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Review

Fucosylated chondroitin sulfate diversity in sea cucumbers: A review

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ABSTRACT

Fucosylated chondroitin sulfate (FucCS) is structurally distinct glycosaminoglycans found from the sea cucumber body wall consisted of chondroitin sulfate type backbone with attached sulfated or non-sulfated fucose side chain. Structurally this compound plays an important role in maintaining the body wall integrity and possesses a wide spectrum of biological activities. Recently several glycosaminoglycans' structures have been solved to elucidate its physicochemical activity. The purpose of this review paper is to elaborate existing structural properties and functions, reporting over 30 years and systematically discussion herein.

Contents

1. Introduction

The compound fucosylated chondroitin sulfate (FuCS) is a characteristic compound none other than the echinoderm sea cucumber (Yamada, Sugahara, & Ozbek, 2011). Structurally, holothurian glycosaminoglycan is composed of a common polysaccharide found in mammalian chondroitin sulfates but the uronic acid residue has an unusual sulfated fucosyl branch *O*-linking to its carbon-3 (Fig. 1) (Vieira & Mourão, 1988). This holothurian glycosaminoglycan has demonstrated various biological activities such as antithrombotic, antitumor and anticancer, immunostimulatory, wound healing, artherosclerosis prophylaxis and apheresis, hypolipidemic, renal fibrosis attenuation, anti-hyperglycemia, angiogenesis, antibacterial, and antiviral (Bastos et al., 2014; Hu et al., 2013; Liu, Ko, & Hu, 2002; Masre, Yip, Sirajudeen, & Farid, 2010; Melo-Filho et al., 2010; Patar, Jaafar, Syed-Mohsin, & Abdullah, 2012; Patar, Syed-Mohsin, Jaafar, & Abdullah, 2012; Yang, Shou, Lian, Xiao, & Fu, 2013; Zhang et al., 2009). Moreover, the compound has showed superiority in terms of biological activities over its comparisons with known established therapeutics (Pomin, 2014). Many of these activities are the result of the anticoagulant properties.

To date, others known source of glycosaminoglycan are mammalian glycosaminoglycans such as dermatan sulfate, keratan sulfate, heparin, and chondroitin sulfate while known source of fucans are from the marine algae bearing fucoidans. The unique of this compound is begun to fetch interest on other sea cucumber species. Subsequently such compound is isolated from *Ludwigothurea grisea* two decades ago. This unusual sulfated glycosaminoglycan is attached to collagen fibrils in the extra cellular matrix (ECM) of the body wall but the biological relevance of this compound is still unclear (Mourão, 2007). The presence of fucose

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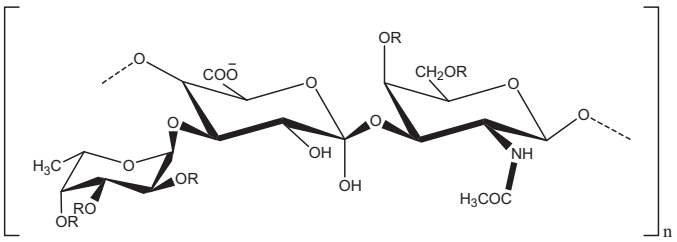

Fig. 1. The structural motif of fucosylated chondroitin sulfate where R represents sulfate esters (Pomin, 2014).

side chain is probably to avoid digestion of the polysaccharide by chondroitinase excreted by marine microorganisms. It is also suggested that the highly sulfate polysaccharide which increases the charge density is required to function in marine environment. Based on studies of the mutability of holothurian connective tissue, proteoglycans on the surface of collagenous fibres dictates the physical state (i.e., stiff or compliant). These proteoglycans are responsible in the linking and unlinking of long, elastic collagen running throughout the body wall.

As far as we know, there is lacking in comprehensive review article discussing on the compound's structural variability. This article aims to review the diversity of fucosylated chondroitin sulfate by focusing on structural conformation of different sea cucumber species.

