

Metabolite profiling of polar steroid constituents in the Far Eastern starfish *Aphelasterias japonica* using LC–ESI MS/MS

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Abstract Numerous polar steroids are characteristic metabolites of starfish which determine physiological activities of their extracts. The Far Eastern starfish *Aphelasterias japonica* is a rich source of different steroid glycosides and polyhydroxysteroids. For detailed analysis of complicated mixture of steroids from this species, isolated by solid-phase extraction, a liquid chromatography–electrospray tandem mass spectrometry (LC–ESI MS/MS) approach was selected and applied. The characteristic fragmentations in ESI MS/MS spectra of steroid glycosides allowed determining types of aglycones, presence of sulfate groups, sugar sequences as well as branching. In addition, main structural features of polyhydroxylated polar steroids including position of hydroxylation and level of sulfation were also established. Totally, 68 metabolites, comprising of 33 asterosaponins, 28 sulfated polyhydroxysteroid mono- and biosides and 7 sulfated polyhydroxysteroid compounds were found by this method. In addition to 15 previously isolated compounds from *A. japonica*, many new steroid glycosides including asterosaponins with unusual carbohydrate chains were discovered and characterized by their ESI product ion mass spectra. Some details of biosynthesis of polyhydroxylated steroids and their conjugated forms in the species studied such as a role of

sulfation and order of introduction of hydroxyl group in *A. japonica* were proposed.

Keywords Starfish · *Aphelasterias japonica* · Steroid · Glycoside · Liquid chromatography/tandem mass spectrometry · Metabolite profiling

1 Introduction

Starfish represent an outstanding source of steroidal secondary metabolites of a great structural diversity with such biological activities as ichthyotoxic, cytotoxic, analgesic, antiviral, antibacterial, anti-inflammatory, antibiofouling, and neurotogenic effects. The secondary metabolites from different representatives of this class of Echinodermata were proved to be polyhydroxylated steroids and steroid glycosides (Minale et al. 1993; Stonik 2001; Stonik et al. 2008; Ivanchina et al. 2011). Steroid glycosides are characteristic compounds of the majority of starfish. Besides very rare glycosides with cyclic carbohydrate chains, these invertebrates contain two main structural groups of steroid glycosides, namely asterosaponins and glycosides of polyhydroxysteroids. The glycosides of polyhydroxysteroids have a steroid nucleus with four to nine hydroxyl groups and, as a rule, one or two monosaccharide units attached either to the side chains or to the steroid nucleus or to the steroid nucleus and the side chain of aglycones simultaneously. The most common sugar residues in these substances were proved to be xylose, its derivatives, which can be methylated or sulfated, and arabinose. Asterosaponins present themselves as steroid oligoglycosides containing carbohydrate chains with four to six sugars attached to C-6. Usually carbohydrate chains of asterosaponins have branching at the second monosaccharide unit. Hexoses

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(glucose, galactose), pentoses (arabinose, xylose), and deoxyhexoses (fucose, quinovose) are the most common sugar residues in asterosaponins. Individual representatives of asterosaponins differ from each other in their steroid side chains and/or sugar moieties, demonstrating a significant natural diversity, while the 3-*O*-sulfated 3 β ,6 α -dihydroxysteroid tetracyclic nucleus with a 9(11)-double bond is common structural feature for the vast majority of asterosaponins.

It is believed that asterosaponins defend starfish against predatory fish mainly due to their strong hemolytic and ichthyotoxic properties (Minale et al. 1993). Asterosaponins also exhibit other biological activities, such as capabilities to inhibit cell colony formation and tumor cell proliferation (Kicha et al. 2011; Ivanchina et al. 2012), activate tubulin polymerization (Tang et al. 2009) and affect the sodium channel activity (Yang et al. 2007). Possibly, polyhydroxylated steroids as well as their mono- and biosides participate in digestion processes like bile acids and bile alcohols do in higher animals (Kicha et al. 2001a). These compounds are less toxic and often show neurotrophic properties (Palyanova et al. 2013).

Usually, steroidal metabolites in starfish extracts form very complicated mixtures difficult for separation into pure compounds by chromatographic and other modern methods. Many minor metabolites remain to be largely unstudied, although knowledge about their chemical structures is important for understanding of the biosynthesis of polar steroids in these invertebrates. In our opinion the metabolomic studies on total fractions of polar steroids with indication of previously unknown constituents and the determination of distribution of individual compounds within this class of animals are of great interest, and lead to a deeper understanding of secondary metabolism processes in starfish. Moreover, these studies may allow further isolation of new individual polar steroids for bioassays to be performed.

Mass spectrometry has been playing an important role in the structural analysis of complex mixtures of natural products mainly due to its high sensitivity, rapid analysis time and selectivity. Recently, electrospray ionization tandem mass spectrometry has demonstrated its great advantages for the structural analysis of steroid glycosides from starfish (Sandvoss et al. 2001; Maier et al. 2007). Liquid chromatography combined with sequential mass spectrometry have been extensively applied to the analysis of complex mixtures of many natural compounds, and proved to be the appropriate analytical tool to obtain the necessary structural information for a great diversity of natural products. LC–MS technique allows the rapid and selective screening of metabolomes, the determination of qualitative and quantitative composition of metabolites and

changes in metabolomic profiles in response to environmental and other stimuli.

Although LC–MS approach is a well-established method for profiling of a wide range of compounds from various natural sources such as phenolic glycosidic conjugates (Stobiecki et al. 2006), saponins from plants (Chapagain and Wiesman 2008; Pollier et al. 2011), saponins from sea cucumbers (Bondoc et al. 2013), lipids (Subramaniam et al. 2011) and others, the number of studies on the profiling of steroid metabolites from marine invertebrates is very limited. Only two times the LC–MS approach was used for rapid screening and for studying of the body distribution of starfish steroid metabolites, namely asterosaponins from starfish *Asterias rubens*, however other steroids from this species were not analyzed in these studies (Sandvoss et al. 2001; Demeyer et al. 2014).

In this paper, we describe the application of liquid chromatography—electrospray mass spectrometry for profiling and characterization of steroid polar metabolites from the Far Eastern starfish *Aphelasterias japonica*.

2 Materials and methods

2.1 Animal material

Specimens of the starfish *A. japonica* (order Forcipulatida, family Asteroidea) were collected from a depth of 3–10 m at the Posyet Bay, the Sea of Japan, in August 2013. Species identification was carried out by Dr. B.B. Grebnev (G.B. Elyakov Pacific Institute of Bioorganic Chemistry of the Far Eastern Branch of Russian Academy of Sciences, Vladivostok, Russia).

