Sulphated and sialylated N-glycans in the echinoderm Holothuria atra reflect its marine habitat and phylogeny

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Running title: Sea cucumber N-glycome

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Amongst the earliest deuterostomes, the echinoderms are an evolutionary important group of ancient marine animals. Within this phylum, the holothuroids (sea cucumbers) are known to produce a wide range of glycoconjugate biopolymers with apparent benefits to health; therefore, they are of economic and culinary interest throughout the world. Other than their highly modified glycosaminoglycans (e.g. fucosylated chondroitin sulphate and fucoidan), nothing is known about their protein-linked glycosylation. Here, we used multi-step N-glycan fractionation to efficiently separate the anionic and neutral N-glycans before analyzing the N-glycans of the black sea cucumber (Holothuria atra) by MS in combination with enzymatic and chemical treatments. These analyses showed the presence of various fucosylated, phosphorylated, sialylated and multiply sulphated moieties as modifications of oligomannosidic, hybrid and complex-type N-glycans. The high degree of sulphation and fucosylation parallels the modifications previously observed on holothuroid glycosaminoglycans. Compatible with its phylogenetic position, H. atra not only expresses vertebrate motifs such as sulpho- and sialyl-Lewis A epitopes, but displays a high degree of anionic substitution of its glycans as observed in other marine invertebrates. Thus, as for other echinoderms, the phylum- and order-specific aspects of this species’ N-glycosylation reveal both invertebrate- and vertebrate-like features.

Introduction

Sea cucumbers (holothuroids) are a group of organisms living in the benthic zones of seas across the world. As one of the five clades of the Echinodermata (Figure 1), sea cucumbers are primitive deuterostomes and are thus related to the ancestors of vertebrates. Around 100 of the 1500 extant sea cucumber species are consumed by humans, in part due to their intrinsic nutritional value and the proposed beneficial effects of many of the constituent biopolymers (1). Overexploitation in some regions has led to the spread of sea cucumber fishing throughout the world with unknown ecological repercussions, as these animals ingest and process detritus from the sea floor and thus play an active role in sea grass and coral reef ecosystems (2). There is also interest in these organisms as regeneration models due to their capability for asexual reproduction by fission.
followed by morphollaxis to produce a smaller but functional complete animal (3). Over the years, various glycosylated biopolymers from sea cucumbers have been analysed: in particular, glycolipids (4,5), fucosylated chondroitin sulphates (6) and glycosylated terpenoids (7); all of which have been claimed to be bioactive (e.g., anti-inflammatory and anti-tumorigenic (8)). On the other hand, as for echinoderms in general, there is no report regarding the standard N-linked oligosaccharides of these species. However based on O-glycan analyses of the distantly related sea urchins, sialic acids can be expected (9).

Here, we analyzed the N-glycome of Holothuria atra (commonly called black sea cucumber or lolly fish) which is possibly the most abundant of its genus and widespread in the tropical Indo-Pacific region. The N-glycans were released with PNGase-F then PNGase-A and subsequently analysed by high performance liquid chromatography (HPLC), mass spectrometry (MS) and enzymatic and chemical treatments (10).

Results
Workflow and analytical strategy:
N-glycans of Holothuria atra were released by serial digestion with PNGase-F then PNGase-A resulting in free N-glycan pools which were sub-fractionated on graphitized carbon into neutral and anionic sub-pools prior to pyridylamination (PA). The initial survey of the complete N-glycome showed some mass shifts of 78 Da compatible with the presence of either phosphate or sulphate modifications (Figure 1). Subsequently, the neutral and anionic PNGase F-released N-glycans were subject to reverse phase (RP) and normal phase (NP) HPLC; all fractions were analysed by MALDI-ToF MS in positive and negative modes (Figure 2 and Supplementary Figure 1). This off-line LC-MS approach revealed that the neutral N-glycan pool was relatively simple, containing primarily well-known oligomannosidic structures, while the anionic pool contained numerous, but unusual, charged hybrid and complex N-glycans.

Oligomannosidic-type N-glycans:
The neutral pool contained a series of Hex$_4$-$\text{HexNAc}_2$ glycans (m/z 1151-2447). These could be assigned as isomers of Glc$_0$-$\text{Man}_4$-$\text{GlcNAc}_2$ due to their retention time on RP/NP-HPLC as well as positive MS/MS fragmentation patterns before and after mannosidase digestions. As these common isomers (see Supplementary Table 1 for a comparison of elution times) were identified previously in other organisms (11,12), they are not discussed further.

There were also unusual N-glycans observed in both positive and negative modes and so predicted to be phosphorylated forms of Hex$_{10-12}$HexNAc$_2$ (m/z 2203, 2365 and 2527; Figure 3 A/E and Supplementary Figure 2 A-C). The most abundant of these (m/z 2527) was dephosphorylated with either alkaline phosphatase or HF yielding an MS/MS spectrum and retention time characteristic of the basic ER Glc$_3$Man$_9$GlcNAc$_2$ N-glycan precursor (m/z 2447). The thereby predicted P$_1$Glc$_3$Man$_9$GlcNAc$_2$ glycan was also treated with α-mannosidase, resulting in loss of up to 5 mannose residues regardless of whether the phosphate had been removed or not, and with endo-α2-mannosidase, which removed a...
P1Glc3Man1 unit (Figure 3 and Supplementary Figure 2); these data indicated that the phosphate residue is on the tri-glucosylated A-arm. Considering also the MS/MS B1-ions at m/z 241 (P1Hex1) in negative mode, we concluded that the terminal glucose residue is the location of the phosphate modification.

