unchanged, indicating that the substitution was quite stable to alkaline media.

We tried to purify the different sialic acids liberated using mild acid hydrolysis and separation on a DEAE-cellulose column. All the material reacting with the resorcinol reagent was eluted using 0.4 M KCl (acid elution was not retained in order to avoid intense de-acetylation during the elution). In this ion-exchange chromatographic system, it was theoretically expected that sulphated sialic acids would be eluted using higher ionic strength (0.8 M KCl was used). Because of the

Table 2
Sialic acid percent composition of the CMI and CM fractions obtained after mild acid hydrolysis

	CMI direct	CMI purif	CMI alk	CM
Hydrolysis				
Neu5Ac	0.00	0.00	0.00	0.00
Neu5Gc	1.81	2.44	3.13	1.56
X	2.82	3.03	6.85	0.00
Y	1.79	1.77	6.48	0.00
Neu5Gc8Me	5.67	75.52	33.97	49.15
Neu4Ac5Gc8Me	19.92	10.06	27.12	0.00
Neu7Ac5Gc8Me	67.18	7.17	22.45	49.28
Neu4.7Ac$_2$5Gc8Me	0.80	0.00	0.00	0.00
Methanolysis				
Neu	4.96	nd	Nd	1.59
Neu8Me	95.04	nd	Nd	98.41

Note the absence of derivatives of Neu5Ac in all samples. nd = not determined; CMI direct: analysis of sialic acids without purification shown in Fig. 2A; CMI purif: sialic acids recovered after ion-exchange chromatography shown in Fig. 2C; CMI alk: sialic acids recovered after mild alkaline hydrolysis (due to a partial de-N-acylation, compounds with or without the N-glycolyl group were combined).

absence of resorcinol-reactive material in this fraction, it was concluded that sulphated sialic acids were absent from the *A. rubens* gonads. However (Fig. 2C), this procedure could not avoid *O*-de-acetylation. Indeed, the peak of Neu4,7Ac$_2$5Gc8Me was absent and those of Neu4Ac5Gc8Me and Neu7Ac5Gc8Me were extremely reduced in parallel with a considerable increase of the peak of Neu5Gc8Me (representing now more than 75% of the total sialic acid). Although these data were not a priori expected, they demonstrated the presence of the mono- and di-*O*-acetylated derivatives of Neu5Gc8Me. These data also indicated that the acetylation in position 4 was more stable than that in position 7. Compounds **x** and **y** were not significantly affected, indicating also the quite stability of their substituents.

Based on these results, our data significantly differed from those previously published [10]. Our data indicated that Neu5Ac and Neu5Gc (and their *O*-acetyl-derivatives) were extremely minor compounds (around 5% instead of 23%). It was agreed that Neu5Gc8Me derivatives were the major sialic acids found in *A. rubens* as previously described [10], but we were unable to detect any trace of Neu5Ac as reported earlier by the same authors. Furthermore, the major compounds were Neu4Ac5Gc8Me and Neu7Ac5Gc8Me and not Neu5Gc8Me. These discrepancies could be easily explained by the fact that in previous methodologies (for a review see Ref. [1]), the GC/MS [29] or HPLC [30,31] analyses of sialic acids could only be performed on purified sialic acids, the purification involving ion-exchange chromatography under more severe conditions than those used here. As demonstrated in Fig. 2C, the major sialic acids were not Neu5Gc8Me but Neu4Ac5Gc8Me and Neu7Ac5Gc8Me.

These considerations cannot explain that we did not detect 9-*O*-acylated sialic acid reported previously [10], because these compounds could be securely identified by specific fragment ions using our methodology [12]. Chromatogram reconstitution for the expected ion at $m/z = 117$ (representative of the $C_{(8)}$–$C_{(9)}$ substituents; equivalent of the ion at $m/z = 271$ for HFB derivatives) was never detected. A possible explanation could be that this ion (actually present on GC/MS chromatograms of poly-TMS derivatives of sialic acids [29] was not issued from a 9-*O*-acetylated-compounds but from a weak intensity ion of the *O*-TMS derivatives of all sialic acids [$Si(CH_3)_3COO^+$] corresponding to the TMS derivatisation of the carboxyl group in position 2. Furthermore, Neu9Ac5Gc, as reported by Bergwerff et al. [10] in other tissues was clearly identified in our analytical system [12]. An additional explanation may be that the purification steps needed in previous methods led to a significant degradation of the 4- and 7-*O*-acetylated compounds with the concomitant appearance of an excess of Neu5Gc8Me, which was previously described as a major compound, but was found a minor compound in our study. But another possibly interesting explanation could be that *A. rubens* studied here were collected in the North-Friesian part of the North Sea, whereas *A. rubens* from the previous study were collected in the Baltic Sea.

4. Conclusion and perspectives

Based on the present data we conclude that Neu4Ac5Gc8Me and Neu7Ac5Gc8Me are the major sialic acids present in the gonads of *A. rubens* collected in the North Sea, compounds which were never detected before. Since the gonads of *A. rubens* preferentially synthesise Neu5Gc8Me derivatives, this starfish is considered as a good experimental model for studying the sialate 8-methyltransferase [11]. Based on our data, it would be also a very good model for studying the sialate 4- and 7-*O*-acetyl-transferases, these enzymes being of difficult access in mammalian tissues [4]. Isolation and sequencing of these *A. rubens* enzymes may be of importance for identifying the corresponding mammalian enzymes. Indeed, 7-*O*-acetylation of Neu5Ac was systematically found in human tissues [32,33,1,34], and 4-*O*-acetylation was also present, although at a very low level [32]. In some bacteria, in mammals and in many other vertebrate species the acetic acid ester group is mainly found at C-9 of sialic acid [1]. Experimental evidence was obtained showing the initial *O*-acetylation of sialic acid occurs at the C-7 preferably [4,35], Srinivasan et al. (unpublished). This ester group can then migrate spontaneously to C-9 [36,4]. It can only be speculated that this isomerisation is hindered by (prior?) methylation at C-8 of the *A. rubens* sialic acid. It would be of interest to know the nature of the molecules bearing these sialic acids and if these "strange" sialic acids are important in the process of fertilisation and/or development of *A. rubens*.

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