2.2 Sample preparation and solid-phase extraction (SPE)

The five fresh animals were chopped and extracted with ethanol. The 500 μ l of ethanol extract was centrifuged and the supernatant was subjected SPE extraction. Solid-phase extraction (SPE) cartridges (Strata-X, 33 μ m, 60 mg/3 ml, polymeric reversed phase, Phenomenex, USA) were fitted into stopcocks and connected to a vacuum manifold. The sorbent was conditioned with 5 ml of acetonitrile followed by 5 ml water. Care was taken that the sorbent did not become dry during conditioning. With the stopcocks opened and the vacuum turned on, the samples were loaded onto the column. The 100 μ l of extract were loaded into the SPE cartridge by drops. After sample addition, the SPE cartridge was washed with 2.5 ml of water. Steroid glycosides and related compounds were eluted with 1.5 ml of 100 % acetonitrile. These acetonitrile extracts were dried and dissolved in 400 μ l 50 % ACN in water (v/v) and

subjected liquid chromatography–electrospray tandem mass spectrometry (LC–ESI MS) analyses.

2.3 LC–ESI MS analysis

Analysis was performed with an Agilent 1200 series chromatograph (Agilent Technologies, Santa Clara, USA) connected to an Agilent 6510 Q-TOF LC/MS mass spectrometer (Agilent Technologies, Santa Clara, USA). Zorbax 300SB-C18 column (1.0 × 150 mm, 3.5 μm, Agilent Technologies, Santa Clara, USA) with Zorbax SB-C8 guard-column (2.1 × 12.5 mm, 5 μm, Agilent Technologies, Santa Clara, USA) was used for chromatographic separation. The mobile phases were 0.1 % acetic acid in H₂O (eluent A) and 0.1 % acetic acid in ACN (eluent B). The gradient program was as follows: isocratic at 30 % of eluent B from start to 3 min, from 30 to 70 % eluent B from 3 to 35 min, from 70 to 100 % eluent B from 35 to 36 min, and isocratic at 100 % of eluent B from 36 to 45 min. After returning to the initial conditions, the equilibration was achieved after 20 min. Chromatographic separation was performed at a 0.05 ml/min flow rate at 40 °C. Injection volume was 0.5 μl.

The mass spectrometer was equipped with Dual-Spray ESI ionization source. Optimized ionization parameters were as follows: a capillary voltage of ± 3.5 kV, nebulization with nitrogen at 2 bar, and dry gas flow of 5 l/min at a temperature of 325 °C, fragmentor voltage of 250 and 360 V in negative and positive ion mode, respectively. Metabolite profiles in positive ion mode were registered using post-column addition of 1×10^{-4} M sodium chloride at 0.3 ml/h flow rate for obtaining stabilized sodium adduct ions. Post-column infusion was performed with syringe pump via T-mixing tee. According to the results of preliminary experiments, the mass spectra were recorded within *m/z* mass range of 100–1,500 and 70–1,500 for MS/MS spectra (scan time 1 s).

Collision induced dissociation (CID) product ion mass spectra were recorded in auto-MS/MS mode using a collision energy ranging from 50 to 95 V, depending on the molecular masses of precursor ions. The precursor ions were isolated with an isolation width of 1.3 *m/z*.

The mass spectrometer was calibrated using the ESI-L Low Concentration Tuning Mix (Agilent Technologies, Santa Clara, USA). High resolution LC–MS analysis was performed using Reference Mix (Agilent Technologies, Santa Clara, USA) provided through reference sprayer in Dual-Spray ESI ionization source.

The instrument was operated using the program MassHunter Data Acquisition and data were analyzed using the MassHunter Qualitative Analysis and MassHunter Quantitative Analysis Software (ver.02.00, Agilent Technologies, Santa Clara, USA).

2.4 Acid hydrolysis

A solution of 1.4 mg of desalted by SPE extract in 1 ml TFA (2 M) was placed in a vial and heated at 100 °C for 2 h with mixing. After evaporation of the solution under reduced pressure, compounds were purified by SPE as described above. The acetonitrile extract containing sulfated aglycones was analyzed by LC–ESI MS (experimental conditions are identical to those described above).

2.5 Chemicals

Acetonitrile (quality HPLC gradient grade) and water (quality UV-HPLC grade) were obtained from Panreac (Barcelona, Spain). All other chemicals and reagents were of analytical grade or equivalent. Standard polar steroids were previously isolated from the starfishes *A. japonica* (Ivanchina et al. 2000; Kicha et al. 2001b; Ivanchina et al. 2005; Popov et al. 2013), *Asterias rathbuni* (Ivanchina et al. 2001), *Patiria* (= *Asterina*) *pectinifera* (Ivanchina et al. 2013) as individual compounds and their structures were established using different methods including high resolution NMR.

3 Result and discussion

The previous investigations of the starfish *A. japonica* have led to isolation of 17 steroid compounds, comprising 8 sulfated glycosides of polyhydroxysteroids (including 3 “shortened” asterosaponins with the only one monosaccharide units at C-6), 3 “classical” asterosaponins and 6 sulfated polyhydroxysteroids (Fig. 1) (Finamore et al. 1992; Ivanchina et al. 2000; Kicha et al. 2001b; Ivanchina et al. 2005; Popov et al. 2013). Several of these compounds were first isolated from the Japanese population of *A. japonica*, while the rest—from several populations inhabiting Russian waters of Japan and Okhotsk Seas.

However, ESI negative and positive ion mass spectra of the total ethanol extract of starfish have shown the presence of much larger number of various types of sulfated steroid glycosides and related compounds in the studied population when compared with previously known. The negative ion mass spectra showed characteristic peaks of series $[M-Na]^-$ within *m/z* range from 1,100 to 1,450 a.m.u. corresponding to decationized asterosaponin molecules and peaks of decationized polyhydroxylated steroids including their mono- or biosides within *m/z* range from 400 to 800 a.m.u. In addition, mass spectra of some compounds showed doubly decationized ion peaks at *m/z* 368 and 318.

In the positive ion mode, observed ions were mainly sodium-coordinated. Although both positive and negative