Neutral hybrid-type N-glycans: Within the H. atra glycome, there were five neutral glycans with predicted compositions of Hex4-6HexNAc3Fuc0-2 (m/z 1500-1970). These potentially hybrid structures were analysed using HPLC, MS and exoglycosidase treatments to define the nature of their antennae. For instance, a 2D-HPLC purified form of Hex6HexNAc3 (m/z 1678; Figure 4 A, I) was sensitive to β3-galactosidase (Figure 4 G, J; loss of one Gal) and α-mannosidase (Figure 4 H, K; loss of 2-3 Man), but not to β4-galactosidase (Figure 4 F). This indicated the presence of a type 1 antenna (neo-LacNAc, Galβ3GlcNAcβ-R) on a hybrid backbone, a conclusion confirmed by comparison to a later eluting isomer with a type 2 antenna (LacNAc, Galβ4GlcNAcβ-R; prepared by in vitro β4-galactosylation; Figure 4 B). The β3-galactosylated hybrid structure appeared to be the basis for a number of sialylated and sulphated glycans as desulphation of S1Hex6HexNAc3 (m/z 1756) and desialylation of NeuGc1Hex6HexNAc3 (m/z 1985) resulted in a co-eluting Gal1Man5GlcNAc3 structure (Figure 4 C-D).

Sulphated hybrid-type N-glycans: Mono-sulphated fucosylated structures of S1Fuc1Gal1Man3GlcNAc3 were analysed via negative MS and MS/MS (Figure 5 A, F, H, K, M), whereby key B-ions and neutral losses aided definition of the sulphate and fucose positions. For instance, the two fucosylated isomers of S1Fuc1Gal1Man5GlcNAc3 (m/z 1902; eluting at 4.7 g.u. and 7.8 g.u.) exhibited different negative MS/MS fragmentation patterns (Figure 5 H, K): the first one with m/z 590 (S1Fuc1Gal1GlcNAc1) and 1603 (loss of GlcNAc2-PA) is concluded to possess an antennal fucose, while the second one with m/z 444 (S1Gal1GlcNAc1) and 1457 (loss of Fuc1GlcNAc1-PA) is rather core α6-fucosylated as it was released by PNGase F. The related di-fucosylated m/z 2048 structure not only presented an m/z 590 B-fragment, but also an m/z 1603 ion indicative of loss of Fuc1GlcNAc1-PA (Figure 5 M). Treatment of the antennally fucosylated isomer with either β3/4-galactosidase (also no digestion even after α-fucosidase), α-mannosidase (loss of 2-3 Man), α3/4-fucosidase and HF (both resulting in loss of 1 Fuc) (Figure 5 A-E) aided definition of the A-arm as Lewis motifs with sulphated galactose residues. An alternative position for sulphation (rather than on galactose) is concluded for a hybrid-type S1Fuc1Gal1Man3GlcNAc3 glycan, which was β3-galactosidase sensitive; particularly, the m/z 241 and 1213 fragments indicated that the sulphate is in this case on mannose (Supplementary Figure 3).

For sulphated structures in general (13), positive mode MS/MS of [M-SO3]+ pseudomolecular ions is useful to define the underlying backbones. Here, positive mode Y-ions at 446 (Fuc1GlcNAc1-PA), 1297/1459 (Fuc1Man4/5GlcNAc2-PA) and m/z 503-1313 (Man0-5GlcNAc2-PA) as well as the B-ions at m/z 366/512 (Gal1GlcNAc1Fuc0/1) provided full sequence coverage of the core and the antennae of the hybrid structures (Figure 5 I-J, L and N).

Therefore, considering the presence of β3-galactose on non-fucosylated glycans (Figure 4) and the available LC-ESI-MS data (Supplementary Figure 4), the antennal motif for these Lewis-modified glycans is concluded to be (HSO3)4Galβ3(Fucα4)GlcNAc-R, i.e., sulpho-Lewis A.
For di-sulphated $S_2Fuc_1-2Gal_1Man_3GlcNAc_3$ structures ($m/z$ 1842-2150; Figure 6 A-D), diagnostic negative B-fragment ions are consistent with two sulphonates substituting either the Gal, GlcNAc or $\alpha3$-Man residues on the A-arm. Negative mode MS/MS could indeed distinguish di-sulphated $m/z$ 2150 isomers (eluting at 42.5 and 48.4 min on NP-HPLC): the pattern for the first isomer with $m/z$ 241 ($S_1Gal_1$), 854 ($S_2Fuc_1Gal_1GlcNAc_1Man_1$) and 1537 ($S_2Fuc_1Man_3GlcNAc_2$-PA) suggests the presence of sulphate on both the terminal Gal and lower $\alpha3$-Man residues (Figure 6 H). For the second one, the occurrence of fragments of $m/z$ 241 ($S_1Gal_1$) and 692 ($S_2Fuc_1Gal_1GlcNAc_1$), together with the loss of the latter upon HF treatment while retaining both sulphate residues, is compatible with the presence of a di-sulpho-Lewis motif (Figure 6 D, E, I and J); thereby possibility of sulphation of the fucose is excluded, but the sulphation of both the Gal and GlcNAc residues is confirmed.

For the disulphated $S_2Fuc_2-3Gal_2Man_3GlcNAc_4$ structures ($m/z$ 2515 and 2661 as [M-2H+Na]$^-$; eluting at 50 min on NP-HPLC), two mannoses were removed by $\alpha$-mannosidase treatment (Supplementary Figure 5 A-B), proving their hybrid-type backbones. MS/MS analyses of the parental ion, as well as the [M-SO$_3$]$^-$ and [M+H-2SO$_3$]$^+$ ions resulting from source loss of sulphate, indicated the presence of two sulpho-Lewis-type antennae, presumably $\beta1,2$- and $\beta1,4$-linked to the $\alpha3$-linked mannose (Supplementary Figure 5 C-E).

Tri-sulphated $S_3Fuc_0/2Gal_1Man_5GlcNAc_3$ structures eluted rather late on the NP-HPLC column (65 - 68 min) and were best detected when supplementing the matrix with sodium acetate (Supplementary Figure 6 A-B and G-H; $m/z$ 1960 and 2252). Negative and positive mode MS/MS of the ‘real’ $[M-H_n+Na_{(n-1)}]$$^-$ and ‘in source’ parent ions yielded fragments (Supplementary Figure 6 C-F and I-L) consistent with the three sulphation positions on the A-arm (terminal Gal, antennal GlcNAc and $\alpha3$-Man) as established for the aforementioned di-sulphated species.