2. Structural diversity

There have been relatively few studies on characterizing the molecular structure of the sulfated polysaccharide base on biological activities. The structure of FuCS is slowly developed adjunct to modern spectroscopic techniques development. Among the reports, FuCS from sea cucumber species has been demonstrated by methylation and NMR analysis (Kariya, Watabe, Hashimoto, & Yoshida, 1990; Vieira & Mourão, 1988; Yoshida & Minami, 1992). In general, the molecular structure from these studies complement one another where the three parts of FuCS: uronic acid, amine sugar and fucose are heterogeneously sulfated and the backbone is very similar to mammalian chondroitin sulfate (CS) where the addition of α-L-fucopyranose (Fuc) branches can be found attached up to several position on the backbone (β-D-glucuronic acid (GlcAβ) and β-D-galactosamine (GalNAcβ)). In addition, these fucose branches have distinguishable sulfation pattern clustering along the CS chain (Mourão et al., 1996) and sulfation patterns of the fucans are generally at carbon-2, 3 and 4 although some reported existence of fucosyl-fucosyl glycosidic linkage type α(1–2, 1–3 or 1–4) and the glycosidic linkage of these fucans to the CS backbone is also at the carbon 3 of glucuronic acid. To date, the preponderance chemical structure of several species acquires a degree of structural variability by extensive modifications involving sulfation and branch component (Fig. 2).

Base on the monosaccharide composition from each species, existing literature showed almost same proportion between D-glucosamine and D-glucuronic acid but the sulfate and fucose ratio have larger changes. As noted by Zhao et al. (2012), the difference in monosaccharide component does not restrict to different species but also due to extraction method. In addition, Chen et al. (2011) had discussed seasonal and habitat structure might influence variation of FuCS. In Table 1 emphasizes on monosaccharide ratio of known references. When these data are collected, curiously, all extractions came from the same tissue origins even in same species. Based on what was known in classification of glycoconjugates of vertebrate origin, there should be investigation on other tissue especially the visceral part, which is often discarded. The monosaccharide composition isolate from cuvierian tubule of *Holothuria forskali* from the works of Isemura, Zahn, and Schmid (1973) and Van der Meer, Kamerling, Vliegenthart, Schmid, and Schauer (1983) showed very similar profile compared to the monosaccharide composition of FCF-3 from *Cucumaria frondosa* body wall reported by Kale et al. (2013). Perhaps one might find different composition of glycosaminoglycan from other tissue in future. The difference in monosaccharide composition also tells us the possibility of species having specific mixture of congeners. *Pearsonothuria graeffei*, another species recently explored for the same compound appears to have similar monosaccharide ratio from the same group of researcher utilizing the same extraction protocol (Chen et al., 2011; Wu, Huang, et al., 2012).

2.1. FuCS backbone and fucose branch structural characterizations

In the works of Vieira and Mourão (1988), holothurian glycosaminoglycan studies have been using mild acid hydrolysis, enzymatic degradation, methylation analysis, and NMR spectroscopy to elucidate the structure. In order to the backbone structure study, mild acid hydrolysis is applied to remove the fucose branch obstructing chondroitinase ABC and AC digestion (Mourão et al., 1996; Vieira & Mourão, 1988; Vieira, Mulloy, & Mourão, 1991). Indeed the groups have reported disparity fractions of the polysaccharide and the FuCS structure is gradually corrected from the group's review studies. Mourão et al. (1996) found that mild acid treatment removed fucose branch at the non-reducing terminal and thus cleaved by chondroitin lyase while the fucose branch from the reducing terminal is only partially removed. Interestingly, the sulfation pattern of the fucose released between non-reducing terminal and reducing terminal are distinguishable. ^{1}H NMR spectroscopy of Mourão et al. (1996) have revealed the anomeric protons of the fucose residue (belonging to the reducing region) signal at 5.22 ppm is still present in the acid hydrolysis and chondroitin lyase digestion fractions while the signal of the released fucose portions at 5.60 ppm is become absent in their time-course NMR scans of acid resistant fragments and chondroitin lyase resistant fragments. Fucose released from the non-reducing terminal consist of monosulfate (49% is 4-*O*-sulfated) and disulfate (20% is 2,3-*O*-disulfated and 17% is 3,4-*O*-disulfated) while fucose from reducing terminal exhibit possibility of disaccharide units of fucosyl-fucosyl linked either 1–4 or 1–2 and also these fucose are likely be sulfated at the 4-*O*-position.