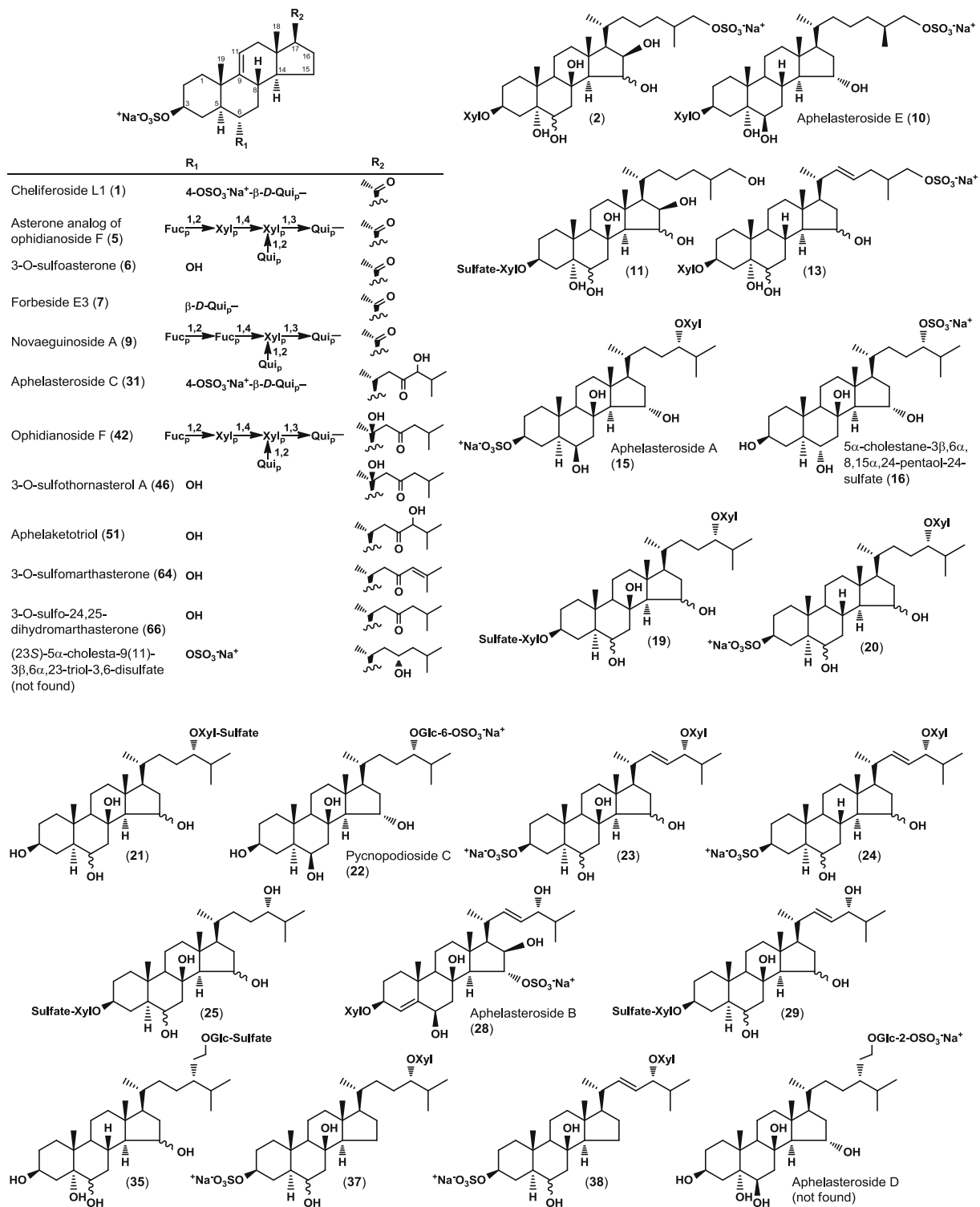


Fig. 1 Structures of steroid compounds identified (1, 5, 6, 7, 9, 10, 15, 16, 22, 28, 31, 42, 46, 51, 64, and 66) and proposed (2, 11, 13, 19, 20, 21, 23, 24, 25, 29, 35, 37, and 38) from the Far Eastern starfish *A. japonica* by LC-MS/MS method

ion mass spectra were good enough to characterize the sulfate steroid glycosides, the negative ion mode contained peaks of higher intensities and was more suitable for analysis.

Profiling of steroid compounds from the starfish *A. japonica* using LC-MS approach allowed a multitude of different steroid glycosides and related compounds to be characterized. LC-MS in negative ion mode resulted in

chromatogram including 68 components—33 asterosaponins, 28 sulfated glycosides of polyhydroxysteroids and 7 sulfated polyhydroxylated steroids (Tables 1 and 2, the numbers of the compounds correspond to the numbers of peaks of (–)LC–MS chromatogram).

Assignment of these compounds in sample analyzed was based on high resolution LC–MS and LC–MS/MS performed both in negative and positive ion modes. The elemental composition of ions was estimated on the basis of the m/z values registered with about 5 ppm accuracy. Elemental composition, determined on the basis of high resolution data, fragmentation in MS and MS/MS spectra, as well as chromatographic behavior of the corresponding compounds, allowed their structures to be proposed.

The LC–MS analysis has shown some chromatographic peaks of the same m/z values that is the evidence for existence isomeric or/and isobaric compounds in the studied mixture of metabolites. For example, compounds with m/z 1,241 gave several peaks on single ion chromatogram, including four of them belong to different asterosaponin isomers (Fig. 2). Such ambiguities can be resolved by tandem MS in the cases when the compounds have different aglycone and/or variable sugar sequence.

Analyzed metabolite profile revealed at least 33 asterosaponins, including 31 pentaosides and 2 (compounds **30** and **36**) hexaosides. Asterosaponins were detected by $[M-Na]^-$ and $[M+Na]^+$ peaks in negative and positive ion mass spectra, respectively. The 31 asterosaponins from them were characterized by tandem MS (Table 1). As it is known, different epimeric monosaccharides as well as types of bonds between sugars can't be strictly distinguished by MS (Zaia 2010). However, taking into account that the earlier isolated asterosaponins from these species have general architectures with the same β -1,3, β -1,4 and β -1,2 sequence of glycosidic bonds in the linear part of carbohydrate chains and β -1,2-bond in the branching at the second monosaccharide as well as the fact that carbohydrate chains of these compounds is closely related to each other (the first monosaccharide unit is quinovose and the second monosaccharide unit is xylose), the same structural features may be suggested for some newly indicated glycosides (**30**, **39**, **43**, **45**, **49**, and **60**), which contain deoxyhexose and pentose as the first and the second monosaccharide units in accordance with MS sequencing. Some of them differ from each other in aglycone moiety mainly.

Several typical mass losses between the precursor and the fragment ions were detected in negative product ion spectra of asterosaponins. These typical mass losses are related to the aglycone, the sulfate group and oligosaccharide chain losses and give information about such important structural features of asterosaponins as aglycone, number of sulfate groups and sequence of monosaccharide

units in carbohydrate chains. For all asterosaponins (–)MS/MS provided an intense Y-type product ion series (nomenclature according to Domon and Costello (1988), see Fig. 3) arising from the cleavages of glycosidic bonds and corresponding sequential losses of a monosaccharide units with charge located on aglycone. In the MS/MS spectra of some asterosaponins, a mass loss of 100 Da between the precursor and the most intense fragment ion was detected. This corresponds to the loss of $C_6H_{12}O$ molecule arising from C-20 to C-22 bond cleavage and 1H transfer and is characteristic for asterosaponins containing an aglycone with a 20-hydroxy-cholestan-23-one side chain (Minale et al. 1993). Additionally, fragment ions at m/z 96.9 and 79.9 were detected in negative ion MS/MS spectra, which indicate to the presence of $[HSO_4]^-$ and $[SO_3]^-$ ions.