Sialylated hybrid-type N-glycans:
The results of off-line LC-MS/MS led us to predict a number of sialylated glycans in the H. atra N-glycome (Figure 2 B and Supplementary Figure 1 B). In order to resolve some of these, a 2D-HPLC approach was applied. Thereby, NP-HPLC-fractionated mono-sialylated structures ($NeuGc_1Fuc_0/1Gal_1Man_5GlcNAc_3$; $m/z$ 1985/2131) were re-injected onto RP-HPLC before or after $\alpha3$-sialidase S treatment and so isomers with different sialylation and sialidase sensitivity were identified (Figure 7 A-B and E-F). Only rather subtle differences in positive and negative mode MS/MS between the mono-sialylated isomers could be observed, with the main diagnostic sialylated negative/positive B-ions at $m/z$ 306/308 ($NeuGc_1$) and 671/673 ($NeuGc_1Hex_1HexNAc_1$) for NeuGc-modified antennae being shared (Figure 7 K-M, R). In case of a sialidase S-resistant isomer, $\beta3$-galactosidase treatment resulted in the loss of one galactose residue and best revealed a diagnostic $m/z$ 511 NeuGc$_1$GlcNAc$_1$ fragment (Figure 7 G-J). Thus, the conclusion was that there were two positions for sialylation (‘externally’ on Gal or ‘internally’ on GlcNAc), whereby sialidase S only removed the former, but not the latter.

There were also related di-sialylated NeuGc$_2Fuc_0/1Gal_1Man_5GlcNAc_3$ glycans ($m/z$ 2292 and 2438); the former was also re-chromatographed before and after $\alpha3$-sialidase S treatment. Removal of only the terminal NeuGc occurred and resulted in altered retention time (Figure 7 C-D) and minimal changes in the MS/MS fragmentation (Figure 7 P, R). Negative
mode MS/MS of the m/z 2292 glycan and positive MS/MS of the core fucosylated m/z 2438 structure resulted in the detection of either an m/z 996 C-fragment or an m/z 980 B-fragment compatible with di-sialylation of Hex1HexNAc1 (Figure 7 Q, S). Some sialylated glycans were also sulphated and MS/MS (Figure 7 N, O, T) could show the presence of either a sulpho-sialyl-Lewis-containing motif (B-ion at m/z 897; S1NeuGc1Fuc1Gal1GlcNAc1) or a sulphated NeuGc (B-ion at m/z 386; S1NeuGc1).

Complex-type N-glycans:
MS predicted a large number of complex-type N-glycans in H. atra, but the relatively low abundance of these structures ‘overloaded’ with fucose and sulphate residues meant that their analysis was challenging. On RP-HPLC, glycans such as S3-4Fuc2-4Hex5-6HexNA4-5 (m/z 2439-3052) were particularly concentrated in the fraction eluting at 14.5 min (Figure 2 B), while on NP-HPLC many eluted after 60 min (Supplementary Figure 1 B). The RP-HPLC fraction was analysed by negative MS before and after digestion with β3/4-galactosidase and α-fucosidase, which resulted in no loss of the sulphated galactose residues, but in removal of up to four fucoses (Figure 8 A-D). MS/MS spectra of such bi- and tri-antennary complex-type N-glycans (see Figure 8 E-J and Supplementary Figure 7 for examples) showed the presence of similar B-ions (e.g., sulpho-Lewis A at m/z 590) as described above; however, possibly to their low abundance, no multi-sulphated fragments were detected as was the case for di- or tri-sulphated hybrid glycans, but positive mode MS/MS facilitated definition of the core and antennal fucose residues. Unlike the hybrid structures, desulphation of multi-sulphated glycans was inefficient and led to some unspecific hydrolysis, thus an unambiguous definition of all galactose linkages (β3 or β4) was not possible; however, where mono-sulphated complex glycans were present in certain fractions, loss of galactose residues could be observed upon β3-galactosidase treatment, especially when the glycome pool had been previously defucosylated with HF (Supplementary Figure 8).

Core α3-linked N-glycans:
The glycopeptides remaining after PNGase-F digestion were treated with PNGase-A in order to identify possible core α3-fucosylated N-glycans. This residual pool was also separated in neutral and anionic sub-pools prior to labelling and injection onto RP-HPLC. While some of the masses in the fractions were the same as those previously identified in the PNGase F digest, two HPLC fractions contained hybrid or complex N-glycans displaying the presence of an additional fucose (i.e., S1Fuc3Gal3Man3GlcNAc5 at m/z 2194 and S1Fuc5Gal3Man3GlcNAc5 at m/z 2893; Figure 9 A and C). While HF treatment resulted in loss of all fucoses except the core α6-linked one (Figure 9 B and D), negative mode MS/MS of the hybrid structure (m/z 2194) showed neutral losses of the di-fucosylated core as well as the B-ions showing the occurrence of a sulpho-Lewis motif (Figure 9 E). On the other hand, positive MS/MS of the corresponding [M-SO3]+ pseudomolecular ion (m/z 2116) yielded a core Y-ion at m/z 592 (Fuc2GlcNAc1-PA), which is a further proof of di-fucosylation of the innermost core GlcNAc (Figure 9 F).

Discussion
The N-glycome of Holothuria atra, the first to be described of any sea cucumber, is characterized by 74% of neutral structures (mainly oligomannosidic-type N-glycans) and 26% of anionic structures (1% phosphorylated, 24% sulphated and 1%
sialylated), as judged by RP-HPLC fluorescence and MS intensities (Figure 10 and Supplementary Table 2). The relatively high amount of sulphated hybrid and complex-type N-glycans were enriched in the anionic pool, while isomers with different positions of the fucose (core or antennal), sulphate (four different positions, i.e., either on Gal, GlcNAc, Man or NeuGc) or sialic acid residues (on Gal or on GlcNAc) could be resolved by NP- or RP-HPLC (Supplementary Figure 1). The enrichment and separation, as well as addition of Na⁺ to enhance sulphate detection by MS, proved crucial for the in-depth sulpho- and sialo-glycomic investigation, as isolation, separation and detection of anionic glycans remains a challenging task, for which special specific protocols involving either fluorescent labelling (14-17) or permethylation (18) have been previously employed.

