Glycosylation at an evolutionary nexus: the brittle star *Ophiactis savignyi* expresses both vertebrate and invertebrate N-glycomic features

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**Running title: Brittle star N-glycome**

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**Abstract**

Echinoderms are amongst the most primitive deuterostomes and have been used as model organisms to understand chordate biology due to their close evolutionary relationship to this phylogenetic group. However, there are almost no data available on the N-glycomic capacity of echinoderms, which are otherwise known to produce a diverse set of species-specific glycoconjugates, including ones heavily modified by fucose, sulphate and sialic acid residues. In order to increase the knowledge of diversity of carbohydrate structures within this phylum, here we conducted an in-depth analysis of N-glycans from a brittle star (*Ophiactis savignyi*), as an example member of the class Ophiuroidea. To this end, we performed a multi-step N-glycan analysis by HPLC and various exoglycosidase and chemical treatments in combination with MALDI-TOF MS and MS/MS. Using this approach, we found a wealth of hybrid and complex oligosaccharide structures reminiscent of those in higher vertebrates as well as some classical invertebrate glycan structures. Some 70% of these N-glycans were anionic carrying either sialic acid, sulphate or phosphate residues. In terms of glycophylogeny, our data position the brittle star between the invertebrates and vertebrates and confirm the high diversity of N-glycosylation in lower organisms.

**Introduction**

The Echinodermata represent the second largest grouping within the deuterostomes, after the Chordata, which also include vertebrates. This lineage has separated from the protostomes around 500-600 million years ago and so are at the nexus between invertebrates and vertebrates (1). Living representatives of the echinoderms include classes of Crinoidea (feather stars), Echinoidea (sea urchins), Holothuroidea (sea cucumbers), Asteroidea (starfishes) and Ophiuroidea (brittle stars). Due to their close relationship to vertebrates, members of this phylum have been intensively studied in terms of the basics of development conserved as compared to vertebrates as well as of species-specific fertilization and regeneration (2,3).

The evolutionary position of Ophiuroidea, with some 2000 living species (4), makes this class of organisms an interesting target to study the phylogeny of protein-linked glycans of not only the echinoderms, but...
also of the vertebrates. To date, a major focus of glycostructural studies on echinoderms has been on their O-glycans and glycolipids, as well as some proteoglycan-like polymers involved in species-specific induction of the acrosome reaction during fertilisation (5-15). From these studies, it appears that, in contrast to protostomes, sialic acids are probably a frequent component of echinoderm glycoconjugates and, as compared to ‘higher’ vertebrates, occur also in sulphated and methylated forms (16).

Furthermore, core α1,3/6-difucosylation is a known recurring feature of protostome N-glycomes (17), but is absent from vertebrates and has not been identified in an echinoderm species before. Although there are a number of studies on the importance of N-glycan biosynthesis for sea urchin development (18,19), there is little exact information regarding the N-glycosylation capacity of echinoderms. Indeed, in terms of actual structures, there is seemingly a single report on the mass spectrometric analysis of oligomannosidic N-glycans (20).

As part of our ongoing efforts to establish a glycophylogeny of animal species, we analysed the N-glycans of *Ophiactis savignyi*, an omnivorous small tropical marine organism, once claimed as being the most common brittle star on the planet, potentially invasive in some areas and capable of both asexual and sexual reproduction (21). We had hypothesised that echinoderm species would display a mixture of invertebrate and vertebrate structural features; indeed, we found many N-glycans with antennal sialic acid or sulphate, but also others displaying difucosylation of the core. Furthermore, as compared to our other studies on echinoderms (accompanying study (22) and unpublished data), there are also unique motifs, which define the individual N-glycome of this species.

**Results**

**Overall glycomic approach:** Our initial hypothesis regarding echinoderm N-glycosylation was that there would be a substantial degree of modification of the oligosaccharides with anionic moieties as in vertebrates, but that there may be vestiges of invertebrate-type N-glycan features such as difucosylation of the asparagine-bound chitobiosyl core. Therefore, the glycomic workflow for *Ophiactis savignyi* was aimed at not only separating neutral from anionic N-glycans, but also at release of structures with core α1,3-fucose. The serial use of PNGase F and A followed by solid phase extraction on non-porous graphitised carbon allowed isolation of three pools of glycans (Supplementary Figure 1; enriched in neutral, anionic and core α1,3-fucosylated structures), all of which were fluorescently labelled and analysed by off-line HPLC-MALDI-TOF-MS. The overall predicted N-glycan compositions, based on MS and MS/MS before and after chemical or enzymatic treatments, are summarised in Supplementary Table 1. Furthermore, O-glycan analysis of the residual glycopeptide fraction was performed by LC-ESI-MS following reductive β-elimination.

**Neutral N-glycans:** The neutral PNGase F-released pool contains primarily oligomannosidic and hybrid structures (see Figure 1 for a normal phase profile) and their isomeric status could be evaluated on the basis of RP-HPLC elution times (Supplementary Figure 2) and MS/MS fragmentation as compared to previous studies. Our initial interest was in determining the nature of the potential galactosylation of an example hybrid structure (m/z 1678 as [M+H]+) as well as of a low-abundance biantennary glycan (m/z 1865). Based on use of linkage-specific galactosidases (Figure 2), β1,3-
galactosylation (type 1 chain, Galβ1-3GlcNAcβ1-R) as found in a number of invertebrates, rather than the β1,4-form (type 2 chain, Galβ1-4GlcNAcβ1-R) dominant in mammals, is concluded to be a feature of this echinoderm. Antennal β1,3-galactosylation was also a basic feature of the anionic glycans.

In later neutral normal phase fractions, also glucosylated oligomannosidic structures were found, as were glycans with unexpected Hex13-16HexNAc2 compositions. These were partially susceptible to digestion with jack bean α-mannosidase, but were also sensitive to a bacterial endo-α-mannosidase, as judged by shifts in the MS and MS/MS spectra (Figure 3 and Supplementary Figure 3). As Hex13HexNAc2 (m/z 2610) lost five hexoses upon either endo-mannosidase or exo-mannosidase digestion and no glucosidase or galactosidase tested removed the extra hexose residues, the Hex13-16HexNAc2 glycans are predicted to be Glc3Man9GlcNAc2 structures carrying unknown hexose residues on the glucosylated arm.