Positive ion MS/MS spectra of asterosaponins were used for verification of data, obtained from (–)MS/MS. In (+)MS/MS spectra, signal from ions resulting from mass loss of 120 Da and corresponding $[M-NaHSO_4]^+$ fragment, and signal at m/z 142.9 corresponding to $[Na_2.HSO_4]^+$ fragment which is related with dissociation of the sulfate group were detected. Also (+)MS/MS provided Y-type product ion series corresponding losses of a monosaccharide units and B- and C-type product ion series arising from the cleavages of glycosidic bonds with charge located on saccharide fragment (Domon and Costello 1988).

The three asterosaponins, namely asterone analog of ophidianoside F (**5**), ophidianoside F (**42**), and novaeguinose A (**9**), were isolated in previous investigations of the starfish *A. japonica* (Ivanchina et al. 2005; Popov et al. 2013).

Negative product ion spectrum of $[M-Na]^-$ precursor at m/z 1,213 exhibits extensive fragmentation including the cleavages of glycosidic bonds and sequential losses of sugar units attached to the aglycone along with neutral loss of $C_6H_{12}O$ molecule at m/z 1,113 $[M-Na-100]^-$, 967 $[M-Na-100-dHex]^-$, 835 $[M-Na-100-dHex-Pent]^-$, 821 $[M-Na-100-2 \times dHex]^-$, 689 $[M-Na-100-2 \times dHex-Pent]^-$, 557 $[M-Na-100-2 \times dHex-2 \times Pent]^-$, 411 $[M-Na-100-3 \times dHex-2 \times Pent]^-$, 393 $[M-Na-100-3 \times dHex-2 \times Pent-H_2O]^-$. Also product ion spectra demonstrated Y_n fragment series at m/z 1,067 $[M-Na-dHex]^-$, 935 $[M-Na-dHex-Pent]^-$, 921 $[M-Na-2 \times dHex]^-$, 789 $[M-Na-2 \times dHex-Pent]^-$, 657 $[M-Na-2 \times dHex-2 \times Pent]^-$, 511 $[M-Na-3 \times dHex-2 \times Pent]^-$, 493 $[M-Na-3 \times dHex-2 \times Pent-H_2O]^-$ and fragments at m/z 96.9 $[HSO_4]^-$ and 79.9 $[SO_3]^-$. These fragmentation patterns match precisely with the structure of ophidianoside F. Product ion series of compound **5** (m/z 1,113) were similar to the previous except the Y_n-100 ion series and also showed neutral

Table 1 Profile of asterosaponins in extract of the Far Eastern starfish *A. japonica* detected by LC-ESI MS

No ^a	R _c	Elemental composition	Sugar chains and aglycones ^b	<i>m/z</i> ^{c, d}	[M-Na] ⁻							C, μg/ml			
					[M-Na-X] ^{-e}	Y ₃ or Y _{2b}	Y _{2a}	Y ₃ /Y _{2b}	Y _{2a} /Y _{2b}	Y ₁	Y ₀		Z ₀	[HSO ₄] ⁻	[SO ₃] ⁻
3	5.7	C ₅₀ H ₇₉ O ₂₇ SNa	dHex-Pent-Hex(-dHex)-dHex-AG I	1,143.4519	-	997.4	865.3	851.3	719.3	557.2	411.1	393.1	96.9	79.9	0.13
4	5.9	C ₅₁ H ₈₁ O ₂₇ SNa	dHex-dHex-Hex(-dHex)-dHex-AG I	1,157.4683	-	1,011.4	865.3	865.3	719.3	557.2	411.1	393.1	96.9	79.9	0.24
5	6.2	C ₄₉ H ₇₇ O ₂₆ SNa	Fuc-Xyl-Xyl(-Qui)-Qui-AG I	1,113.4408	-	967.4	835.3	821.3	689.3	557.2	411.1	393.1	96.9	79.9	0.05
9	6.9	C ₅₀ H ₇₉ O ₂₆ SNa	Fuc-Fuc-Xyl(-Qui)-Qui-AG I	1,127.4564	Lack of product ions in MS/MS spectra										0.03
18	10.8	C ₅₅ H ₈₉ O ₂₈ SNa		1,229.5247	Lack of product ions in MS/MS spectra										0.05
26	14.3	C ₅₇ H ₉₁ O ₂₈ SNa	dHex-dHex-Hex(-dHex)-dHex-AG III	1,255.5430	1,157.5	1,011.4	865.3	865.3	719.3	557.2	411.1	393.1	96.9	79.9	0.29
30 ^f	15.5	C ₆₂ H ₁₀₁ O ₃₃ SNa	Hex-dHex-Hex-Pent(-dHex)-dHex-AG II	1,405.5908	1,305.5; 1,243.5 (Y ₄); 1,143.5 (Y ₄ -100)	1,097.4 (Y ₃ , Y ₄); 997.4 (Y ₃ -100, Y ₄ Y _{2b} -100); 1,159.5 (Y _{2b})	835.3	851.3	689.3	557.2	411.1	393.1	96.9	79.9	0.41
32	16.1	C ₅₇ H ₉₁ O ₂₈ SNa	dHex-dHex-dHex(-dHex)-DXU-AG III	1,255.5400	1,157.5; 1,139.4	1,011.4; 993.4	865.3; 847.3	865.3; 847.3	701.3	573.2; 555.2	411.1	393.1	96.9	79.9	0.42
36 ^f	17.7	C ₆₂ H ₁₀₁ O ₃₃ SNa	Hex-dHex-Hex-Pent(-dHex)-dHex-AG IV	1,405.5935	1,243.5 (Y ₄)	1,097.4 (Y ₃ , Y ₄ Y _{2b}); 1,259.5 (Y _{2b})	935.4	951.4	789.3	657.3	511.1	493.1	96.9	79.9	0.35
39	19.6	C ₅₆ H ₉₁ O ₂₈ SNa	dHex-Hex-Pent(-dHex)-dHex-AG II	1,243.5422	1,143.5	1,097.4; 997.4	935.4; 835.3	951.4; 851.3	789.3; 689.3	657.3; 557.2	511.2; 411.1	493.2; 393.1	96.9	79.9	2.53
40	20.5	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-dHex-Hex(-dHex)-dHex-AG II	1,257.5563	1,157.5	1,111.5; 1,011.4	965.4; 865.3	965.4; 865.3	819.3; 719.3	657.3; 557.2	511.2; 411.1	493.2; 393.1	96.9	79.9	1.55
41	22.1	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-Hex-Hex(-dHex)-dHex-AG IV	1,273.5525	-	1,127.5	965.4	981.4	819.3	657.3	511.2	493.2	96.9	79.9	5.09
42	22.6	C ₅₅ H ₈₉ O ₂₇ SNa	Fuc-Xyl-Xyl(-Qui)-Qui-AG II	1,213.5296	1,113.4	1,067.4; 967.4	935.4; 835.3	921.4; 821.3	789.3; 689.3	657.3; 557.2	511.2; 411.1	493.2; 393.1	96.9	79.9	0.60
43	23.1	C ₅₆ H ₉₁ O ₂₈ SNa	dHex-Hex-Pent(-dHex)-dHex-AG IV	1,243.5431	-	1,097.4	935.4	951.4	789.3	657.3	511.2	493.2	96.9	79.9	1.03
44	24.0	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-dHex-DXU(-dHex)-dHex-AG II	1,257.5564	1,157.5; 1,139.5	1,111.5; 1,011.4; 993.4	965.4; 865.3; 847.3	965.4; 865.3; 847.3	819.3; 701.3	657.3	511.2; 411.1	493.2; 393.1	96.9	79.9	4.12