Four possible sulfated species of the galactosamine (GalNAcβ) moiety: nonsulfated GalNAc (31%), GalNAc4S (4%), GalNAc6S (53%) and GalNAc-4/6diS (12%) were yield from chondroitin lyase treatment (Vieira & Mourão, 1988; Vieira et al., 1991). The FuCS structure elucidated by the team is generalized by having the fucose branch is mainly attached to glucuronic acid residue and others at position 4 and/or 6 of the galactosamine residue. Released fucose branch from the non-reducing region was from the carbon 4 and/or 6 of the GalNAcβ. As for the glucuronic acid moiety, large amount of sulfated and nonsulfated fucose is attached to the carbon 3 of the GlcAβ (Mourão et al., 1996). A residue of 3-sulfo-β-D-glucuronosyl (GlcA3S) is suggested in the reducing region from the positive immunoreaction using the specific leu-7 monoclonal antibody (specifically recognizes 3-sulfoglucuronic acid residues).

The notion of mild acid hydrolysis which expects only cleave the branch linkage connecting to the CS core later was corrected. Intriguingly, mild acid hydrolysis treatments even cleave the glycosidic bond of a single galactosamine moiety (Mourão et al., 1996). The cleaving of this linkage separates the non-reducing and reducing fractions which the non-reducing fractions are subsequently defucosylated since glycosidic linkage of the fucan branch is more labile to mineral acid degradation. The reducing fractions still

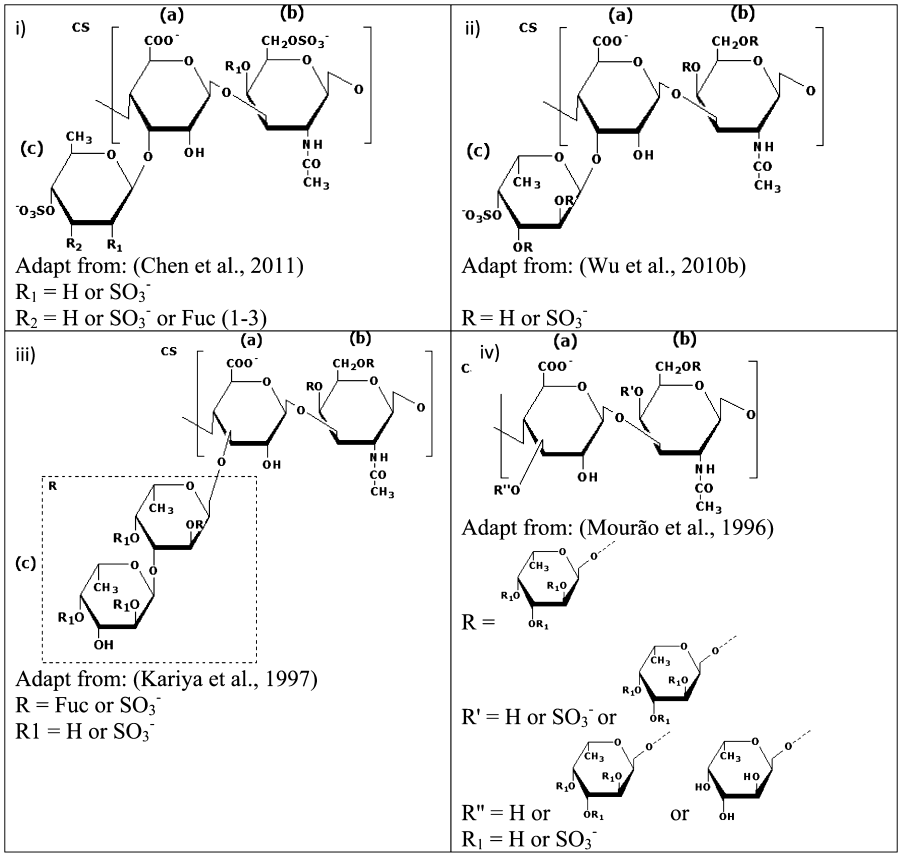

Fig. 2. Preponderance structure of fucosylated chondroitin sulfate; (a) β-D-glucuronic acid, GlcAβ. (b) N-acetyl-β-D-galactosamine, GalNAcβ. (c) The fucose branch, α-L-fucose.

Table 1
Monosaccharide components of FuCS and their sulfate groups of several sea cucumber body walls.

