Conference paper

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New insight on the structural diversity of holothurian fucosylated chondroitin sulfates

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Abstract: Fucosylated chondroitin sulfates (FCS) are unique glycosaminoglycans isolated from body walls of sea cucumbers (holothuria). These biopolymers are composed of a chondroitin core [→4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→]$_n$ bearing fucosyl branches and sulfate groups. Structural variations of FCS are species specific and depend on type, amount and position of branches, as well as on degree and pattern of sulfation of a backbone and branches. A wide spectrum of biological properties was determined for these polysaccharides including anticoagulant, antithrombotic, antitumor, anti-inflammatory activities. Structural features of FCS influence significantly on their biological effect. In this review recent data about structural variations within holothurian FCS are summarized. The NMR data of the key building blocks are presented, which may be used for the analysis of new FCS.

Keywords: fucosylated chondroitin sulfate; glycosaminoglycan; holothuria; ICS-29; NMR; structure.

Introduction

Glycosaminoglycans (GAG) are known to play an important role in many biological processes including blood coagulation, thrombosis, vessel formation, oncogenesis, as well as recognition, adhesion and migration of cells [1–5]. Structural features of GAG such as molecular weight, type of monosaccharide units in a backbone, order of glycosidic bonds, pattern and degree of sulfation influence significantly their biological effect. In 1988 a new type of GAG built up of D-glucuronic acid, N-acetyl-D-galactosamine and L-fucose was found in the body wall of the sea cucumber (holothuria) *Ludwigothuria grisea* [6]. This polysaccharide was resistant to chondroitinase degradation, whereas, after defucosylation, it was partially degraded by the enzyme. Additional desulfation step led to the polysaccharide which was almost totally degraded by chondroitinases AC or ABC. These results together with the methylation analysis suggested that the native polymer contained a typical chondroitin core [→4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→]$_n$, in which sulfated difucosyl units α-L-Fuc3S,4S-(1→2)-α-L-Fuc4S-1→ were linked to O-3 of approximately one-half of the glucuronic acid residues (repeating block **I**), while another part of GlcA units was 3-O-sulfated (repeating block **II**) [7] (Fig. 1). This type of GAG was named fucosylated chondroitin sulfates (FCS).

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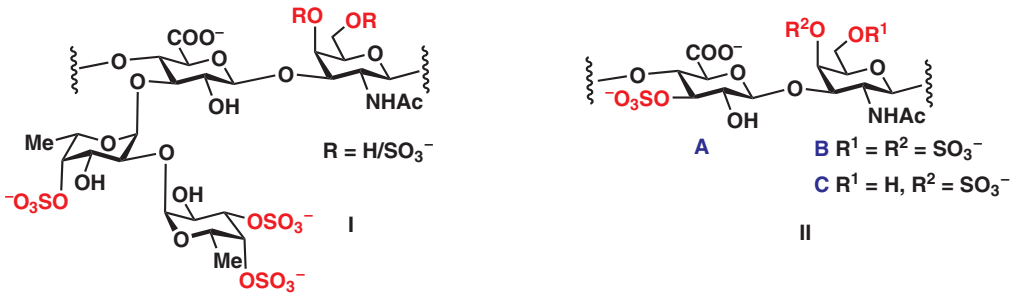

Fig. 1: The structural fragments of FCS from the sea cucumber *Ludwigothuria grisea* determined in 1988–1991 [6, 7].

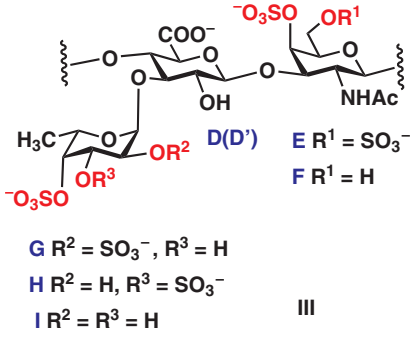

Fig. 2: The structural fragments of FCS from different species of sea cucumbers. Unit **D** bears Fuc2S4S (**G**), whereas unit **D′** bears Fuc3S4S (**H**) or Fuc4S (**I**).