Overall, our data suggest that at least four sulphates can modify the N-glycans of *H. atra* and indeed most LacNAc-like antennae are not just sulphated, but are most commonly fucosylated; sulphated forms of β3-galactose, β-GlcNAc, α3-mannose and α3-sialic acid residues could be proven by MS/MS. Sulphation of galactose is similar to that in the oyster (19), but the relative dominance of sulphation of α3-mannose is in contrast to insects, in which sulphation of α6-mannose or core fucose is more common (13). Unlike the highly sulphated keratan-like N-glycans of unfertilized eggs of a fish, *Tribolodon hakonensis* (20) with repetitive sulphated neo-LacNAc motifs, no obvious repeating units were detected in this study. Although the function of the glycans in echinoderms is unclear, sulphation is implicated as a critical determinant mediating a diverse range of biological recognition functions on N- and O-glycans (21).

Other hybrid and complex N-glycans in *H. atra* are sialylated and some structures are even carrying antennal sialic acid in combination with sulphate and/or fucose modifications. Interestingly, like mammalian fetuin (22), the sialylation occurs on either antennal galactose or antennal GlcNAc residues; there may, of course, be biosynthetic competition with sulphation for these positions. In the proven β3-galactosylated/α4-fucosylated structures in *H. atra*, the sialyl-Lewis A element corresponds to the human CA19-9 epitope with roles in cancer (23). Potentially such motifs have a role in cell-cell interactions; in the case of echinoderms, it is conceivable that a sialylated glycan would be important for regeneration. In comparison to the brittle star (24), sialylation is less common in *H. atra* (Figure 10). Nevertheless, the ability of this species to sialylate N-glycans on two different residues (α2,3 on Gal or α2,6 on GlcNAc) would correlate with the expansion of the sialyltransferase gene family in the echinoderms (25). As compared to the evolutionarily more primitive protostome phyla (Figure 10), nematodes have no sialylation capacity at all, while most insect species have single homologues of α2,6-sialyltransferase and CMP-NeuAc synthase (26,27); only for *Drosophila* are there MS data indicative of sialylation of N-glycans in a non-engineered insect system (28). However, to date, glucuronic acid and sulphate have proven to be the most recurrent anionic modifications of invertebrate N-glycans (29).

All sialylated N-glycans proposed in *H. atra* contain NeuGc rather than NeuAc, even though both have been previously reported on glycolipids from other sea cucumbers (4,5,30). Certainly, the CMP-NeuAc hydroxylase required for NeuGc transfer is known in echinoderms (31) and NeuGc occurs also in many higher deuterostomes, including cephalochordates, fish and mice (32-34),

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but not humans (35). However, unlike the brittle star, there is an apparent lack of methylated NeuGc on \textit{H. atra} N-glycans. A rather unusual anionic feature detected in \textit{H. atra} is phosphorylation of three oligomannosidic-type N-glycans with a triglucosylated A-arm (P$_3$Glc$_3$Man$_7$GlcNAc$_2$) carrying the phosphate on the terminal glucose; such an N-glycan modification has not been previously reported, in contrast to the ‘famous’ mannose-6-phosphate involved in intracellular cell trafficking via the cognate receptor for lysosomal enzymes (36). The terminal localization of the glucose-6-phosphate could have an important role for glycoprotein folding regulation during calnexin/calreticulin cycles in the endoplasmic reticulum (37). This phosphorylation position contrasts with the presence of phosphate on antennal GlcNAc residues of the brittle star as described in the accompanying study (24). The fucosylation level in \textit{H. atra} is very high in the hybrid and complex sub-N-glycomes, with many of the sulphated and/or sialylated glycans displaying antennal fucosylation; as the galactose residue on the hybrid glycans is clearly β3-linked, this means that the fucose residue on such antennae is α4-linked, a feature found on complex plant N-glycans as well as some human glycans (38,39). Furthermore, a small minority of glycans are core α3-fucosylated, a feature known to be common in nematodes, insects and plants (40), while core α6-fucosylation in \textit{H. atra} is frequent. Thus, there must be at least three fucosyltransferases capable of modifying N-glycans in this echinoderm species.

Another obvious difference to the brittle star is the relative dominance of hybrid structures as compared to complex forms in the sea cucumber. Also, the maximal number of branches appears to be three in \textit{H. atra} rather than four. This would suggest low processing by Golgi α-mannosidase II, but also the presence of GlcNAc-transferases I, II and IV in the sea cucumber; some of the hybrid glycans actually are displaying processing by both GlcNAc-transferases I and IV which results in disubstitution of the α3-mannose (Supplementary Figure 4), as observed also in, e.g., insects or birds (41,42). The high abundance of the same hybrid β3-galactosylated ‘backbone’ in \textit{H. atra}, regardless of whether the N-glycans are sialylated or sulphated, suggests that these classes of structure are biosynthetically related and are not random contaminants from the diet.

In conclusion, the N-glycome of \textit{H. atra} contrasts with that of the brittle star, but galactosylation, sialylation and sulphation of the antennae are common features. The presence of fucose, sulphate and sialic acid have also been reported in other glycoconjugates of various sea cucumbers, such as glycolipids with a fucose-modified tri-sialylated glucosyl-ceramide, chondroitin sulphate with sulphate-modified di-fucose branches and triterpene glycosides which can also be sulphated (4-7). The glycome of \textit{H. atra} may reflect a high expression level of sulpho- and fucosyl-transferases as well as their associated metabolites; thus, if genetic manipulation becomes possible, it could prove a good model to study regulation, mechanisms and functions of fucosylation and sulphation. From an evolutionary perspective, the occurrence of β3-galactosylation and core α3-fucosylation on one hand, but of sialylation or antennal α4-fucosylation on the other, shows that this echinoderm species does present both invertebrate- and vertebrate-like features in its glycome.
**Experimental Procedures**