Table 1 continued

No ^a	R _c	Elemental composition	Sugar chains and aglycones ^b	<i>m/z</i> ^{c, d}	[M-Na-X] ^e										C, μg/ml	
					[M-Na] ⁻	[M-Na-X] ^{-e}	Y ₃ or Y _{2p}	Y _{2z}	Y ₃ /Y _{2p}	Y _{2z} /Y _{2p}	Y ₁	Y ₀	Z ₀	[HSO ₄] ⁻		[SO ₃] ⁻
45	26.0	C ₅₆ H ₉₁ O ₂₇ SNa	dHex-dHex-Pent(-dHex)-dHex-AG II	1,227.5463	1,127.5	1,081.4; 981.4	935.4; 835.3	935.4; 835.3	935.4; 835.3	789.3; 689.3	657.3; 557.2	511.2; 411.1	493.2; 393.1	96.9	79.9	0.19
48	27.4	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-dHex-dHex(-dHex)-DXU-AG II	1,257.5560	1,239.5; 1,157.5; 1,139.5	1,111.5; 1,093.4; 993.4	965.4; 847.3	965.4; 847.3	965.4; 847.3	819.3; 701.3	673.3; 555.3	511.2; 411.1	493.2; 393.1	96.9	79.9	3.52
49	28.6	C ₅₆ H ₉₁ O ₂₇ SNa	dHex-dHex-Pent(-dHex)-dHex-AG IV	1,227.5475	-	1,081.5	935.4	935.4	935.4	789.3	657.3	511.2	493.2	96.9	79.9	0.13
50	29.4	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-dHex-dHex(-dHex)-DXU-AG II	1,257.5563	1,239.5; 1,157.4; 1,139.5	1,111.4; 1,093.5; 993.4	965.4; 847.3	965.4; 847.3	965.4; 847.3	819.3; 701.3	673.3; 555.3	511.2; 411.1	493.2; 393.1	96.9	79.9	0.93
52	31.1	C ₅₇ H ₉₃ O ₂₇ SNa	dHex-dHex-dHex(-dHex)-dHex-AG II	1,241.5606	1,141.5	1,095.5; 995.4	949.4; 849.3	949.4; 849.3	949.4; 849.3	803.3; 703.3	657.3; 557.2	511.2; 411.1	493.2; 393.1	96.9	79.9	0.09
53	31.9	C ₅₇ H ₉₃ O ₂₇ SNa	dHex-dHex-dHex(-dHex)-dHex-AG II	1,241.5611	-	1,095.5	949.4	949.4	949.4	803.3	657.3	511.2	493.2	96.9	79.9	0.42
54	32.3	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-dHex-dHex(-dHex)-dHex-AG IV	1,221.5365	1,121.5	976.4	829.3	829.3	829.3	-	637.2; 537.2	411.2	393.1	96.9	79.9	0.09
55	33.1	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-Hex-dHex(-dHex)-DXU-AG VI	1,255.5415	1,237.5	1,109.5; 1,091.5	947.4; 929.4	947.4; 929.4	947.4; 929.4	801.3; 783.3	655.3; 637.3	493.2	475.2	96.9	79.9	0.46
56	33.2	C ₅₇ H ₉₃ O ₂₈ SNa	dHex-dHex-dHex(-dHex)-126-AG IV	1,221.5358	-	1,075.4	929.4	929.4	929.4	-	637.3	511.2	493.2	96.9	79.9	0.21
57	33.6	C ₅₇ H ₉₃ O ₂₇ SNa	dHex-dHex-Hex(-dHex)-dHex-AG VI	1,239.545	-	1,093.5	947.4	947.4	947.4	801.3	639.3	493.2	475.2	96.9	79.9	0.40
58	34.2	C ₅₇ H ₉₃ O ₂₇ SNa	dHex-dHex-dHex(-dHex)-DXU-AG VII	1,237.5301	1,219.5	1,091.5; 1,073.4	945.4; 927.4	945.4; 927.4	945.4; 927.4	781.3	653.3; 635.3	491.2	473.2	96.9	79.9	0.41
59	34.2	C ₅₇ H ₉₃ O ₂₇ SNa	dHex-dHex-dHex(-dHex)-DXU-AG VI	1,239.5449	1,221.5	1,093.5; 1,075.4	947.4; 929.4	947.4; 929.4	947.4; 929.4	801.3; 783.3	655.3; 637.3	493.2	475.2	96.9	79.9	1.30
60	35.2	C ₅₆ H ₉₁ O ₂₇ SNa	dHex-Hex-Pent(-dHex)-dHex-AG V	1,227.5457	-	1,081.4	919.4	919.4	919.4	773.3	641.3	495.2	477.2	96.9	79.9	0.11
61	35.2	C ₅₈ H ₉₅ O ₂₇ SNa	dHex-dHex-dHex(-dHex)-171-AG II	1,266.5564	1,239.5; 1,166.5; 1,139.5	1,120.5; 1,093.5; 993.4	974.4; 874.3; 847.3	974.4; 874.3; 847.3	974.4; 874.3; 847.3	828.3; 728.3; 701.3	682.3; 582.2; 555.2	511.2; 411.1	493.2; 393.1	96.9	79.9	0.37
62	35.3	C ₅₇ H ₉₃ O ₂₇ SNa	dHex-dHex-Hex(-dHex)-dHex-AG V	1,241.5617	-	1,095.5	949.4	949.4	949.4	803.3	641.3	495.2	477.2	96.9	79.9	0.28