Since that time about 30 different species of holothuria have been studied as sources of FCS. The main repeating blocks of these biopolymers was found to be the trisaccharide structures of type **III** built of substituted chondroitin disaccharide →4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→ in which the glucuronic acid unit contained a fucosyl branches at O-3 (Fig. 2) [8–15]. The sulfate groups were located at O-4 and/or O-6 of GalNAc residues and at different positions of fucosyl branches. Structural variations of FCS from different species were connected with the different positions of sulfate groups in GalNAc and Fuc residues. Usually several fucosyl branches (**G**, **H**, **I**) were detected in FCS from a certain sea cucumber species. A wide spectrum of biological properties was determined for this type of GAG including anticoagulant, antithrombotic, antitumor, anti-inflammatory activities [9–15]. In 2014 two reviews have been published, where the species specific structural variations of these biopolymers were regarded [8] and their biological properties were described [9].

After 2014 a number of papers have been published where new unusual fragments of FCS were reported. It was shown that structural variations could be determined not only by pattern of sulfation of GalNAc and Fuc residues, but also by sulfation of GlcA units, fucosylation of GalNAc residues, and the presence of more complex difucosyl branches. In this review the recent data about the structural features of holothurian fucosylated chondroitin sulfates are summarized.

Recent data about the structural diversity of holothurian fucosylated chondroitin sulfates

Interesting examples of FCS discovered recently with structural motive **III** were the polysaccharides from the sea cucumbers *Stichopus chloronotus* and *Stichopus horrens* [16] bearing only one type of branches, namely, 2,4-di-O-sulfated fucosyl units (**G**), as well as the polysaccharide from *Massinium magnum* containing only

3,4-di-O-sulfated fucosyl branches (**H**) [17] (Fig. 2). These biopolymers had well resolved 1D and 2D NMR spectra, which led to define their structure unambiguously. The data of ^{1}H and ^{13}C NMR spectra of these polysaccharides are presented in Table 1.

In 2015 the structure of the first studied FCS from *Ludwigothuria grisea* was reinvestigated using 1D and 2D NMR spectroscopy [18]. It was found that, instead of difucosyl branch described previously and shown in repeating unit **I**, there was the fragment α-L-Fuc-(1→2)-α-L-Fuc3S-1→ (see repeating unit **IV**, Fig. 3). Moreover, repeating block **III** was also detected, and the presence of **II** in a structure of this biopolymer was confirmed.

In 2018 another type of a difucosyl branch was found in FCS from *Holothuria lentiginosa* [19]. It was a fragment α-L-Fuc-(1→3)-α-L-Fuc4S-1→ with the unusual (1→3)-glycoside bond (see repeating unit **V**, Fig. 4). Also the repeating blocks **III** were determined in this FCS. The ratio of the blocks **V**:**III** was about 1:2.

In 2017 detailed analysis of anionic polysaccharides from the sea cucumber *Eupentacta fraudatrix* showed the presence of two structurally different FCS **EF1** and **EF2** which were successfully separated by ion-exchange chromatography [20]. Both polysaccharides together with typical monofucosyl branches contained difucosyl fragment α-L-Fuc-(1→2)-α-L-Fuc3S4S-1→ linked to O-3 of GlcA (see repeating block **VI**) (Fig. 5). Moreover, disaccharide repeating unit →4)-β-D-GlcpA2S3S-(1→3)-β-D-GalpNAc6S-(1→ was determined in a backbone of **EF2** (repeating block **VII**). The presence of structurally different fucosylated chondroitin sulfates in one species of a sea cucumber was rather unusual and was described for the first time.

In 2018 the NMR study of FCS from the holothuria *Cucumaria djaconovi* revealed the presence in a backbone (along with the trisaccharide blocks **III**) GlcA residues unsubstituted both at O-2 and O-3 (see repeating block **VIII**) (Fig. 6) [21]. The ratio of blocks **VIII**:**III** was about 2:3. Hence, fragments characteristic to chondroitin sulfates A, C and E could be presented in a structure of holothurian chondroitin sulfates.

In 2016 FCS **CJ** isolated from the holothuria *Cucumaria japonica* was characterized in terms of monosaccharide content, degree of sulfation, molecular weight, and the data of ^{1}H and ^{13}C NMR spectra [22]. It was found that the polysaccharide contained, together with repeating block **III**, structural fragment **II** →4)-β-D-GlcA3S-(1→3)-β-D-GalNAc-(1→ devoid of fucose (Fig. 1), identified previously in FCS from *Ludwigothuria grisea*. This was the second example of the presence of 3-O-sulafated GlcA unit in a structure of FCS.