Upon PNGase A treatment of glycopeptides remaining after PNGase F release, a mixture of neutral structures was obtained. Only fucosylated paucimannosidic glycans as well as ‘residual’ oligomannosidic ones were present in this pool. Of the identified structures, two had elution times (8 g.u.), compositions and MS/MS patterns (i.e., a Y-ion at m/z 592) indicative of core α1,3/α1,6-difucosylation (Supplementary Figure 4). Due to its early elution, also the monofucosylated structure at 5 g.u. (m/z 1135, Hex3HexNAc2Fuc1) is concluded to be core α1,3-fucosylated, which is a feature known from plants and invertebrates, but not from vertebrates.

Separation of anionic N-glycans

Based on fluorescence intensity of the various N-glycan pools, it was estimated that some 70% of the structures are anionic and initial mass spectrometric data suggested that both sulphated and sialylated glycans were present (Supplementary Figure 1). ‘Sialylation-friendly’ RP-HPLC conditions (pH 6) in a first dimension yielded eleven pools, which were then subject to charge/size-based separation using a HIAx column; thereby, almost 120 2D-HPLC-separated glycan-containing fractions were isolated (Figure 4 and Supplementary Figure 5).

Sulphated and phosphorylated N-glycans:

Although sulphation and phosphorylation both result in an 80 Da modification as compared to ‘parental’ neutral N-glycans, their properties differ (23); for instance, ‘in-source’ loss in positive mode is a hallmark of sulphate, while sensitivity to hydrofluoric acid is a characteristic of phosphate. Thereby, we were able to conclude that both sulphated and phosphorylated N-glycans are synthesised by O. savignyi.

Based on negative ion mode MS/MS and the results of exoglycosidase treatments, three different types of sulphated monosaccharides were observed: core α1,6-fucose, antennal galactose or antennal GlcNAc. Sulphation of core α1,6-fucose has previously been found in insects and hallmarks are the m/z 225 sulphated fucose fragment and resistance to bovine fucosidase treatment (Figure 5 A-E, K and M). In comparison, an isomeric glycan with sulphation of an antennal GlcNAc was distinguishable on the basis of the m/z 282 B-fragment and the loss of only one HexNAc upon serial galactosidase/hexosaminidase digestion (Figure 5 F-J and N). Sulphation of galactose was associated with a dominant m/z 241 fragment (sulphated hexose; Figure 5 L) and galactosidase resistance. Disulphation of type 1 antennae was indicated by the
MALDI-TOF-MS/MS and LC-ESI-MS\(^n\) data with respectively \(m/z\) 545 [M-2H+Na]\(^-\) or 261 [M-2H]\(^2-\) fragments (Hex\(^3\)HexNAc\(^4\)S\(^2\); Figure 5 O-Q) being observed; the galactose is proposed to be 4-sulphated (24), while the GlcNAc may be 4- or 6-sulphated.

Other glycans with an 80 Da modification were observed in both positive and negative modes and so were concluded to be phosphorylated on HexNAc residues as judged by the \(m/z\) 284 [M+H]\(^+\) fragment ions (Figure 6 A-E). In the case of two hybrid structures, this was verified by sensitivity to jack bean \(\beta\)-hexosaminidase only after hydrofluoric acid treatment; subsequently one or two hexose residues could be removed upon jack bean \(\alpha\)-mannosidase treatment (Figure 6 F-I).

Sialylated N-glycans: A number of glycans contained modifications of either 307 or 321 Da as defined by results of positive and negative mode MS/MS. Relevant B-fragments in either positive or negative ion modes indicated the presence of N-glycolyneuraminic acid or its methylated form on terminal HexHexNAc motifs (Figure 7 A-G and Supplementary Figure 6). As there were multiple isomers for some masses amongst the 2D-HPLC fractions (e.g., two, three or even four each of mono- and biantennary structures with \(m/z\) 1807, 1821, 2172 or 2333; see Supplementary Figure 5), a major question was whether there were multiple positions for sialylation. Therefore, selected glycans were incubated with sialidases and/or mild acid.

As shown for the four isomeric monosiallylated biantennary Hex\(^3\)HexNAc\(^4\)Fuc\(^1\)NeuGc\(^1\) glycans (Figure 8; 2171 Da), two structures only lost the NeuGc residue upon acid treatment, while two others were sensitive to \(\alpha\)2,3-specific sialidase S. On the other hand, the sialidase-resistant glycans lost two hexose residues upon \(\beta\)1,3-galactosidase treatment, while only one hexose was removed from those sensitive to sialidase S (Figure 8 B, G, R and W). Thus, as sialic acids are known to modify either terminal galactose or antennal GlcNAc residues in, e.g., mammalian fetuin, we concluded that this also occurs in the brittle star (see below); indeed some structures with NeuGc on both the Gal and GlcNAc of the same antenna were also found (Figure 7E-G).

In order to determine which antennae of the 2171 Da isomers were sialylated, serial chemical or enzymatic treatments were then performed. By comparison of the RP-HPLC retention time of digestion products to standards, the two isomers with sialylated GlcNAc could be distinguished regarding the NeuGc substitution on either the \(\alpha\)1,6- or \(\alpha\)1,3-antenna (Figure 8 A-P). On the other hand, despite similar fragmentation patterns and only slightly different 2D-HPLC elution properties, the two isomers with sialylated galactose were shown to differ, in terms of which antenna carried the sialic acid residue, by performing \(\beta\)1,3-specific galactosidase treatment followed by incubation with the arm-specific insect FDL hexosaminidase (25) (Figure 8 Q-Z).

A similar approach was employed on two glycans with putative NeuGcMe modifications. In the case of a terminal NeuGcMe residue, sialidase S treatment resulted in loss of 321 Da, but the glycan was resistant to \(\beta\)1,3-galactosidase unless desialylated (Supplementary Figure 7 A-C and E); in contrast, the isomeric glycan with a later NP-HPLC retention was resistant to sialidase S, but sensitive to \(\beta\)1,3-galactosidase and to acid treatment (Supplementary Figure 7 G-J), consistent with an \(\alpha\)2,6-sialyl substitution of GlcNAc. Based on treatment of the desialylated/degalactosylated structures with insect FDL, both glycans possessed
sialylated lower arm Galβ1,3GlcNAc motifs (Supplementary Figure 7 D and K). The MS/MS fragmentation patterns were very similar (Supplementary Figure 7 F and L), but the trace ion at m/z 525 (i.e., HexNAcNeuGcMe) verified that the sialidase S-resistant structure was modified by NeuGcMe on the antennal GlcNAc residue. Some glycans were predicted to contain both sulphate and sialic acid; in the case of two isomeric structures (Hex5HexNAc4Fuc1NeuGc1S1) with different 2D-HPLC elution properties, fragments at either m/z 282 or 387 were observed and correlated with loss of either 307 or 387 Da upon sialidase S treatment. This suggested that the sulphate residues were respectively either on an antennal GlcNAc or covalently bound to the terminal sialic acid residue (Figure 7 H-M).