Table 1 continued

No ^a	R _t	Elemental composition	Sugar chains and aglycones ^b	<i>m/z</i> ^{c, d}	Sugar chains and aglycones ^b							C, μg/ml			
					[M-Na] ⁻	[M-Na-X] ^{-e}	Y ₃ or Y _{2β}	Y _{2α}	Y ₃ /Y _{2β}	Y _{2α} /Y _{2β}	Y ₁		Y ₀	Z ₀	[HSO ₄] ⁻
63	35.8	C ₅₇ H ₉₃ O ₂₇ SiNa	dHex-dHex-dHex(-dHex)-DXU-AG V	1,241.5605	1,223.5	1,095.5; 1,077.4	949.4; 931.4	949.4; 931.4	803.3; 785.3	657.3; 639.3	495.2	477.2	96.9	79.9	0.24
65	37.3	C ₅₇ H ₉₃ O ₂₆ SiNa	dHex-dHex-dHex(-dHex)-dHex-AG V	1,225.5655	-	1,079.4	933.4	933.4	787.3	641.3	495.2	477.2	96.9	79.9	0.05
67	38.1	C ₅₈ H ₉₉ NO ₂₆ SiNa	dHex-dHex-dHex(-dHex)-171-AG VI	1,248.5462	1,221.5	1,102.5; 1,075.4	956.4; 929.4	956.4; 929.4	810.4; 783.4	664.3; 637.3	493.2	475.2	96.9	79.9	0.27

^a The numbers of the compounds correspond to the peaks on (-)LC-MS chromatogram

^b AG = aglycone, AG I for aglycones of the group I, AG II for aglycones of the group II, etc

^c The most intense ion series are highlighted bold

^d Y_i-ions corresponding the losses of sugar units from [M-Na]⁻ or [M-Na-X]⁻

^e [M-Na-X]⁻ denotes fragment ions where X = 18 (H₂O) for compounds **55**, **58**, **59**, and **63**; X = 98 for compound **26**; X = 100 for compounds **30**, **39**, **40**, **42**, **45**, **52**, **54**, and **61**; X = 118 (100 + H₂O) for compounds **32**, **44**, **48**, **50**; X = 27 for compounds **61** and **67**

^f Y₄ ions corresponding the losses of fifth sugar unit of oligosaccharide chain of hexosides **30** and **36**

losses of 3 dHex and 2 Pent. Thus product ion series of oligosaccharide moieties and aglycone in MS/MS spectra allowed asterosaponins **5** as asterone analog of ophidanoside F and **42** as ophidanoside F (Fig. 1) to be identified. No informatively useful product ion spectra of compounds **9** (*m/z* 1,127) were registered due to not enough amount of this compound in analyzed sample. However, asterosaponin **9** may be identified as novaeguinose A by comparison of its accurate mass (and so elemental composition) and chromatographic behavior with an authentic sample obtained from this starfish in the previous study (Popov et al. 2013).

It should be noted that the majority of detected asterosaponins were found to be clustered in characteristic retention time window according to their aglycone type. Variations within the oligosaccharide chains have a small effect on retention time whereas modifications of the side chains of aglycone affect retention time significantly. For example, asterosaponins **5** and **42** have the same oligosaccharide chains, but **42** contains a longer side chain in the aglycone and its retention time shifted significantly (*R_t* of **5** is 6.2 min, and *R_t* of **42** – 22.6 min). In accordance with this fact, all asterosaponins can be divided into seven groups (I–VII) according to fragmentation type of aglycones.

The group I include asterosaponins with short-chains aglycone containing CH₃CO groups as side chains (**3**, **4** and **5**). The (-)MS/MS spectra of these compounds provided Y₀ and Z₀ product ions at *m/z* 411 and 393, respectively, which are specific for asterosaponins with 3-*O*-sulfoasterone (3β,6α-dihydroxy-5α-pregn-9(11)-en-20-one) aglycone (Minale et al. 1993).

The group II includes more asterosaponins. There are compounds **30**, **39**, **40**, **42**, **44**, **45**, **48**, **50**, **52**, **54**, and **61**. Fragmentation of these compounds under CID conditions produces intense characteristic product ion series [M–100] (M = molecular ions and Y-type ions) corresponding lose of C₆H₁₂O molecule, Y₀, Z₀ product ions at *m/z* 511 and 493 and Y₀-100, Z₀-100 product ions at *m/z* 411 and 393. These product ions are characteristic for asterosaponins having 3-*O*-sulfothornasterol A aglycone with 20-hydroxy-cholestan-23-one side chain (Minale et al. 1993).

MS/MS spectra of the group III (asterosaponins **26** and **32**) produced intense product ion series [M–98] (M = molecular ions and Y-type ions), Y₀, Z₀ ions at *m/z* 509 and 491 and Y₀-98, Z₀-98 ions at *m/z* 411 and 393. Aglycones of this group are very similar to those of group II and most likely contain one double bond at the end of 20-hydroxy-cholestan-23-one side chain. The double bond position may be localization either at 24(25) or at 25(26). According literature data one isomeric aglycone of this type having Δ²⁴⁽²⁵⁾-20-hydroxy-cholestan-23-one structure of side chain (Minale et al. 1993) is known, while aglycons

Table 2 Profile of glycosides of polyhydroxysteroids and polyhydroxysteroids in extract of the Far Eastern starfish *A. japonica* detected by LC-ESI MS