Structural fragment **II** was also revealed in FCS from the holothuria *Cucumaria frondosa* [23]. Notably, this polysaccharide together with the repeating fragments **II** and **III** contained an unusual fucosyl branch (**W**) at O-6 of GalNAc unit (see the repeating block **IX**) (Fig. 7). The fucosyl branch at O-6 of GalNAc was also determined in FCS from the sea cucumbers *Holothuria mexicana* [24] and *H. scabra* [25].

Usually FCS isolation procedure requires the treatment of the material with papain to destroy proteins linked to carbohydrate chains [7]. The anionic molecules are precipitated from solution as cetyltrimethylammonium salts, which then are transformed into water-soluble sodium salts [22]. To our knowledge, further successful separation of the crude polysaccharides could be achieved by anion-exchange chromatography followed by gel permeation chromatography [20–23].

NMR spectroscopy was shown to be the most informative method for the analysis of fine structure of polysaccharides [26–28]. Application of additive schemes was found to be useful for the assessment of the signals in NMR spectra of these compounds [29–31]. Therefore, the data of ^{1}H and ^{13}C NMR spectra of the key building blocks **A**-**W** summarized in Table 1 may be applied for the structural characterization of new FCS.

Analysis of the data of Table 1 revealed the presence of characteristic signals for all building blocks **A**-**W**. These are the signals of H-1 and C-1 of almost all units, the signals of C-2 (52.3–52.8 ppm) of GalNAc units, and the signals of C-6 (15.3–17.2 ppm) of Fuc residues. The presence of sulfate group was determined by the downfield shift signals of respective proton and carbon atoms. For instance, the H-1 signals of three fucosyl branches **G**, **H** and **I** are distinguished significantly (5.69, 5.34 and 5.41 ppm, respectively), which may be used for the determination of these fragments in FCS structure. Integration of these signals lets to reveal the ratio of the monofucosyl branches. Also the H-1 signals of 3-O-fucosylated (**D, D′**), 3-O-sulfated (**A**) and 2,3-di-O-sulfated (**P**) GlcA units differed sufficiently (4.48, 4.59 and 4.89 ppm) to find out these blocks in a backbone of FCS. The units GalNAc4S and GlaNAc4S6S can be distinguished by the signals of H-6 (3.70–4.07 ppm for GalNAc4S and 4.20–4.35 for GalNAc4S6S) and C-6 (62.3–62.4 ppm for GalNAc4S and 68.5–68.9 for GalNAc4S6S). Integration of the cross-peaks H6-C6 in the HSQC spectrum lets to determine the ratio of these blocks in a backbone.

Table 1: The data of the ^{1}H and ^{13}C NMR spectra of the key building blocks **A-W** of fucosylated chondroitin sulfates (the bold numerals indicate the positions of sulfate).