As mentioned above, the resistance to hydrofluoric acid of the 80 Da moiety would indicate the occurrence of sulphate and when treating the complete anionic pool with this reagent, major peaks at, e.g., m/z 1578 and 1943 were still dominant in the overall spectrum. However, masses of the sialylated glycans shifted by 18 Da (Supplementary Figure 8). This change is akin to that upon lactonisation and suggested that hydrofluoric acid can also stabilise specific sialic acid linkages similarly to combined 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/1-hydroxybenzotriazole treatment; the shift in the NeuGcMeHex1HexNAc1 positive mode fragment from m/z 673 to 655 is also the same (Supplementary Figure 8 C-H). Thereby, there were two proofs that the NeuGc or NeuGcMe on terminal galactose were α2,3-linked.

On the other hand, sialic acid linked to the GlcNAc is concluded to be α2,6-linked, due to (i) the ability to remove it with a non-specific sialidase, (ii) 3-sialylation of GlcNAc being ruled out by the presence of the 3-linked galactose and (iii) LC-ESI-MS3 evidence (Figure 7E).

**Maximum number of N-glycan antennae**

Not only did the results of hydrofluoric acid treatment of the entire anionic pool indicate lactonisation, but we could also detect the presence of trisialylated triantennary structures (m/z 3113 in negative mode; Figure 9). Upon desialylation of the anionic pool using 2 M acetic acid, we could also observe larger glycans (Hexα7HexNAc5,6Fuc1), whose monosulphated forms were detected in negative mode (Figure 9 and Supplementary Figure 9 A-D). As MS/MS analysis indicated that these correspond to tri- and tetra-antennary structures, selected RP-HPLC fractions containing mono-, bi- and tri-antennary glycans were also subject to mild acid hydrolysis prior to degalactosylation (Supplementary Figure 9 E-J) and RP-HPLC, as this method is known to distinguish different triantennary isomers [24, 25]. The underlying structures were compared to asialoagalactoglycans derived from fetuin (Supplementary Figure 9 K-M) for which the β1,2-GlcNAc on the α1,6-mannose and the β1,2/β1,4-disubstitution of the α1,3-mannose have been previously defined. The resulting retention time comparisons (Supplementary Figure 9 N) indicated that the mono-antennary glycans were β1,2-substituted on the α1,3-mannose and the bi-antennary β1,2-substituted on both α-mannose residues, whereas the tri-antennary glycans from Ophiacis savignyi have the same configuration as those in bovine fetuin.

**O-glycans:** When performing LC-ESI-MS after reductive β-elimination of residual glycopeptides (Supplementary Figure 10 and Supplementary Table 2), the two major identified O-glycans were predicted to be Galβ1,3GalNAc, the typical core 1
‘mucin-type’ glycan or T antigen, and Xylα1,3Xylα1,3Glc, a structure known from epidermal growth factor domains (26). Core 1 glycans with additional fucose, NeuAc, NeuGc, hexose, HexNAc or methyl modifications were also identified. Based on MS/MS of the lower abundance O-glycans, also in comparison to the literature (27,28), it can be concluded that also fucosylated core 1 (Fucα1,2Galβ1,3GalNAc), sialyl Tn (NeuAc/NeuGcα2,6GalNAc) and mono- and di-sialylated core 1 (Galβ1-3[NeuAc/NeuGcα2,6]GalNAc, NeuAc/NeuGcα2,3Galβ1,3GalNAc and NeuAcα2,3Galβ1-3[NeuAcα2,6]GalNAc) structures are present in Ophiactis savignyi. Thereby, it is interesting that NeuAc is well represented in the O-glycome, but not detectable in the N-glycome.

Discussion
Due to their close relationship to vertebrates at the nexus of protostomes and deuterostomes, echinoderms are historically useful model organisms for studies on development, fertilisation and embryogenesis. As little is known about their N-glycosylation capacity, we conducted an in-depth analysis of N-glycans from Ophiactis savignyi. Our initial hypothesis was that the N-glycome of an echinoderm would reflect the evolutionary position of this phylum and would thereby have aspects of both invertebrate and vertebrate glycosylation patterns. Indeed, this presumption, based also on older work especially on O-glycans and glycolipids, was more than confirmed in the case of the brittle star, O. savignyi, and a sea cucumber, Holothuria atra (as shown in our companion report). Previously, the occurrence of calcium-binding sulphated N-glycans potentially involved in sea urchin embryonic skeleton formation was postulated (29,30), but their exact structures were never defined, whereas the occurrence of classical oligomannosidic structures in echinoderms was confirmed in a mass spectrometric study on egg jelly glycans from the sea urchin Paracentrotus lividus (20). Therefore, based on our reading of the literature, our two studies are indeed the first in-depth N-glycomic analyses of any echinoderm species. As summarised in Figure 10A, in the brittle star we have found (i) complex and hybrid N-glycans featuring the type 1 antennal motif, (ii) a minor degree of the α1,3/α1,6-difucosylation core modification, (iii) glucosylated oligomannosidic structures with an unknown hexose cap and (iv) various anionic modifications, specifically sulphate, phosphate and sialic acid.