Sea cucumber species	Chemical components (molar ratio) U:N:F:-OSO3-	References
Stichopus japonicus	1:0.88:0.85:3.68	Fan, Chen, and Lin (1980)
	1:1.14:1.06:4.56	Yoshida and Minami (1992)
	1:0.85:2.36:4.63	Kariya et al. (1990)
	1:1.19:2.83:4.39	Kariya, Sakai, Kaneko, Kiyoshi, and Kyogashima (2002)
	1:1:1:4.7	Fan et al. (1983)
	1:1.29:0.18:1.86	Liu et al. (2012)
Stichopus variegatus	1:0.83:1.07:3.82	Chan, Fan, and Xing (1994)
Holothuria leucospilota	1:1.06:0.89:3.83	Fan et al. (1983)
	1:1.04:0.81:2.06	Li, Zhou, Cai, and Zhang (1999)
Holothuria atra	1:0.87:0.69:2.35	Tang, Qiu, Li, Su, and Yan (1999)
Holothuria scabra	1:0.78:0.53:1.34	Chen, Zheng, Xiao, and Lin (2006)
Ludwigothurea grisea	1:0.85:1.85:2.04	Mourão and Bastos (1987)
	1:1.10:1.07:2.93	Vieira and Mourão (1988)
	1:1.30:1.03:2.93	Vieira, Mulloy, and Mourão (1991)
	1:0.92:1.23:2.21	Mourão et al. (1996)
	1:1.1:1.07:2.93	Landeira-Fernandez et al. (2000)
Pearsonothuria graeffei	1:0.8:1.5:2.6	Chen et al. (2011)
	1:0.8:1.3:2.5	Wu, Ye, et al. (2012)
Holothuria vagabunda	1:1.1:0.9:2.7	Chen et al. (2011)
Stichopus tremulus	1:0.8:1.2:3.0	Chen et al. (2011)
Isostichopus badionotus	1:0.7:0.9:3.1	Chen et al. (2011)
	1:0.71.2:ND	Chen, Li, Ye, and Xue (2012)
Thelenota ananas	1:1.09:1.12:3.83	Wu et al. (2010a)
Holothuria nobilis	1:0.96:0.82:2.99	Luo et al. (2013)
Holothuria edulis	1:1.28:0.82:3.5	Luo et al. (2013)
Holothuria (Metriatyla) scabra	1:1.07:0.33:ND (expressed in ug/mg)	Liu, Ko and Hu (2002)
Cucumaria frondosa	1:7.78:9.35:9.32 (expressed as % in molar ratio)	Kale et al. (2013)
Athyonidium chilensis	1:1.1:1.1:2.08 (sulfate expressed as 32.9%)	Matsuhiro, Osorio-Roman, and Torres (2012)
Acaudina molpadioidea	ND:2:1:21.6% (sulfate expressed as 21.6%)	Ye, Xu, and Li (2012)

containing the fucan branch was not digested by chondroitin ABC or AC lyase and was referred to as the chondroitin lyase-resistant fragments. Generally, the depolymerization of this compound after defucosylation produces disaccharides, oligosaccharides and the chondroitin lyase-resistant fragments with the later having lower proportion.

The FuCS structure elucidated from *Stichopus japonicus* is different from *L. grisea*. Classical studies for *S. japonicus* utilized the transformation of fucosylated chondroitin sulfate into its sugar alditol configuration and the structure was eluted through gas chromatography tandem with mass spectrometer (GCMS). Kariya et al. (1990) adopted the mild acid treatment and chondroitinases plus sulfatases like that in Vieira and Mourão (1988) works and found chondroitin (11.2%), chondroitin-4-sulfate (10.4%), chondroitin-6-sulfate (56%) and chondroitin sulfate E (22.4%). Later on the same group found about 20% of fucans were linked through the 3-*O* position of the GlcAβ while the remaining 80% of fucose branch were intact at carbon 4 and/or 6 in the GalNAcβ moiety; In the 80% GalNAc moiety, 60% have fucose attached on the 4-*O* while its 6-*O* is sulfated; 10% are 4-*O*-sulfated while 6-*O* is fucan attached and; 10% are both 4-*O* and 6-*O* fucosylated. Moreover, the group's interpretation on the formation of 2,3,4-tri-*O*-methyl-fucitol and 2,4-di-*O*-methyl-fucitol in equimolar approximate indicates the glycosidic linkage of the fucose branch containing two fucopyranosyl is linked α(1–3) (Kariya, Watabe, Kyogashima, Ishihara, & Ishii, 1997). Cleaving on the α(1–3) glycosidic bond in this fucan yield several types of partially permethylated alditol acetates (PMAA). The inner fucopyranosyl will either yield non-sulfate or 2,4-disulfate type while the outer fucopyranosyl yield four types: 2,3,4-trisulfated, 2,3-disulfate, 3,4-disulfate and 3,4-monosulfate. The fucosylation pattern on the disaccharide core reflects the percentage of sulfation of CS types and the team also deduced the disaccharide core is of type CS E if the 3-*O* position of the GlcAβ is fucosylated. Apart from the findings of Kariya's team on the *S. japonicus* FGAG structure, Yoshida and Minami (1992) further reported three types of sulfated fucans patterns (Type 1: 2,4-disulfate, Type 2: 3,4-disulfate, Type 3: 4-*O* monosulfate) with molar ratio of 5:3:1 exist in the 3-*O* position of GlcAβ moiety.