*Enzymatic release of N-glycans*

3 g of wet weight black sea cucumber (*Holothuria atra* adult form) shredded into 2-4 mm cubes were suspended in boiling water and denatured for 5 min, prior to the addition of 0.1 M ammonium bicarbonate at pH 8.0, 20 mM CaCl₂ and 3 mg of thermolysin in a final volume of 15 mL. Proteolysis was allowed to proceed for 2 hours at 70°C then the sample was centrifuged to remove residual insoluble material. The resulting glycopeptides were enriched by cation-exchange chromatography (Dowex AG50; Bio-Rad) and gel filtration (Sephadex G25; GE Healthcare), yielding 30 mg of purified glycopeptides. N-glycans were released using peptide:N-glycosidase F (PNGase-F; Roche) in 100 mM ammonium carbonate, pH 8, overnight at 37°C; remaining glycopeptides were then digested using peptide:N-glycosidase A (PNGase-A; Roche) in 50 mM ammonium acetate, pH 5, overnight at 37°C. PNGase-F and PNGase-A released N-glycan fractions were further purified by a second round of cation-exchange chromatography (Dowex; Biorad); then separated by a non-porous graphitized carbon column (NPGC; Merck) using 40% acetonitrile to elute the neutral glycans followed by 40% acetonitrile with 0.1% trifluoroacetic acid to elute the anionic glycans (43). All N-glycan fractions were then pyridylaminated (PA), as described previously (44). As compared to the RP-HPLC fluorescent signal of 10 pmol of a purified PA labelled N-glycan from commercial source (30 mV at the detector gain used), the yield of total labelled N-glycans was 7 nmol for the neutral pool and 3 nmol for the acidic pool. Sea cucumbers feed on planktonic algae, amoebae and small animals; as no pentose-containing glycans were detected, we conclude that no algae were co-analysed.

**MALDI TOF MS analysis**

The pyridylaminated N-glycans were fractionated by reversed-phase (RP) or normal-phase (NP) HPLC columns and profiled by MALDI-TOF MS (Autoflex Speed, Bruker Daltonics, Germany) in positive and negative ion modes using FlexControl 3.4 software. All HPLC peaks were collected, freeze dried, re-dissolved in 10 µL and examined by MALDI-TOF MS, using 6-aza-2-thiothymine as matrix (45). Sample and matrix solution (1 µL each) were sequentially spotted and dried under vacuum. In order to enhance formation of [M+H]+ or [M-Hn+Na-n-1]- ions either 1 µL of 20 mM ammonium sulphate or 1 µL of 10 mM sodium acetate were spotted on top of the matrix. MS/MS to confirm the composition of all proposed structures was performed by laser-induced dissociation (precursor ion selector was generally set to ±0.6%). The detector voltage was generally set at 1977 V for MS and 2133 V for MS²; 500-1000 shots from different regions of the sample spots were summed. Spectra were processed with the manufacturer’s software (Bruker FlexAnalysis 3.3.80) using the SNAP algorithm with a signal/noise threshold of 6 for MS (unsmoothed) and 3 for MS² (four-times smoothed). All MS and MS² spectra were manually interpreted on the basis of the mass fragmentation pattern and results of chemical and enzymatic treatments; isomeric structures present in different RP-HPLC or NP-HPLC fractions were defined on the basis of comparisons of the aforementioned parameters. At least four MS² fragment ions were used to aid definition of each of the structures, which are depicted according to the Symbol Nomenclature for Glycans (46). For further details, also refer to the Supplementary Information.

**HPLC purification of N-glycans**

Separation of PA-labelled glycans was carried out on a Shimadzu HPLC system...
equipped with a fluorescence detector (RF-20AXS) using a Kinetex 5 μm RP-column (XB-C18 100A, 250 × 4.6 mm; Phenomenex®, Torrance, CA), with a gradient of methanol in 0.1 M ammonium acetate, pH 4, up to 16.5% over 44 min applied at a flow rate of 0.8 mL/min as follows: 0–30 min, 0–9% methanol; 30–35 min, 9–12% methanol; 35–40 min, 12–16.5% methanol; 40–44 min, 16.5% methanol; and 44–50 min, return to 0% methanol. For separation based on size and charge, an HIAX IonPac AS11 NP-column (Dionex) was used with 800 mM ammonium acetate, pH 3.85 (buffer A) and 80% (v/v) acetonitrile (buffer B). The following gradient was applied at a flow rate of 1 mL/min: 0-5 min 99% B; 5-50 min 90% B; 50-65 min 80% B; 65-85 min 75% B. PA-labelled glycans were detected by fluorescence with excitation/emission wavelengths of 320/400 nm. The RP-HPLC column was calibrated daily in terms of glucose units (g.u.) using a pyridylaminated dextran hydrolysate whereas the NP-HPLC column was calibrated daily using a mixture of pyridylaminated N-glycans (Man₅GlcNAc₂) derived from white beans; the order of elution of the standards was confirmed by MALDI-TOF MS of collected calibrant fractions (43).

**Structural elucidation using exoglycosidases and chemical treatment**

The following glycosidases were employed: recombinant *Aspergillus niger* β3/4-galactosidase (prepared in-house (47)); *Xanthomonas manihotis* β3-galactosidase (New England Biolabs); *Xanthomonas manihotis* β4-galactosidase (New England Biolabs); Bovine kidney α-fucosidase (Sigma-Aldrich); almond α3/4-fucosidase (NEB); Jack bean α-mannosidase (Sigma-Aldrich); purified recombinant *Bacteroides xylanisolvens* BxGH99 α2-endomannosidase, which catalyzes the removal of a disaccharide from Glc1Man₅GlcNAc₂ but not from unglucosylated Man₅GlcNAc₂ (48); recombinant *Aspergillus soitoi* α2-mannosidase (Prozyme); *Streptococcus pneumoniae* α3-sialidase S (New England Biolabs). In general, 10% of an HPLC fraction (1 µL) were incubated overnight at 37 °C with 0.8 µL of 100 mM ammonium acetate at pH 5.0 and 0.2 µL of a glycosidase (see above). For removal of phosphate or α3/4-linked fucose, 30% of an HPLC fraction (3 µL) were dried under vacuum and incubated overnight on ice with 3 µL of 48% (w/v) hydrofluoric acid (HF) prior to drying again. For removal of sulphate, 30% of an HPLC fraction (3 µL) were dried under vacuum and incubated 4 h at 37 °C with 20 µL of 0.05 M methanol-HCl (methanolysis) prior to drying again. Enzymatically or chemically treated N-glycans were generally re-analysed by MALDI-TOF MS and MS/MS without further purification, unless rechromatographed by RP-HPLC (see relevant figure legends). The β4-galactosylated Hex₆HexNAc₃ standard was generated by treatment of a Man₅GlcNAc₂ structure with bovine milk galactosyltransferase (Fluka) in the presence of UDP-Gal and Mn(II) ions (49).