No ^a	R _t	Elemental composition	m/z		C (μg/ml)
			Precursor ions [M-Na] ⁻	Product ions in MS/MS spectra	
1	4.1	C ₂₇ H ₄₀ O ₁₃ S ₂ Na ₂	318.0952 ^b	96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.82
			637.1974 ^c	96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	
			659.1795 ^d	539.2 [M ₂ Na-Na-NaHSO ₄] ⁻ ; 411.1 [M ₂ Na-Na-dHexSO ₃ Na] ⁻ ; 393.1 [M ₂ Na-Na-dHexSO ₃ Na-H ₂ O] ⁻ ; 225.0 [dHexSO ₃] ⁻ ; 164.9 [C ₄ H ₅ O ₅ S] ⁻ ; 136.9 [C ₃ H ₅ O ₄ S] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	
2	5.4	C ₃₂ H ₅₅ O ₁₄ SNa	695.3297	677.3 [M-Na-H ₂ O] ⁻ ; 563.3 [M-Na-Pent] ⁻ ; 545.3 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.30
6	6.4	C ₂₁ H ₃₁ O ₆ SNa	411.1848	96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.09
7	6.6	C ₂₇ H ₄₁ O ₁₀ SNa	557.2532	Lack of product ions in MS/MS spectra	0.07
8	6.7	C ₃₁ H ₅₁ O ₁₂ SNa	647.3116	Lack of product ions in MS/MS spectra	0.22
10	8.1	C ₃₂ H ₅₅ O ₁₂ SNa	663.3416	645.3 [M-Na-H ₂ O] ⁻ ; 531.3 [M-Na-Pent] ⁻ ; 513.2 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.50
11	8.3	C ₃₂ H ₅₅ O ₁₄ SNa	695.3328	210.9 [PentSO ₃] ⁻ ; 192.9 [C ₅ H ₅ O ₆ S] ⁻ ; 150.9 [C ₃ H ₅ O ₅ S] ⁻ ; 122.9 [C ₂ H ₃ O ₄ S] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.15
12	8.3	C ₃₁ H ₅₁ O ₁₂ SNa	647.3119	Lack of product ions in MS/MS spectra	0.13
13	9.2	C ₃₂ H ₅₃ O ₁₂ SNa	661.3258	643.3 [M-Na-H ₂ O] ⁻ ; 529.2 [M-Na-Pent] ⁻ ; 511.2 [M-Na-Pent-H ₂ O] ⁻ ; 493.2 [M-Na-Pent-2 × H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.13
14	9.5	C ₃₁ H ₅₁ O ₁₁ SNa	631.3147	210.9 [PentSO ₃] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.18
15	10.1	C ₃₂ H ₅₅ O ₁₂ SNa	663.3408	645.3 [M-Na-H ₂ O] ⁻ ; 531.3 [M-Na-Pent] ⁻ ; 513.2 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.20
16	10.3	C ₂₇ H ₄₇ O ₈ SNa	531.2982	Lack of product ions in MS/MS spectra	0.05
17	10.7	C ₃₃ H ₅₅ O ₁₂ SNa	675.3413	543.3 [M-Na-Pent] ⁻ ; 525.3 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.25
19	11.5	C ₃₇ H ₆₃ O ₁₆ SNa	795.3844	663.3 [M-Na-Pent] ⁻ ; 645.3 [M-Na-Pent-H ₂ O] ⁻ ; 210.9 [PentSO ₃] ⁻ ; 150.9 [C ₃ H ₅ O ₅ S] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.32
20	12.1	C ₃₂ H ₅₅ O ₁₁ SNa	647.3479	629.3 [M-Na-H ₂ O] ⁻ ; 515.3 [M-Na-Pent] ⁻ ; 497.3 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.24
21	12.6	C ₃₂ H ₅₅ O ₁₂ SNa	663.3407	210.9 [PentSO ₃] ⁻ ; 194.9 [C ₅ H ₇ O ₆ S] ⁻ ; 168.9 [C ₃ H ₅ O ₆ S] ⁻ ; 150.9 [C ₃ H ₅ O ₅ S] ⁻ ; 138.9 [C ₂ H ₃ O ₅ S] ⁻ ; 122.9 [C ₂ H ₃ O ₄ S] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	1.47
22	12.6	C ₃₃ H ₅₇ O ₁₃ SNa	693.3511	675.3 [M-Na-H ₂ O] ⁻ ; 241.0 [HexSO ₃] ⁻ ; 198.99 [C ₄ H ₇ O ₇ S] ⁻ ; 180.99 [C ₄ H ₅ O ₆ S] ⁻ ; 168.98 [C ₃ H ₅ O ₆ S] ⁻ ; 150.9 [C ₃ H ₅ O ₅ S] ⁻ ; 138.97 [C ₂ H ₃ O ₅ S] ⁻ ; 122.9 [C ₂ H ₃ O ₄ S] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.31
23	12.6	C ₃₂ H ₅₃ O ₁₂ SNa	661.3239	643.3 [M-Na-H ₂ O] ⁻ ; 529.3 [M-Na-Pent] ⁻ ; 511.2 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.23
24	13.7	C ₃₂ H ₅₃ O ₁₁ SNa	645.3326	513.3 [M-Na-Pent] ⁻ ; 495.2 [M-Na-Pent-H ₂ O] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.11
25	14.1	C ₃₂ H ₅₅ O ₁₂ SNa	663.3409	210.9 [PentSO ₃] ⁻ ; 192.9 [C ₅ H ₅ O ₆ S] ⁻ ; 168.9 [C ₃ H ₅ O ₆ S] ⁻ ; 150.9 [C ₃ H ₅ O ₅ S] ⁻ ; 138.9 [C ₂ H ₃ O ₅ S] ⁻ ; 122.9 [C ₂ H ₃ O ₄ S] ⁻ ; 96.9 [HSO ₄] ⁻ ; 79.9 [SO ₃] ⁻	0.45

Table 2 continued

No ^a	R _t	Elemental composition	m/z		C (μg/ml)
			Precursor ions [M–Na] [–]	Product ions in MS/MS spectra	
27	14.4	C ₃₅ H ₅₉ O ₁₃ SNa	719.3668	701.3 [M–Na–H ₂ O] [–] ; 241.0 [HexSO ₃] [–] ; 198.99 [C ₄ H ₇ O ₇ S] [–] ; 180.9 [C ₄ H ₅ O ₆ S] [–] ; 164.9 [C ₄ H ₅ O ₅ S] [–] ; 150.9 [C ₃ H ₃ O ₅ S] [–] ; 138.9 [C ₂ H ₃ O ₅ S] [–] ; 122.9 [C ₂ H ₃ O ₄ S] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.30
28	14.8	C ₃₂ H ₅₁ O ₁₃ SNa	675.3076	Lack of product ions in MS/MS spectra	0.21
29	15.0	C ₃₂ H ₅₃ O ₁₂ SNa	661.3271	210.9 [PentSO ₃] [–] ; 194.9 [C ₅ O ₆ H ₇ S] [–] ; 150.9 [C ₃ H ₃ O ₅ S] [–] ; 138.9 [C ₂ H ₃ O ₅ S] [–] ; 122.9 [C ₂ H ₃ O ₄ S] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.26
31	15.6	C ₃₃ H ₅₂ O ₁₄ S ₂ Na ₂	368.1408 ^b 737.2886 ^c 759.2703 ^d	96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–] 657.3 [M _{Na+H} –Na–SO ₃] [–] ; 225.0 [dHexSO ₃] [–] ; 164.9 [C ₄ H ₅ O ₅ S] [–] ; 136.9 [C ₃ H ₅ O ₄ S] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–] 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	13.53
33	16.5	C ₃₂ H ₅₅ O ₁₂ SNa	663.3400	Lack of product ions in MS/MS spectra	0.19
34	16.7	C ₃₃ H ₅₅ O ₁₂ SNa	675.3415	543.3 [M–Na–Pent] [–] ; 525.3 [M–Na–Pent–H ₂ O] [–] ; 507.2 [M–Na–Pent–2 × H ₂ O] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.16
35	17.3	C ₃₅ H ₆₁ O ₁₃ SNa	721.3832	703.3 [M–Na–H ₂ O] [–] ; 685.3 [M–Na–2 × H ₂ O] [–] ; 241.0 [HexSO ₃] [–] ; 198.9 [C ₄ H ₇ O ₇ S] [–] ; 180.9 [C ₄ H ₅ O ₆ S] [–] ; 164.9 [C ₄ H ₅ O ₅ S] [–] ; 150.9 [C ₃ H ₃ O ₅ S] [–] ; 138.9 [C ₂ H ₃ O ₅ S] [–] ; 122.9 [C ₂ H ₃ O ₄ S] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.28
37	18.2	C ₃₂ H ₅₅ O ₁₁ SNa	647.3461	515.3 [M–Na–Pent] [–] ; 497.2 [M–Na–Pent–H ₂ O] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.26
38	18.7	C ₃₂ H ₅₃ O ₁₁ SNa	645.3324	513.3 [M–Na–Pent] [–] ; 495.2 [M–Na–Pent–H ₂ O] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.19
46	26.9	C ₂₇ H ₄₃ O ₇ SNa	511.2734	Lack of product ions in MS/MS spectra	0.10
47	27.0	C ₃₃ H ₅₅ O ₁₁ SNa	659.3470	527.3 [M–Na–Pent] [–] ; 509.2 [M–Na–Pent–H ₂ O] [–] ; 96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.13
51	30.1	C ₂₇ H ₄₃ O ₇ SNa	511.2737	96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.21
64	36.4	C ₂₇ H ₄₁ O ₆ SNa	493.2636	96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.06
66	38.0	C ₂₇ H ₄₃ O ₆ SNa	495.2792	96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.03
68	38.9	C ₂₈ H ₄₇ O ₆ SNa	511.3101	96.9 [HSO ₄] [–] ; 79.9 [SO ₃] [–]	0.06