In Kariya et al. (1990) study, the use of milder condition in the hydrolysis treatment (0.1 N H_2SO_4 at 80 °C) showed lower sulfate loss from intact fucose branch as compared to the mild acid treatment from that reported in Vieira and Mourão (1988). They have found two processes of hydrolytic reaction, one releasing sulfate plus fucose is eluted rapidly and the other release fucose only is slower. The rapid eluted fractions have approximately two times the fucose content of the slower fraction. This had prompt by Kariya et al. (1990) to suggest the fucose from rapid eluted fractions are sulfated and is a disaccharide unit compared to the slower fraction which is a non-sulfated fucose.

Wu, Ye, et al. (2012) attempted to elucidate quite similar structure of low molecular weight GAGs from *Thelenota ananas* using free-radical depolymerization. The chemical compositions of free-radically depolymerized holothurian glycosaminoglycans (DHG) remain almost identical to intact GAG by comparing their ^{13}C and ^{1}H NMR spectra (Wu, Xu, Zhao, Kang, & Ding, 2010a). Furthermore, HPLC analysis of enzymatic digestion on the native polysaccharide had elucidate four separate peaks of unsaturated disaccharide. This then concluded that the backbone of the *T. ananas* GAG is composed of 27.8% α-ΔUA-1→3-GalNAc, 56.5% α-ΔUA-1→3-GalNAc(6SO_4), 9.7% α-ΔUA-1→3-GalNAc (4SO_4) and 6% α-ΔUA-1→3-GalNAc (4,6-diSO_4) (Wu, Xu, Zhao, Kang, & Ding, 2010b). Physicochemical of the fucose branch was reported differ from species to species. The molar ratio for types of fucose branch found in *T. ananas* is 25:22:53 for 3-monosulfate, 4-monosulfate and 2,4-disulfate fucose unit respectively and the latter confers highest anticoagulant activities among than the other two type (Wu, Ye, et al., 2012). Despite the physicochemical potential of FuCS residue bearing 2,4-disulfated fucose is known, specific backbone structure bearing 2,4-disulfate fucose branch is not clearly informed by some authors (Chen et al., 2011; Wu, Huang, et al., 2012; Yoshida & Minami, 1992).

In order to better understand this compound's physicochemical function, Gao et al. (2012) have studied the hydroxyl group role in anticoagulant activity by preparing *O*-acylated derivatives. Accordingly, the depolymerized FuCS is homogeneous with an average 2.5 hydroxyl group per trisaccharide monomer. Their result showed that propionoylation with increased temperature and double volume of propionic anhydride (60 °C, 0.4 ml) afforded 95% derivatization compare to lower parameter condition. Nevertheless, the group deduced the formation of partial acylated products was due to the conformation of FuCS bearing heavy sulfate esters creating steric hindrance to substitution agent and also requirement of higher energy barrier by breaking a hydrogen bond formed between sulfate esters and adjacent hydroxyl groups. Based on NMR spectra of both partial acylated and 95% acylated FuCS, preferential acylation on the fucose side chain is more obvious than the CS backbone. Moreover, partially acylated product has showing preferential acylation on the 2-OH of type α-L-Fuc4S whereas 4-OH of type α-L-Fuc2S. Since there are no acylation on the 3-OH position, this partial acylation pattern justifies the steric influence of sulfate esters on either sides exerted on the 3-OH in the α-L-Fucose. In other words, the repulsion forces of sulfate esters shield the 3-OH from the acylating agent and thus attack the consecutive hydroxyl group. In addition, the preferential acylation is more on α-L-Fuc4S than α-L-Fuc2S. This is possible since 4-(*N*,*N*-dimethylamino) pyridine (DMAP) is known to exhibit regioselective acylation in carbohydrate analysis (Kurahashi, Mizutani, & Yoshida, 2002). Another possible explanation is the covalent bond energy of 2-OH position nearer to the anomeric carbon is easier to cleave compared to the 4-OH.