**Conflicts of interest:**
The authors declare that they have no conflicts of interest.

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References:


**Figures:**

Figure 1: MALDI-ToF MS analyses of PNGase F-released neutral and anionic N-glycans of after pyridylamination. Total PNGase F released N-glycans from neutral and anionic pools were analysed by MALDI-ToF MS in positive ([M+H]+) and negative modes ([M-H]-) (A-C). The neutral pool (A) contains mainly oligomannosidic-type N-glycans while the anionic pool (B-C) shows a wide range of structures modified with phosphate, sialic acid and/or sulphate. MALDI-ToF MS analyses were performed with ATT as matrix supplemented with ammonium sulphate (A-C) which enhanced the occurrence of [M+H]+ or [M-H]- ions, but impaired the formation of sodiated ions such as [M+Na]+ and [M-Hn+Na-n-1]-, which leads to an underestimation of the degree of sulphation of some structures as compared to Figure 2. Asterisks (*) indicate the presence of multiple structural isomers. Example structures are annotated using the Symbol Nomenclature for Glycans: ○ mannose; ○ galactose; ○ glucose; □ N-acetylglucosamine; ▲ fucose; ◆ N-glycolylneuraminic acid; S sulphate; P phosphate. The right panel indicates a simplified phylogenetic tree indicating the different classes of echinoderms in comparison to the chordates (including vertebrates).
Figure 2: RP-Kinetex HPLC of neutral- or anionic-enriched pools of PNGase-F released N-glycans from *Holothuria atra*. Pyridylaminated neutral (A) and anionic (B) pools are annotated with proposed structures as confirmed by MS, MS/MS and enzymatic and chemical treatments; ‘+nS’ indicates ambiguity as to which residues are sulphated. The insets show proposed linkages, also for disialylated antennae, while the annotated observed m/z values are either positive mode [M+H]+ for neutral, sialylated or phosphorylated glycans or negative mode [M-H]- for monosulphated and [M-H]+Na(n-1)- for multiply-sulphated glycans. Retention times are given in minutes and the annotated structures for each fraction are shown in order of abundance with the most abundant uppermost; calibration is in terms of glucose units (g.u.). It can be noted that antennal fucosylation and multiple sulphation results in earlier RP-HPLC elution than core α1,6-fucosylation or mono-sulphation.
Figure 3: Analysis of phosphorylated oligomannosidic-type N-glycans. The phosphorylated oligomannosidic-type N-glycans (P<sub>1</sub>H<sub>10-12</sub>N<sub>2</sub>; m/z 2203, 2365 and 2527) in the neutral enriched pool (see Figure 2 A and Supplementary Figure 1 A) were analysed by MALDI-ToF MS (A-D) and MS/MS (E-H) in positive mode as [M+H]<sup>+</sup> in conjunction with enzymatic and chemical treatments. The 80 Da modification was shown to be phosphate due to its sensitivity to alkaline phosphatase treatment (A-B) as monitored also by MS/MS (E-F), while the probable position of the phosphate was deduced from α-mannosidase and endo-α-mannosidase digestions (C-D and G-H) removing respectively five mannoses or a P<sub>1</sub>Gl<sub>c</sub>Man<sub>1</sub> unit. Note that the digestions with phosphatase or endomannosidase (B and D) result in a shift to sodiated adducts (+22 Da), while the m/z 2225 species in the mannosidase digest (C) is a contaminant generally found when using the jack bean enzyme preparation. Losses of phosphate (-P) or hexoses (-H1, -H2, etc.) as well as B-ion Hex<sub>i</sub>P fragments (H1P, etc.) are indicated. Based also on all the evidence, including mannosidase treatment and HPLC analysis after hydrofluoric acid treatment (see Supplementary Figure 2), it is concluded that the underlying backbone of the P<sub>1</sub>H<sub>12</sub>N<sub>2</sub> glycan (m/z 2527) is a standard Glc<sub>3</sub>Man<sub>9</sub>GlcNAc<sub>2</sub> structure with the same RP-HPLC elution properties as a previously published structure from <i>P. pacificus</i> (50).
Figure 4: HPLC and MALDI-TOF MS analysis of hybrid-type N-glycan. The 19.9 min NP-HPLC fraction containing Gal1Man5GlcNAc3 (m/z 1678 as [M+H]+) was re-injected onto RP-HPLC and eluted at 21.9 min (A; 7.2 g.u.); subsequently, positive MS before (E and I) and after β4-galactosidase (F; no digestion), β3-galactosidase (G and J; loss of 1 Gal) and jack bean α-mannosidase (H and K; loss of up to 3 Man) proved the presence of a neo-LacNAc (Galβ3GlcNAc) antenna on a hybrid Man5-type backbone. In contrast, an isomeric Gal1Man5GlcNAc3 structure (generated by enzymatic remodelling with β4-galactosyltransferase and carrying a LacNAc Galβ4GlcNAc antenna) had a later RP-HPLC elution time (B; 23.8 min or 8.2 g.u.), which proves the distinct linkage on the *H. atra* glycan.