Invertebrate characteristics of both the brittle star and sea cucumber glycomes are reflected in the occurrence of some core difucosylated N-glycan structures of the type known from a wide range of insect, mollusc, trematode and nematode species (31). The modification of antennal GlcNAc residues with β1,3-linked galactose is also shared with oysters and insects (17,32-34), but LacdiNAc (GalNAcβ1,4GlcNAc; found in many invertebrates) was lacking (Figure 10B). While β1,3-galactose is also present in mammals, the typical vertebrate modification with β1,4-galactose is absent in the brittle star as are digalactose motifs, whether these be Galα1,3/4Gal or Galβ1,4Gal as found in many mammals, birds or fish (35-37), and histo-blood group motifs. In contrast, the sea cucumber H. atra has sulpho- and sialyl-Lewis A motifs (22). On the other hand, there are certainly triantennary N-glycans and a trace of tetra-antennary glycans indicative of the presence of a number of branching N-acetylglucosaminyltransferases, including GlcNAc-TIV (38), an enzyme also predicted to occur in cnidarians and some
nematodes as well as insects and vertebrates (Supplementary Figure 11). Interestingly, where there is only one Galβ1,3GlcNAc antenna, this is always on the α1,3-mannose, which is an indication that there is no dominant FDL-like hexosaminidase (25) removing the β1,2-linked GlcNAc from the lower arm as known from many invertebrates.

The high degree of ‘charged’ N-glycans in O. savignyi (estimated as 70%; as summarised in Figure 10A) results in a more ‘vertebrate-like’ characteristic, although we previously found approximately a 50% degree of N-glycan sulphation in oyster plasma (33). Indeed, the predominance of sialylation differentiates it from typical invertebrates, but the sialic acid modifications (methylation and sulphation) are different as compared to vertebrates. N-glycolyneuraminic acid is the most abundant sialic acid on N-glycans of the brittle star with either α2,3-sialylated β1,3-linked galactose residues or α2,6-sialylation of antennal GlcNAc. The latter modification is known from bovine fetuin (39) and a corresponding mammalian GlcNAc-6-sialyltransferase activity has been reported (40). Recently this ‘internal’ sialylated motif has also been found in mice brain and named ‘sialyl Lewis C’ (41). This epitope, which interacts with Siglec-H, has been found in developing mice brains and may mediate interactions between dendritic spines and microglia (42).

Although the glycoproteins and their binding partners still need to be identified in echinoderms, it is known that their neuronal systems do display similarities to those in chordates (43).

We have also examined O-glycans and find a number of simple structures in the brittle star, some of which are sialylated. The previous literature on echinoderm O-glycans is a bit richer than for N-glycans and di-, oligo- or polysialylated structures have been found in sea urchins. Specific examples include mucin-type O-glycans from sperm flagella containing terminal 8-O-sulphated α2,9-linked polyNeu5Ac (12,13) or other sperm and egg O-linked glycoproteins with α2,8-sialic acid and α2,5-sialic acid (14,15,44). Also, sialylated glycolipids from various echinoderms have been described (14,45-47). Thereby, a range of sialyltransferases should be present in echinoderms and indeed a number of α2,3-, α2,6- and α2,8-sialyltransferase homologues are encoded by the genome of the sea urchin Strongylocentrotus purpuratus (48-50). However, considering the lack of enzymatic characterisation of the various homologues in any echinoderm, it is difficult to predict which sialic acid linkages are theoretically possible in each species. Thus, we do not know whether our detection of only monosialylated motifs in the brittle star is either due to a lack of α2,5/6/8/9-sialyltransferases in the brittle star or to preparative/analytical factors.

In terms of types of sialic acid, it is remarkable that the NeuGc and its methylated form are the only sialic acid residues on the N-glycans of O. savignyi, while the O-glycans carry both NeuAc and NeuGc. As CMP-NeuGc biosynthesis requires the prior formation of CMP-NeuAc, an absence of NeuAc from N-glycans seems curious. However, the methylation and sulphation of NeuGc seems curious. However, the methylation and sulphation of NeuGc match with reports on starfish and sea cucumber O- and lipid-linked glycans (8,12,51-53), and two starfish enzymes required for the generation of methylated NeuGc (CMP-NeuAc hydroxylase and 8-O-methyltransferase) have already been identified (54,55).

O-acetylation of sialic acid is known from other echinoderms, but is notoriously difficult to detect due to instability and we did not define any acetylated structures, whereas sulphated sialic acid was found only on a small subset
of brittle star N-glycans. As NeuGc and acetylated NeuAc have been reported to be less easily cleaved by bacterial and viral sialidases than NeuAc (56-58), it can be speculated that the abundance of NeuGc or its methylated or sulphated forms on the N-glycans of O. savignyi may provide a first line of defence against pathogens. In terms of anionic modifications other than sialylation, sulphation and phosphorylation were detected; as mentioned above, there was some sulphation of sialic acid residues, but also sulphated galactose, GlcNAc and fucose were observed. Here there are parallels to the sulphation of galactose in oysters and of core fucose in insects (32,33). LC-MS suggested that the galactose residues are 4-O-sulphated, unlike the 3-O-sulphation more common in mammals; nevertheless, 4-O-sulpho-modifications of fucose and GalNAc are known as modifications of sea cucumber chondroitin chains (59) and 4-sulphated GalNAc is a component of mammalian chondroitin and dermatan sulphates (60). On the other hand, phosphorylation of GlcNAc was found, rather than of mannose as known from N-glycans of mammalian lysosomal enzymes or slime mould glycoproteins (61,62), but no zwitterionic modifications were detected (23).

Glycosylation is a species-specific process, which is especially reflected in the distinct set of glycans involved in the fertilization process (63). These differences were also reflected in our studies, as only low amounts of Man$_{1-3}$GlcNA$_{2}$-based paucimannosidic glycans and almost no pseudohybrid glycans (i.e., with an antenna only on the α1,6-mannose) were found in O. savignyi. Noteworthily, the anionic N-glycome of the sea cucumber H. atra is rather biased towards hybrid structures based on Man$_{5}$GlcNA$_{2}$, including sulphated/fucosylated, but more rarely sialylated, HexHexNAc motifs (22). Additionally, our preliminary data on two sea urchin and two starfish species, are each revealing a distinct set of N-glycans. This highlights that individual echinoderm species analysed have a quite different overall N-glycome, even though some motifs overlap. Thus, we conclude that the N-glycome of O. savignyi is unique as compared to other species, even when compared to that of H. atra. Indicative that there is a push to ‘final products’ of certain biosynthetic pathways is that the hybrid and complex glycans of this species are almost all sulphated and/or sialylated (accounting for some 70% of the N-glycome), leaving a neutral subset dominated by oligomannosidic structures, including some with unusual hexose modifications on the ‘lower arm’ triglucose motif. In general, excepting the presence of some core difucosylated structures, the O. savignyi N-glycome is probably nearer to those of vertebrates; further comparative studies will certainly show variations and similarities in the glyco-motifs associated with the emergence of the deuterostome lineage before the Cambrian era.