Fragment	Residue	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
II [22][a]	**A**→4)-β-D-Glc*p*A3*S*-(1→	4.58/	3.60/	**4.38/**	4.03/	3.77/	–
		104.9	73.4	**82.5**	78.2	78.3	176.3
II [22][a]	**B**→3)-β-D-Gal*p*NAc4*S*6*S*-(1→	4.61/	4.08/	3.99/	**4.83/**	3.91/	**4.35, 4.26**
		101.4	52.7	77.5	**77.5**	73.2	**68.5**
II [22][a]	**C**→3)-β-D-Gal*p*NAc4*S*-(1→	4.61/	4.08/	3.99/	**4.83/**	3.91/	3.70, 4.07
		101.4	52.7	77.5	**77.5**	73.2	62.4
III [16][a]	**D**→4)-β-D-Glc*p*A-(1→	4.48/	3.64/	3.71/	3.96/	3.71/	–
		105.0	75.0	78.1	76.6	78.1	176.0
III [16][a]	**D′**→4)-β-D-Glc*p*A-(1→	4.48/	3.60/	3.68/	4.00/	3.71/	–
		105.0	75.0	80.7	76.6	78.1	176.0
III [16][a]	**E**→3)-β-D-Gal*p*NAc4*S*6*S*-(1→	4.58/	4.07/	3.95/	**4.81/**	4.00/	**4.33, 4.20**
		100.9	52.7	77.9	**77.2**	73.2	**68.5**
III [16][a]	**F**→3)-β-D-Gal*p*NAc4*S*-(1→	4.58/	4.07/	3.95/	**4.81/**	4.02/	3.81/
		100.9	52.7	77.9	**77.2**	76.2	62.3
III [16][a]	**G** α-L-Fuc*p*2*S*4*S*-(1→	5.69/	**4.48/**	4.17/	**4.86/**	4.90/	1.37/
		97.7	**76.6**	67.8	**82.5**	67.5	16.9
III [17][a]	**H** α-L-Fuc*p*3*S*4*S*-(1→	5.34/	3.95/	**4.53/**	**5.01/**	4.80/	1.37/
		100.5	67.6	**76.6**	**80.6**	67.6	17.2
III [21][a]	**I** α-L-Fuc*p*4*S*-(1→	5.41/	3.82/	4.04/	**4.77/**	4.80/	1.37/
		99.6	69.7	70.0	**82.4**	67.6	17.2
IV [18][b]	**J**→2)-α-L-Fuc*p*3*S*-(1→	5.42/	4.22/	**4.70/**	4.19/	4.21/	1.36/
		98.5	77.5	**75.4**	68.8	68.7	15.5
IV [18][b]	**K** α-L-Fuc*p*-(1→	5.28/	4.26/	3.97/	4.13/	4.15/	1.22/
		98.0	71.2	67.5	72.3	68.8	15.6
V [19][b]	**L**→3)-α-L-Fuc*p*4*S*-(1→	5.40/	3.83/	4.01/	**4.75/**	4.84/	1.36/
		98.9	72.3	67.9	**81.6**	66.7	16.4
V [19][b]	**M** α-L-Fuc*p*-(1→	5.28/	4.30/	3.99/	4.16/	4.15/	1.36/
		97.9	72.5	72.3	72.5	69.2	16.4
VI [20][a]	**N**→2)-α-L-Fuc*p*3*S*4*S*-(1→	5.41/	4.17/	**4.69/**	**5.01/**	4.91/	1.37/
		99.6	72.6	**76.3**	**80.5**	68.0	17.3
VI [20][a]	**O** α-L-Fuc*p*-(1→	5.33/	3.79/	3.89/	4.07/	4.48/	1.25/
		100.6	69.9	71.0	71.0	69.6	17.0
VII [20][a]	**P**→4)-β-D-Glc*p*A2*S*3*S*-(1→	4.89/	**4.48/**	**4.86/**	4.40/	4.06/	–
		102.5	**78.6**	**78.8**	77.0	79.0	176.1
VII [20][a]	**Q**→3)-β-D-Gal*p*NAc6*S*-(1→	4.72/	4.01/	3.86/	4.20/	3.90/	**4.23/**
		103.1	52.2	81.9	67.7	73.3	**67.1**
VIII [21][a]	**R**→4)-β-D-Glc*p*A-(1→	4.47/	3.38/	3.59/	3.78/	3.70/	–
		105.1	73.9	75.2	81.8	77.9	175.9
VIII [21][a]	**R′**→4)-β-D-Glc*p*A-(1→	4.50/	3.38/	3.59/	3.74/	3.70/	–
		105.3	73.9	75.2	82.9	77.9	175.7
VIII [21][a]	**S**→3)-β-D-Gal*p*NAc4*S*6*S*-(1→	4.59/	4.03/	4.03/	**4.75/**	4.12/	**4.24/**
		102.4	52.8	76.8	**77.6**	74.0	**68.9**
VIII [21][a]	**T**→3)-β-D-Gal*p*NAc4*S*-(1→	4.59/	4.03/	4.03/	**4.75/**	3.83/	3.80/
		102.4	52.8	76.8	**77.6**	75.8	62.3
VIII [21][a]	**U**→3)-β-D-Gal*p*NAc6*S*-(1→	4.56/	4.03/	3.86/	4.18/	3.98/	**4.24/**
		102.6	52.3	81.5	68.9	74.0	**68.9**
IX [23][a]	**V**→3)-β-D-Gal*p*NAc4*S*-(1→	4.60/	4.07/	3.95/	**4.80/**	3.96/	4.17/
		100.8	52.6	78.0	**77.5**	73.3	69.0
IX [23][a]	**W** α-L-Fuc*p*2*S*3*S*4*S*-(1→	5.52/	**4.62/**	**4.86/**	**5.01/**	4.44/	1.35/
		93.4	**73.6**	**73.9**	**80.5**	68.0	17.0

[a]Chemical shifts are relative to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 at 0.0 ppm for ^{1}H and at –1.6 ppm for ^{13}C spectra.
[b]Chemical shifts are relative to sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 at 0.0 ppm for ^{1}H and methanol for ^{13}C spectra.