Furthermore, treatment of the sulphated S1Gal1Man5GlcNAc3 and sialylated NeuGc2Gal1Man5GlcNAc3 hybrid-type N-glycans with respectively methanolysis and sialidase resulted in neo-LacNAc antennae as judged by co-elution with the β3-galactosylated neutral form of Gal1Man5GlcNAc3 (C, D) as well as subsequent enzymatic digestions (data not shown; but similar to Figure E-K). The effects of enzymatic treatments are indicated by red arrows and digested structures are displayed in grey (C and D).
Figure 5: MALDI-TOF MS analysis of mono-sulphated hybrid-type N-glycans. S1Fuc1/2Gal1Man5GlcNAc3 glycans (m/z 1902/2048 as [M-H]) eluting at 4.7 and 6.8 g.u.; see Figure 2 B were analysed by negative MS before (A, F) and after enzymatic or chemical treatments (B-E, G). The hybrid nature and lack of sulphation of fucose were shown by sensitivity to jack-bean α-mannosidase (C, removing three mannoses) and the removal of fucose by either α3/4-fucosidase (D) or HF (E, G) treatments without loss of the negative charge, while the resistance to β3/4-galactosidase (B) is expected for a Lewis-type antenna, regardless of sulphation. Negative and positive MS/MS of these glycans, also in comparison to a core fucosylated isomer eluting at 7.8 g.u., showed a number of key diagnostic variations (H-N). While the presence of a negative mode B-fragment ion at m/z 241 (S1Hex1; H, K and M) is compatible with sulphation of galactose, the position of the fucose on either the antenna or core can be deduced on the basis of the sulphated B-ions at m/z 444/590 (S1Fuc0/1Gal1GlcNAc1); the neutral losses of either -299 (GlcNAc1-PA; -NPA) or -445 (Fuc0/1GlcNAc1-PA; -NFPA) were indicative for the absence (H) or presence (K, M) of core fucosylation. On the other hand, positive mode MS/MS of these glycans and an HF digestion product (I, J, L and N; m/z 1824, 1678 or 1970 resulting from in-source loss of sulphate) reveals the underlying backbones, whereby specific diagnostic Y-ions at m/z 300/446 (Fuc0/1GlcNAc1-PA) and 1151/1297 (Fuc0/1Man4GlcNAc2-PA) are characteristic for core and hybrid-type structures, whereas the m/z 512 B-ions are indicative for fucosylated antennae. Highlighted in green and purple are respectively those fragments aiding definition of the antennae and the core. Asterisks (*) indicate ions [M-179]- deriving from 0.2A cross-ring cleavage of the core PA-labelled GlcNAc as also previously observed in negative mode MS/MS of sulphated N-glycans from lepidopteran species [13]. Losses of Fuc (F), Hex (H), HexNAc (N) or HexNAc-PA (NPA) are indicated.
Figure 6: MALDI-TOF MS analysis of di-sulphated hybrid N-glycans. Di-sulphated hybrid-type \( S_2Fuc_2\) Gal-Man-GlcNAc glycans present in different normal phase fractions (see Supplementary Figure 1 B) were analysed by negative MS (A-D), exhibiting some in-source loss of sulphate (indicated by \( \Delta 102 \) and \( * \)), and MS/MS (F-I); mono-sulphated ([M-H]) and di-sulphated ([M-2H+Na/K]) B-fragment ions were observed at \( m/z \) 241, 282 or 444 \( \left(S_1Gal_0\_GlcNAc_{0-1}\right) \), 546/562 \( \left(S_2Gal_1\_GlcNAc_{1}\right) \), 692/708 \( \left(S_2Fuc_2\_Gal_1\_GlcNAc_{1}\right) \), 708/724 \( \left(S_2Gal_1\_GlcNAc_1\_Man_1\right) \) and 854/870 \( \left(S_2Fuc_1\_Gal_1\_GlcNAc_1\_Man_1\right) \) in addition to Y-ions at \( m/z \) 1375/1537 \( \left(S_1Fuc_1\_Man_{4-5}\_GlcNAc_{2-PA}\right) \) aid definition of sulphation of either Gal, GlcNAc or Man. The fragmentation patterns of the two \( m/z \) 2150 isomers \( (H, I) \) indicated either sulphation on the terminal Gal and lower \( \alpha3\)-Man or two sulphate residues on a Lewis motif (see blue arrows). HF treatment of the latter only removed the antennal \( \alpha4\)-fucose \( (E \text{ and } J) \); whereby the \( m/z \) 546 fragment corresponds to a di-sulphated Hex\_HexNAc; as the 80 Da modification was not lost, it is neither linked to the Lewis-type fucose nor is it an isobaric phosphate. The distinct elution of the disulphated isomers is the result of the spatial arrangement of sulphate residues causing weaker or stronger binding to the HIAAX column. Losses of Fuc \( (F) \), hexose \( (H) \) or reducing terminal Fuc\_GlcNAc\_PA \( (-NPA \text{ or } -FNPA) \) are indicated.
Figure 7: HPLC and MALDI-TOF MS analysis of sialylated hybrid-type N-glycans. NeuGc1Fuc0/1Gal1Man5GlcNAc3 glycans (m/z 1985/2131) eluting on NP-HPLC at 28-31 min were re-injected before or after sialidase S treatment onto RP-HPLC (A, B) which resolved four structures concluded to display either internal or external sialylation of the antenna (see Figure 2 B) and showed that the enzyme specifically removed one NeuGc from the externally-sialylated isomers, compatible with the incomplete desialylation observed by MS analysis of the NP-30.1 fraction (E-F). Positive and negative MS/MS of the externally-sialylated m/z 2131/1985 glycans (K-M) showed the occurrence of NeuGc containing B- and C-fragments at m/z 308/306 (NeuGc1), 673/671 (NeuGc1Gal1GlcNAc1), 853/851 (NeuGc1Gal1GlcNAc1Man1) as