**Experimental Procedures**

**Biological material and glycan preparation:** The Brittle stars *Ophiactis savignyi* (Müller & Troschel, 1842) were sourced from the “Haus des Meeres - Aqua Terra Zoo”, a public aquarium in Vienna, Austria. There, upon first detection in 2013 they were already living in a total of four coral reef exhibition tanks pertaining to two independent seawater systems. Therefore, only gross reconstruction of origin and date of introduction appears possible: during the last decade all imports of coral reef materials came from the Central Indopacific Ecoregion (Philippines, Indonesia and NE-Australia) via a Dutch
wholesaler (DeJongMarinelife Inc., NL). The imports of live corals usually comprised small portions of foundation-rocks. This suggests that the *O. savignyi* founder population had hitch-hiked in shelters found in crevices of such rocks and that they then populated the open-celled polyurethane foam which served as the filter of the aquarium system. A regime of 10 h dim light / 14 h darkness prevailed in the service room. The inorganic composition of the seawater was routinely analyzed employing ICP-OES and IC-VWD/ECD methodology (Oceamo Inc., 1170 Vienna, Austria; Supplementary Table 3). Freshly collected animals were put in small Petri dishes for microphotography (see Figure 1 and Supplementary Figure 12).

10 g of starved brittle stars were hand-picked from aquarium substrates, washed once with deionized water and stored at -80°C. After thawing, the material was heat inactivated for 10 min in boiling water and lyophilized prior to grinding in liquid nitrogen. The powder was suspended in deionized water and the pH adjusted with 100 mM ammonium carbonate buffer to 8.2; CaCl₂ was added to a final concentration of 0.5 mM prior to addition of 10 mg thermolysin (Promega). Proteolysis was allowed to proceed for two hours at 70°C and 37°C over-night for completion. The glycopeptides were purified using standard laboratory protocols (64) prior to release with PNGase F (Roche) overnight at 37°C. After cation exchange chromatography on Dowex, the unbound material was subject to solid phase extraction on non-porous graphitised carbon (ENVIcarb, Supelco) and eluted with 40% acetonitrile or 40% acetonitrile with 0.1% TFA. These fractions were subject to a further solid phase extraction step on a C18 reversed phase resin (LiChroprep; Merck) and the glycans were eluted with water and with stepwise increases in the methanol (15%, 40%, 100% (v/v)) concentration. Glycan-containing fractions, as judged by MALDI-TOF MS, were fluorescently labelled with 2-aminopyridine. The remaining glycopeptides bound to the Dowex resin were gel filtrated (Sephadex G25) prior to incubation with PNGase Ar (recombinant NEB) overnight at 37°C to facilitate release of core α1,3-fucosylated glycans. The glycans released with this enzyme were then no longer bound by Dowex and were subject to the same purification and fluorescent-labelling steps as for the PNGase F released ones. The residual glycopeptides remaining after PNGase Ar digestion were subject to reductive β-elimination. As the animals were starved and no traces of algal glycans (which would be expected to contain pentose residues) were detected, no food contamination of the glycomes occurred.

**N-glycan fractionation:** Complete pyridyl-aminated N-glycomes were fractionated by reversed-phase HPLC (Kinetex 5µ RP-column, XB-C18 100A, 250 × 4.6 mm; Phenomenex®) using two different gradients at either pH 4 or pH 6. With buffer A (0.1 M ammonium acetate, pH 4) and buffer B (30% methanol), a gradient of up to 55% over 44 minutes was applied at a flow rate of 0.8 ml/min as follows: 0-30 min, 0-30% B; 30-35 min, 30-40% B; 35-40 min, 40-55% B; 40-44 min, 55% B; 44-50 min, return to 0% B. For higher sialic acid stability, the following gradient was used: buffer C (0.1 M ammonium formate, pH 6) and buffer B with a flow rate of 0.8 ml/min: 0-5 min, 0-10% B; 5-35 min, 10-40% B; 35-40 min, 40-50% B; 40-45 mins, 50-65% B; 45-49 min, hold at 65%; 49-51 min, return to starting conditions. The RP-HPLC column was calibrated daily in terms of glucose units.
using a pyridylaminated dextran hydrolysate. HIA-X-HPLC (IonPac AS11 column, Dionex, Sunnyvale, USA; 4 × 250 mm, combined with a 4 × 50 mm guard column) was used with a two solvent gradient (buffer D 0.8 M ammonium acetate, pH 3.85 and buffer E 80% acetonitrile LC-MS grade) at a flow rate of 1 ml/min as follows: 0-5 min, 99% E; 5-50 min, 90% E; 50-65 min, 80% E; 65-85 min, 75% E. The HIA-X-HPLC was calibrated using oligomannosidic PA-labelled bean glycans. All manually-collected HPLC glycan fractions were analysed after lyophilization by MALDI-TOF MS and MS/MS.

**Glycan mass spectrometry:** Monoisotopic MALDI-TOF MS was performed using an Autoflex Speed (Bruker Daltonics, Bremen) instrument in either positive or negative reflectron modes with 6-aza-2-thiothymidine (ATT) or 2,5-DHB (2,5-dihydroxybenzoic acid) as matrix. MS/MS was in general performed by laser-induced dissociation of the [M+H]⁺ or [M-H]⁻ ions (except for derivatized sialylated glycan masses detected as [M+Na]⁺ with 2,5-DHB as matrix); typically 2000 shots were summed for MS (reflector voltage, lens voltage and gain respectively 27 kV, 9 kV and 2217 V) and 4000 for MS/MS (reflector voltage, lift voltage and gain respectively 27 kV, 19 kV and 2174 V). 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 MS/MS (four-times smoothed). Glycan spectra were manually interpreted on the basis of the masses of the predicted component monosaccharides, differences of mass in glycan series, comparison with co-eluting structures from other insects, nematodes and other marine organisms and fragmentation patterns before and after chemical treatments or exoglycosidase digestions. A list of theoretical m/z values for each glycan composition is presented in **Supplementary Table 1**.