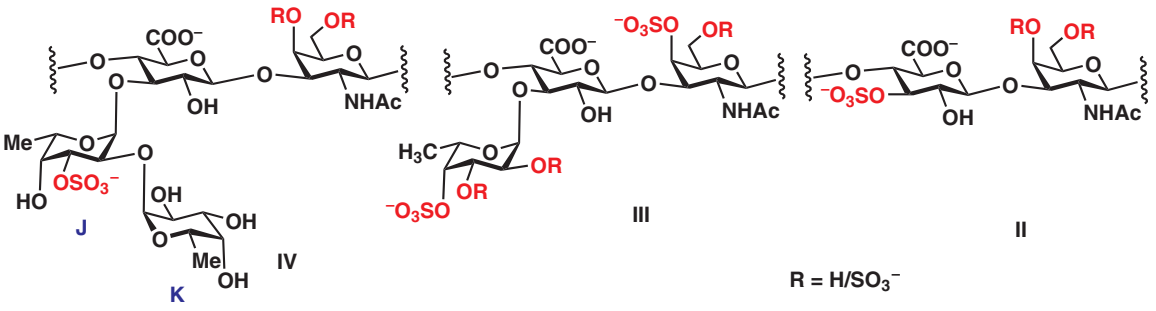

Fig. 3: The structural fragments of FCS from the sea cucumber *Ludwigothuria grisea* found in 2015 [18].

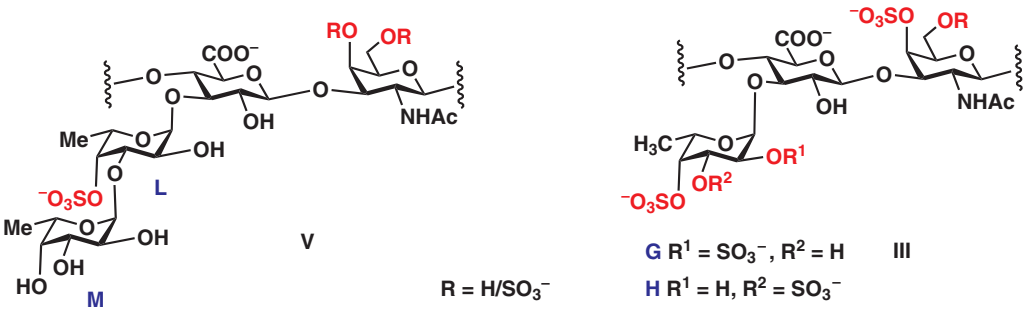

Fig. 4: The structural fragments of FCS from the sea cucumber *Holothuria lentiginosa* [19].

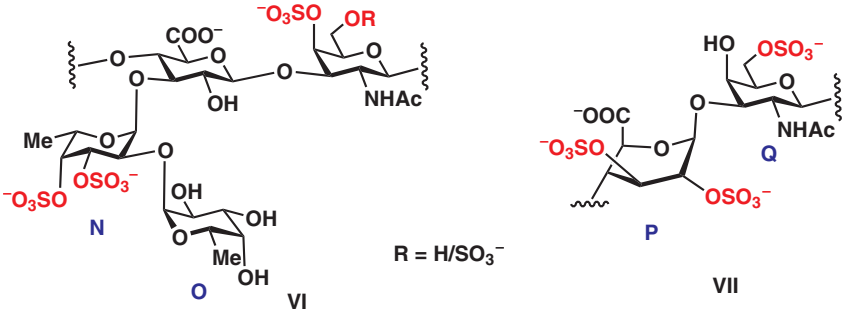

Fig. 5: The structural fragments of FCS from the sea cucumber *Eupentacta fraudatrix* [20].