The complex structure of FuCS produced overlapping NMR ^{1}H and ^{13}C spectra, making it difficult to correctly assign the residue structure. Existing data using ^{1}H spectra from different sea cucumber species are showing α-L-fucopyranosyl signals envelopes at the 5.0–5.7 ppm region. Apparently, distinct signals of the anomeric proton for sulfated fucan residues are found between 5.1 and 5.6 ppm but those signals are overlapping among other residue making it difficult to assign the structure (Mourão et al., 1996). The three major sulfate fucans: 2,4-disulfate fucose, 3,4-disulfate fucose and 4-monosulfate can be distinguished from other fucose residue with the anomeric protons falling at 5.66–5.69 ppm, 5.31–5.35 ppm, and 5.38–5.43 ppm, respectively. Anomeric proton shifts of other minor sulfated fucans, such as 3-monosulfate, 2-monosulfate and non-sulfate fucose are usually recorded at 5.24–5.29 ppm, 5.49 ppm and 5.20–5.22 ppm. The methyl protons from sulfated α-L-Fuc residues are readily assigned between 1.2 and 1.3 ppm as compared to the $COCH_3$ from the GalNAcβ reciting at 2.0–2.1 ppm. Nevertheless, different sulfation patterns of fucose residue affect the chemical shifts of the methyl protons on fucose residue and $COCH_3$ on GalNAcβ (Chen et al., 2011). However, when the compound is defucosylated, one may easily distinguish the chondroitin sulfate type (Fan, Chen, Lu, Hao, & Li, 1983). Sulfation pattern on the galactosamine carbon-4 or carbon-6 are usually detected in the 845 cm^{-1} and 820 cm^{-1} region.

Chen et al. (2011) isolated quite similar molecules from four sea cucumber *Pearsonothuria graeffei*, *Stichopus tremulus*, *Holothuria vagabunda*, and *Isostichopus badionotus* of different geographical origins. The sulfation patterns on the fucose branch were different proportionally among these species and that the backbone is dominantly 4,6-*O* disulfated (CS E). With a considerable number of literatures suggesting CS E found in these holothurians may be the species characteristic particular class Holothuroidea (Kariya et al., 1990; Vieira et al., 1991; Yamada et al., 2011). It is still unclear if

this CS E is a characteristic found in holothurian since this compound is obtained through different methods (Zhao et al., 2012). Moreover, less information is made on studying the geographical difference and habitat preference relating to glycosaminoglycans to reach this conclusion (Chen et al., 2011). As for the types of fucose branch has found, 2,4-*O*-disulfate confers the highest anticoagulant activity and is believed to be common occurring in temperate sea cucumber species consistent from previous literatures (Chen et al., 2011; Kariya et al., 1997) but Wu, Ye, et al. (2012) reported high content of 2,4-*O*-disulfate from *Thelenota ananas* and is responsible to high anticoagulant activity.

3. Conclusion

The molecular structure of sea cucumber has been said to account for its different physicochemical ever since its first discovery more than two decades ago. However, there are only a few species have thorough FuCS profile while the same structure from other newly explored species are only partially characterized. Indeed, Pomin (2014) collected multitude of reference on the structural related medical effects and stressed that structural characterization cannot be done in a single work. Nevertheless, the wonders of fucose branch when attached to the common chondroitin sulfate core are demonstrated. One of the useful knowledge on sulfation patterns on the fucose branch among species is distinct, garners the possibility for new therapeutics. Moreover, many recent studies have described specific congeners of FuCS can be assigned as a valuable chemotaxonomic character for holothurians. In the future, one should expect additional FuCS from other body compartment of the many holothurians using contemporary techniques.

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