Selected RP-HPLC fractions of pyridylaminated N-glycans as well as O-glycans prepared by β-elimination were also analyzed by online LC-MS/MS using a 5 µm porous graphitised carbon column (10 cm × 150 µm, prepared in-house) coupled to an LTQ ion trap mass spectrometer (Thermo Scientific, Waltham, MA). Glycans were eluted using a linear gradient from 0-40% acetonitrile in 10 mM ammonium bicarbonate over 40 min at a flow rate of 10 µl/min. The eluted N-glycans were detected in negative-ion mode with an electrospray voltage of 3.5 kV, capillary voltage of -33.0 V and capillary temperature of 300°C. Specified ions were isolated for MSⁿ fragmentation by collision induced dissociation (CID) with the collision energy set to 30%. Air was used as a sheath gas and mass ranges were defined dependent on the specific structure to be analysed. The data were processed using the Xcalibur software (version 2.0.7, Thermo Scientific). Glycans were identified from their MS/MS spectra by manual annotation using the fragment nomenclature of Domon and Costello (65).

**Enzymatic and chemical treatments:** Aliquots of the isolated HPLC fractions were, based on results of HPLC elution and MALDI-TOF MS and MS/MS data, subject to targeted exoglycosidase digestion and chemical treatment. Either α-mannosidases (jack bean from Sigma, Aspergillus saitoi α1,2-specific from Prozyme, Xanthomonas α1,2/3-specific from NEB or Xanthomonas α1,6-specific from NEB), endo-α1,2-mannosidase (recombinant Bacteroides xylanisolvens BxGH99), α-fucosidases (bovine kidney α1,6-specific from Sigma), β-
galactosidases (recombinant *Aspergillus niger* or *A. oryzae*, prepared in-house, *Xanthomonas* β1,3-specific from NEB or *Bacillus fragilis* β1,4-specific from NEB), β-hexosaminidase (recombinant *Apis mellifera* FDL, prepared in-house, or native jack bean from Sigma) or α-sialidases (Sialidase V, S or A, Prozyme, from respectively *Vibrio cholerae*, *Streptococcus pneumoniae* or *Arthrobacter ureafaciens* or Neuraminidase, NEB, from *Clostridium perfringens*) were used for further treatment of the sample in 50 mM ammonium acetate, pH 5 (pH 6.5 with 5 mM CaCl₂ for sialidases and pH 5.5 with 5 mM CaCl₂ for β1,3-galactosidase), at 37°C for 24 or 48 hours (37°C and 1.5 hours for FDL). The FDL hexosaminidase is under these conditions specific for the β1,2-GlcNAc linked to the α1,3-mannose residue of the trimannosyl N-glycan core. For attempted digestion of unknown hexoses, β-mannosidase (*Helix pomatia* from Sigma, α-galactosidase (green coffee bean from Sigma) or α-glucosidases (*Trichoplusia ni* HighFive cell culture supernatant (66) or rice glucosidase from Sigma) were used. For the removal of phosphate groups, selected fractions were dried and incubated 48 hours at 0°C with 3 ul 48% (v/v) hydrofluoric acid prior to evaporation in a centrifugal concentrator. The samples were diluted in water and re-evaporated, before re-dissolving once again. Under these conditions N-glycolyneuraminic acids were also stabilized by the loss of a water molecule. Otherwise, shrimp alkaline phosphatase (Fermentas) was also employed to remove phosphate residues. As appropriate, treated glycans were re-chromatographed by RP-HPLC (pH 6 conditions) to ascertain retention time shifts prior to MALDI-TOF-MS; otherwise, an aliquot (generally one-fifth) of any digest was analysed by MALDI-TOF-MS without further purification.

**Linkage-specific sialic acid derivatization and mild acid hydrolysis:** Sialic acid specific derivatization was performed as previously described (67). Thereby around one-fifth of a sample was mixed with the carboxylic acid activator EDC (1-ethyl-3-(3-dimethylamino)-propyl)carbodiimide) together with the catalyst 1-hydroxybenzo-triazole (HOBt) as follows: a 0.5 M solution of EDC and HOBt in 100% (v/v) ethanol was added to the sample in a 1:1 ratio; incubation was performed for 1h at 37°C. After adding 100% (v/v) MeCN to a final concentration of around 85% (v/v) the reaction mixture was purified using a HILIC-SPE followed by MALDI-TOF analysis using 2,5-dihydroxybenzoic acid as matrix. Due to resistance of some sialic acid linkages towards sialidases, chemical hydrolysis was employed. Around one-fifth of a selected HPLC peak was mixed with 2M acetic acid and incubated for 2h at 80°C (68). To remove the acetic acid the samples were dried using a SpeedVac and washed twice by adding water and re-evaporation. The de-sialylated samples were directly analysed by MALDI-TOF-MS.

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**Conflict of interest**
The authors declare no conflicts of interest.