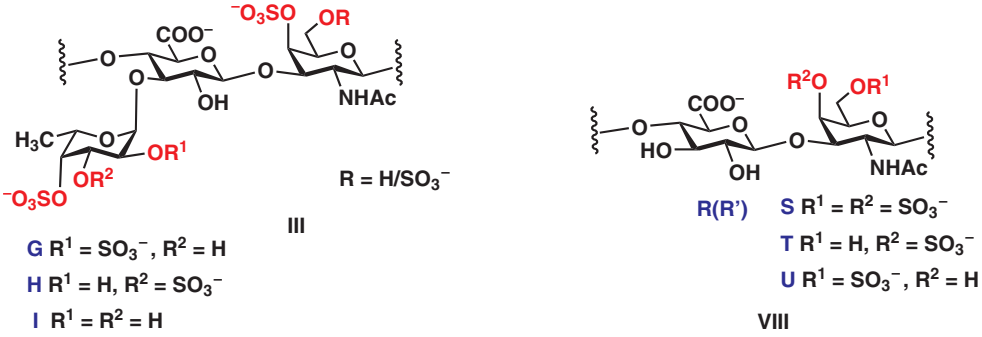

Fig. 6: The structural fragments of FCS from the sea cucumber *Cucumaria djakonovi* [21]. Unit **R** is linked to GalNAc4S6S (S) or GalNAc4S (**T**), whereas unit **R′** is linked to GalNAc6S (**U**).

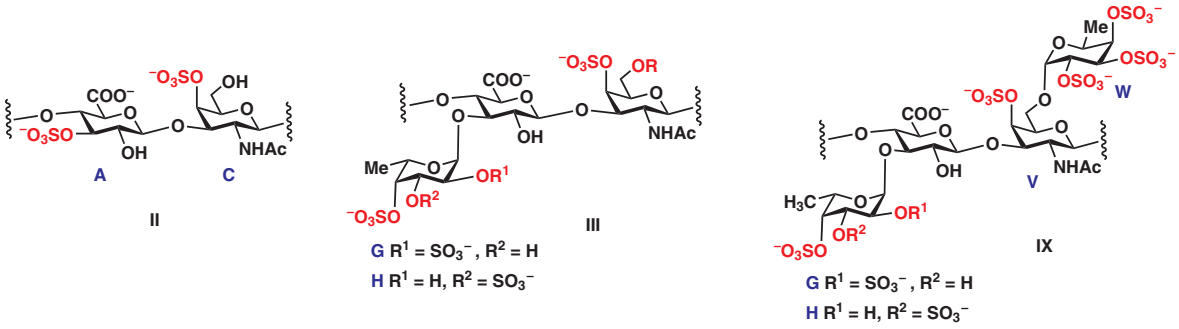

Fig. 7: The structural fragments of FCS from the sea cucumber *Cucumaria frondosa* [23].

The optimal temperature for NMR spectra registration was found to be 333 K, as the signal of HOD was not overlapped with the signals of anomeric protons of GlcA, GalNAc and Fuc units. Variations of pH of the sample solutions were found to be an additional tool in the analysis of overcrowded NMR spectra of FCS [32]. Registration of NMR spectra at different pH was shown to be useful for the polysaccharides contained repeating blocks **II**, **VII** and **VIII**, because the positions of signals of the constituent residues changed significantly and could be separated from the signals of other units.

NMR spectra of synthetic oligosaccharides may help in assignment of chemical shifts in the complex NMR spectra of FCS. For this purpose a series of oligosaccharides related to the repeating blocks **III** and **IX** was synthesized [33, 34]. Besides, synthetic and semisynthetic oligosaccharide fragments of FCS were used as models for determination of the structure-activity relationship within this class of GAG [35–38].

Conclusions

The structural diversity of fucosylated chondroitin sulfates from holothuria is rather wide. To date at least eight types of the repeating blocks were revealed. Structural variations are species specific and depend on amount and position of branches, as well as on degree and pattern of sulfation of a backbone and branches. FCS from a certain species may include one or several types of the repeating blocks in its structure. Moreover, there is an example of the presence of two structurally different FCS in one species of sea cucumber. The NMR data of the key building blocks **A**-**W** are presented in Table 1, which may be used for the analysis of new FCS.

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