well as Y-loss of NeuGc₁. In contrast, internal NeuGc was resistant to sialidase, but the isomer eluting at 24.7 min (A-B) was sensitive to β3-galactosidase (G-H), whose action resulted in a shift in the MS/MS B-ions from \( m/z \) 673 to \( m/z \) 511 indicative of NeuGc linked to GlcNAc (I-J). In case of the di-sialylated hybrid type \( m/z \) 2292 glycan containing both the internal and external NeuGc, only the latter was removed by sialidase S treatment resulting in a shift to a retention time to 21.9 min, i.e., identical to the internally-sialylated \( m/z \) 1985 isomer (C-D); MS/MS of such di-sialylated glycans (P-Q, S) showed little difference to the externally-sialylated glycans, but either low abundance positive \( m/z \) 511 NeuGc₁GlcNAc₁ or \( m/z \) 980 NeuGc₂Gal₂GlcNAc₁ ions were present, in addition to di-sialylated negative B- and C-ions at \( m/z \) 996 and 1788. Negative and positive MS/MS of a sulpho-sialyl-Lewis A containing structure (N-O) revealed diagnostic B-fragments at \( m/z \) 897 (S₁NeuGc₁Fuc₁Gal₂GlcNAc₁) and 819 (NeuGc₁Fuc₁Gal₂GlcNAc₁), while negative mode MS/MS of a sulphated NeuGc containing glycan (T) showed a characteristic ion at \( m/z \) 386 (S₁NeuGc₁) as well as loss of a carboxyl group (-44 Da). Note that the \( m/z \) 424 fragment ion (L) has been observed in other reports on negative mode MALDI-TOF MS/MS of neutral and sialylated N-glycans, being annotated as a \( ^{1,3}A_3 \) ion of the composition Gal–GlcNAc–O–CH=CH–O⁻ (51). Effects of enzymatic treatments are illustrated by red and blue arrows and digested structures are displayed in grey (B and D).
Figure 8: MALDI-TOF MS analysis of multi-sulphated complex-type N-glycans. The highest degree of sulphation of bi-/tri-antennary N-glycans was observed in early-eluting RP-HPLC fractions by negative MS in presence of sodium acetate buffer which allows the detection of \([M-H_{n}+Na_{n-1}]^{-}\) ions corresponding to multi-sulphated structures (A-B; structures containing two to four sulphates are indicated in bold \(m/z\) 2165-3052 and none were digested with a non-specific galactosidase). Due to in-source loss of sodiated sulphates (Δ102), pseudomolecular ions (*) with varying sulphated status (S1-S4) were also observed and fragmented in negative mode as \([M-(SO_{3})_{n}]^{-}\) or fragmented in positive mode as \([M+H(n-1)-(SO_{3})_{n}]^{+}\). Analysis in the presence of ammonium sulphate (C) results in mono-sulphated ions, observed in defucosylated form upon fucosidase treatment (D). Example glycans (see also Supplementary Figure 7) were fragmented in negative mode either as in-source pseudomolecular (F, I) or as parental ions (G, J) showing the presence of sulphated Lewis motifs as well as some neutral losses from the core, while their bi- and tri-antennary nature as well as core and antennal fucosylation were confirmed by positive B- and Y-fragments (E, H) such as \(m/z\) 446 (Fuc\(_{2}\)GlcNAC\(_{1}\)-PA), 512 (Fuc\(_{2}\)Gal\(_{1}\)GlcNAC\(_{1}\)) and 1484/1646 (Fuc\(_{2}\)Gal\(_{1}\)Man\(_{2}/3\)GlcNAC\(_{3}\)-PA); the two \(\beta2/\beta4\)-linked antennae on the lower \(\alpha3\)-Man are assumed in comparison to hybrid N-glycans (see Supplementary Figure 5). ‡ indicates early-eluting reducing-end ManNAC epimers (52) of structures predominantly found in later fractions. Losses of Fuc (F), Hex (H) and HexNAC (N) are indicated.
Figure 9: MALDI-TOF MS analysis of specific PNGase-A released N-glycans. Larger core α3-fucosylated glycans with compositions of S$_1$Fuc$_3$Gal$_1$Man$_5$GlcNAc$_3$ (A; hybrid-type at m/z 2194) and S$_1$Fuc$_5$Gal$_3$Man$_3$GlcNAc$_5$ (C; complex-type, observed with in-source loss of sulphate at m/z 2893) respectively eluted at 5.3 and 3.8 g.u. upon RP-HPLC of the anionic pool of PNGase-A released N-glycans. Both fractions were subject to HF treatment (B, D) which removed all fucoses (see red dashed arrows) except the α6-linked core-fucose, indicative that the second core fucose is α3-linked. Furthermore, negative MS/MS of S$_1$Fuc$_3$Gal$_1$Man$_5$GlcNAc$_3$ (E) clearly yielded B-ions at m/z 1400 and 1603 (resulting in loss of 591 or 794 Da, i.e., of Fuc$_2$GlcNAc$_1$-PA), while the positive mode spectrum of the pseudomolecular [M-SO$_3$]$^+$ ion (F) shows a Y-fragment at m/z 592 revealing the presence of an additional core fucose on the reducing terminus as in many invertebrates (13). In comparison, as shown in Figure 5 M and N, MS/MS of a glycan (S$_1$Fuc$_2$Gal$_2$Man$_5$GlcNAc$_3$) with one core and one antennal fucose shows an m/z 590 negative mode B-ion, but lacks the m/z 592 and 1605 difucosylated positive mode Y-ions.
Figure 10: Semi-quantitative analysis of *Holothuria atra* N-glycome and comparisons within the Deuterostoma. The signal intensities of HPLC and MALDI-ToF peaks containing characterised N-glycans were used to estimate the ratio of each individual class and sub-class of N-glycan to provide an overview of their relative abundance. Particular proven epitopes include variable antennal α4-fucosylation, β3-galactosylation, 4-linked sulphation of galactose, α3-sialylation of galactose, α6-sialylation of GlcNAc, sulphation of GlcNAc (putatively 6-linked, if otherwise not sialylated) and sulphation of mannose; phosphorylation of triligucosylated glycans and core difucosylation of hybrid/complex glycans were also detected (the latter accounting for some 0.3% of the total N-glycome of *H. atra*). For a full list of predicted compositions, refer to Supplementary Table 2. The simplified evolutionary tree (left panel; based on Vaughn et al. (53)) exhibits the division between Protostomes and Deuterostomes in the Animalia (500 million years ago) as well as example resulting species. The depiction of the Deuterostoma (middle panel) shows the phyla of the Echinodermata and Chordata. An overall comparison of N-glycomic features (right panel) of *Holothuria atra* (sea cucumber; this study), *Ophiactis savignyi* (brittle star; accompanying study (24)) and Vertebrata (e.g., human and bovine) shows selected similarities (e.g., di-sialylated motif) and differences (e.g., variation in fucosylation, sulphation and sialylation levels).
Sulphated and sialylated N-glycans in the echinoderm *Holothuria atra* reflect its marine habitat and phylogeny
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