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Figure 1: HIAX-HPLC of the neutral PNGase F pool. Using hydrophilic interaction anionic exchange (HIAX) HPLC, which separates glycans due to size and charge resulted in detection of extended A-arm modified oligomannosidic structures, m/z 2609 – 3096 (Hex13HexNAc2–Hex16HexNAc2; for MS/MS see Supplementary Figure 3). The HIAX column was calibrated with a set of PA-labelled N-glycans derived from beans (H3X-H9, i.e., Man3GlcNAc2Xyl1-Man9GlcNAc2). The chromatogram is annotated with glycan structures (each with the positive mode m/z value for the protonated ion in order of abundance in each fraction with the most abundant uppermost) according to the Symbol Nomenclature for Glycans. Linkages of example glycans are shown in the inset; for further photographs of the organism, refer to Supplementary Figure 12. The simplified evolutionary tree of the Echinodermata and Chordata is based on Vaughn et al. (69).
Figure 2: Antennal motifs are based on β1,3-galactose as proven by glycosidase digests. The biantennary glycan (A) m/z 1865 (Hex5HexNAc4Fuc1) was sensitive to a β1,3-specific galactosidase (B) but resistant to a β1,4-specific galactosidase (C). Treatment of the hybrid glycan (F) m/z 1678 (Hex6HexNAc3) with the β1,3-galactosidase resulted in a loss of one galactose (G) whereas jack bean α-mannosidase slowly removed up to three mannose residues (H). The co-eluting Hex7HexNAc2 structure was resistant to the galactosidase but sensitive to the mannosidase. In the MS/MS spectra of the untreated (D and I) and galactosidase treated glycans (E and J), the losses of galactose (Δ162) are indicated by red lines, e.g. the shifts in B-ions from m/z 366 (Hex1HexNAc1) to m/z 204 (HexNAc1), as well as in Y-ions from m/z 1338 (Hex3HexNAc3Fuc1) or m/z 1192 (Hex3HexNAc3) to ones at m/z 1176 (Hex2HexNAc3Fuc1) or m/z 1030 (Hex2HexNAc3).
Figure 3: Example of enzymatic digests of A-arm elongated N-glycan structures. Both glycans m/z 2447 (Hex$_{12}$HexNAc$_2$) A and m/z 2610 (Hex$_{13}$HexNAc$_2$) G (MS/MS in D and J and Supp. Figure 3 D/E) are sensitive to *B. xylanisolvens* endo-α-mannosidase (B/H) cleaving the glucose substituted α1,2-mannose of the A-arm, thereby releasing four to five hexoses; which is reflected by the shift of the diagnostic fragments from m/z 1637/1799 (Hex$_7$-8HexNAc$_2$) to m/z 989 (Hex$_3$HexNAc$_2$) as shown in the MS/MS of the digest products in E and K. On the other hand an α-mannosidase removed from both structures up to five mannoses from the B- and C-arm, resulting in m/z 1637 or m/z 1799 (C and I) with a dominant m/z 665 fragment as shown in the MS/MS (F and L).
Figure 4: RP-HPLC fractionation of anionic N-glycans. For the first dimension anionic enriched N-glycans were separated on a Kinetex C18 RP-HPLC (pH 6; calibrated in terms of glucose units, g.u.). The colour code and letters indicate pooled peaks whose second dimension HIAX-HPLC profiles are shown in Supplementary Figure 5 A-K. Fractions are annotated with example glycans according to the Symbol Nomenclature for Glycans (whereby Me and S represent respectively methyl and sulphate). The illustrated structures are based on a combination of MS/MS analysis, RP- and HIAX-HPLC elution times as well as enzymatic and/or chemical treatments. Due to the separation properties of the RP-column used as the first dimension, multiple sulphated structures are found in A, modified hybrid structures in B, multiple sialylated bi-antennary structures in C-E, whereas those with methylated N-glycolyneuraminic acid and multi-antennary structures are detected in G-K. The 4 g.u. RP-HPLC peak not subject to 2D-HPLC contains a non-digestable glycan not related to other analysed structures.
Figure 5: Analysis of monosulphated and disulphated N-glycans. Two isomeric sulphated N-glycans (m/z 1943, Hex3HexNAc2Fuc1S1; A/F) separated by 2D-HPLC G-26 min/I-31.2 min (see Figure 4 and Supplementary Figure 5) with key diagnostic fragments of either m/z 225 (Fuc1S1) and 727 (HexNAc2Fuc1S1-PA; K) or m/z 282 (HexNAc1S1) and 444 (Hex1HexNAc1S1; N), were subject to various
exoglycosidase digests. The first isomer was sequentially sensitive to β1,3-galactosidase (B), jack bean β-hexosaminidase (D), jack bean α-mannosidase (E) but resistant to bovine fucosidase (C) thereby confirming the sulphate position on the core fucose. In the case of the second isomer, β1,3-galactosidase removed two galactoses (G), but jack bean β-hexosaminidase only one GlcNAc (H), while the A-arm specific FDL removed none (I) in accordance with the key fragment indicative of sulphate linked to an antennal GlcNAc residue. MS/MS analysis of monosulphated N-glycans (K-N) as [M-H]⁻ or disulphated N-glycans as [M-SO₃]⁻ and [M-2H+Na]⁻ (O/P), yielded fragments indicative of sulphation of either core fucose (m/z 225/524/727, K/M), terminal galactose (m/z 241, L/O), subterminal GlcNAc (m/z 282, N/P); or with the B-ion at m/z 545 indicative of double sulphate substitution of the Galβ1,3GlcNAc motif (P). Fragment ions at m/z 444 and 606 are indicative of sulphation on Gal₁GlcNAc₁Man₄₋₅₁ antennae. For a disulphated hybrid structure, m/z 241, 282, 443 and 545 fragments (Hex₁S, HexNAc₁S and Hex₁HexNAc₁S₂₋₃; O and P) were observed when performing MS/MS on either the [M-2H+Na]⁻ parent or the [M-SO₃]⁻ ion resulting from in-source-loss of one sulphate; actually it appeared that sulphation was most commonly of antennal GlcNAc. LC-ESI-MS³ of a disulphated glycan (Q; Hex₁HexNAc₁Fuc₁S₂) at m/z 828.97 [M-2H]^⁻² indicates sulphate on terminal galactose and subterminal GlcNAc, respectively; some of the singly-charged fragments were also observed in MALDI-TOF-MS/MS, while others are doubly-charged and are indicated in blue with (-2). MS³ of fragment ions at m/z 241 (GalS, inset) resulted in the observation of ions at m/z 181 indicative of 4-sulphation of galactose.

**Figure 6:** Identification of phosphorylated N-glycans. A-E: Positive mode MALDI-TOF MS/MS of hybrid and bi-antennary phosphorylated glycan structures of *O. savignyi*. The diagnostic fragment ion at m/z 284 is compatible with a phosphate substitution on the terminal GlcNAc. Note that the fragment at m/z 446 (in A/B), which refers to the B-ion P-GlcNAc-Man, is isobaric to the Y-ion for Fuc-GlcNAc-PA (see C-E). F-I: The phosphorylated structures at m/z 1272 and 1434 in positive ion mode (m/z 1270/1432 negative ion mode, see inset) were both sensitive to hydrofluoric acid treatment (G) whereby the loss of 80 Da demonstrates the presence of a phosphate. After dephosphorylation, jack bean β-hexosaminidase was able to remove the GlcNAc residues (H) in contrast to the resistance before treatment (data not shown) thus proving the position of the phosphate group on the antennal GlcNAc; (I) the susceptibility to jack bean α-mannosidase proved the hybrid nature of the m/z 1272 and 1434 glycans.
Figure 7: MS/MS analysis of sialylated N-glycans. (A-D) Example positive ion mode MALDI-TOF MS/MS of N-glycans modified with either N-glycolylneuraminic acid (A) or its methylated form (C) with the diagnostic B- (m/z 673 or 687 for NeuGc, Me0; Hex; HexNAc) and Y- ions depicted. Negative ion mode MS/MS of these glycans resulted in diagnostic fragments at m/z 306 (B) or 320 (D) for NeuGc or NeuGcMe. Since the sialic acids are easily lost in the MS/MS experiments, there was no obvious difference in fragmentation pattern between glycans with a NeuGc or NeuGcMe on either terminal galactose or internal GlcNAc; however, enzymatic digests enabled these to be distinguished (see Figure 8 and Supplementary Figure 7). (E) The negative CID ESI-MS/MS of a disialylated glycan (Hex4HexNAc3Fuc1NeuGc2 at m/z 1056.27 [M-2H]2-) indicates one NeuGc residue linked to subterminal GlcNAc (fragment ions at m/z 509); the fragment ions at m/z 937 are concluded to be due to \( \text{X} \) cleavage of NeuGc (indicated in red), which is diagnostic for 2,6-linked sialic acid. (F and G) Positive and negative mode MALDI-TOF MS of the Hex4HexNAc3Fuc1NeuGc2 glycan showing also the m/z 978/980 B-fragments indicative of the sialyl Lewis C motif (NeuGc 2Gal1GlcNAc). (H-M) The two monosulphated isomers of m/z 2250 as [M-H] eluting at 2D-HIAX E-50.5 min and F-51 min (H and K) with MS/MS in J/M indicating a sulphate linked either to antennal GlcNAc (m/z 282) or to the NeuGc (m/z 386) were subject to a 2,3-specific sialidase S which either removed m/z 307 or 386 (NeuGc or its sulphated version I and L) resulting in either a still charged structure with a sulphate at m/z 1943 as [M-H] or a neutral glycan at m/z 1865 as [M+H]. The loss of 45 Da in MS as well as the m/z 341 MS/MS fragment could be the result of decarboxylation of sialic acid (70).
Figure 8: Structural elucidation of four monosialylated isomeric Hex,HexNAc,Fuc,NeuGc, N-glycans. Four 2D-HPLC separated 2171 Da isomers (H-33min/I-35 min/G-33min/H-34min in A/F/Q/V) only
showed subtle differences in their MS/MS fragmentation patterns (E/J/U/Z). In case of the isomers with internal NeuGc, \( \beta \)1,3-galactosidase was able to cleave two residues (B/G), as compared to one galactose for those with one terminal sialic acid residue (R/W); only after the \( \beta \)1,3-galactosidase digest the key fragment for the NeuGc-HexNAc modification, m/z 511 (see inset at B/C and G/H) could be detected. The four isomers also differentiated in their susceptibility to the \( \alpha \)2,3-sialidase S, which only cleaves off terminal NeuGc (S/Y), whereas internal NeuGc was removed with acid hydrolysis (D/I). In order to determine which arms were sialylated, two of the m/z 1338 digest products were reinjected on the HPLC (K/L), and co-eluted with two standards A and B, which also showed different key fragment ions at m/z 1135 as in standard A and digest product H-33 min and m/z 1176 as in standard B and digest product I-35 min (M-P); the other two m/z 2172 structures were subject to lower arm specific FDL hexosaminidase (T/X), whereby the respective resistance or sensitivity proves substitution of the lower or upper arm.

![Figure 9: Impact of mild acid and hydrofluoric acid treatments on sialic acids. (A-C) Incubation of an aliquot of the complete anionic N-glycan pool with 2 M acetic acid removed all sialic acids resulting in residual neutral and sulphated structures. Thereby sulphated tri and tetra-antennary glycans were detected, as verified by the MS/MS analyses represented in the insets. (D) An aliquot of the complete anionic enriched N-glycan pool from *O. savignyi* was incubated with 48% Hydrofluoric acid for 48h at 4°C, facilitating the observation of larger glycan structures. The lactonization (\( \Delta \)18) of sialylated N-glycans seemingly stabilized the structures, thereby changing the mass difference from \( \Delta \)307 (N-glycolyneuraminic acid) to \( \Delta \)289. For MS/MS data refer to Supplementary Figures 8 and 9.](http://www.jbc.org/)
Figure 10: Summary of glycoepitopes and their abundance in the N-glycome of the brittle star *Ophiactis savignyi*. (A) Based on fluorescence intensities, some 30% of the N-glycans are neutral and 70% carry an anionic moiety. Within these classes, some 92% of the neutral N-glycans are classical oligomannosidic Man$_5$-GlcNAc$_2$ structures, but also neutral glycans with extra glucose and unknown hexose residues are present, in addition to neutral core α1,3-fucosylated, hybrid and biantennary glycans; antennal galactosylation was solely detected in a β1,3-linkage. Within the acidic N-glycan pool, sialylation (solely N-glycolylneuraminic acid) is the major anionic modification with up to three sialylated antennae being detected; 20% of the sialylated structures are disialylated (one on Gal, one on GlcNAc) to result in “sialyl Lewis C” epitopes (see grey box), 5% of the sialylated structures are also sulphated and 20% of the NeuGc residues are methylated; glycans with just sulphation are less abundant and those displaying phosphorylation account for just 1% of the acidic pool. (B) A simplified glyco-evolutionary scheme showing the occurrence of selected antennal elements in protostomes and deuterostomes, whereby the phylogeny is adapted from Vaughn et al. (69); depicted are Lewis X, sulphated blood group A, glucuronylated LacdiNac (also modified by phosphorylcholine), glucuronylated/sulphated type I, sulphated Lewis A, NeuGcMe-modified type I and NeuAc-modified type II antennae from respectively *Schistosoma mansoni*, *Crassostrea virginica*, *Dirofilaria immitis*, *Anopheles gambiae*, *Holothuria atra*, *O. savignyi* and *H. sapiens* (17,22). Note that anionic N-glycans are unknown in *S. mansoni*, but that *D. immitis* is the only nematode for which glucuronylated N-glycans have been proven.
Glycosylation at an evolutionary nexus: the brittle star *Ophiactis savignyi* expresses both vertebrate and invertebrate N-glycomic features

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