^a The numbers of the compounds correspond to the peaks on (–)LC–MS chromatogram

^b The mass of the precursor ion corresponds to [M–2 × Na]^{2–} ion

^c The mass of the precursor ion corresponds to [M_{Na+H}–Na][–] ion

^d The mass of the precursor ion corresponds to [M_{2Na}–Na][–] ion

with Δ²⁵⁽²⁶⁾-20-hydroxy-cholestan-23-one side chains were not found before. That is why we suggest that these asterosaponins contain the Δ²⁴⁽²⁵⁾-20-hydroxy-23-one structures of side chains in aglycones.

The additional information about the aglycone structures were provided by LC–MS analysis of mixture of sulfated aglycones, obtained by particular hydrolysis of steroidal sum (see “Materials and methods”). Aglycones of

asterosaponins of groups II and III are labile and hydrolyzed to the 3-*O*-sulfoasterone. The LC–MS analysis showed the presence of 3-*O*-sulfoasterone in sample after hydrolysis and no presence of 3-*O*-sulfothornasterol A and its unsaturated derivative. It confirms the presence of 20-hydroxy-23-oxo group in their aglycones.

Fragmentation of the group IV of asterosaponins (**36**, **41**, **43**, **49**, **53**, and **56**) gives product ions Y₀, Z₀ at m/z 511 and

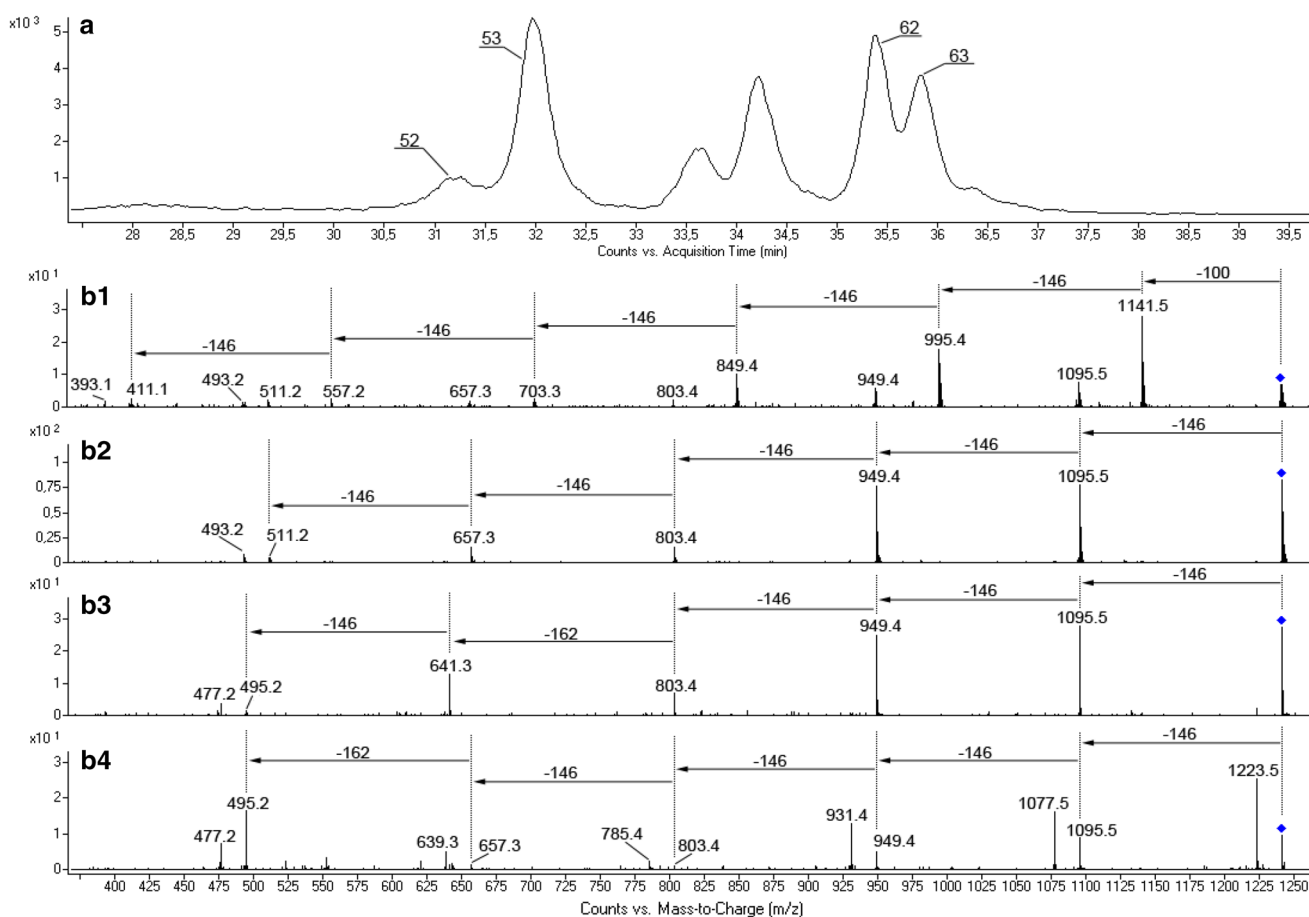


Fig. 2 Single ion chromatogram of $[M-Na]^+$ ions at m/z 1,241 for isomers **52**, **53**, **62**, **63** presented in extract of starfish *A. japonica* (**a**). MS/MS spectra registered for isomeric compounds **52**, **53**, **62**, **63**

separated on LC (**b1**, **b2**, **b3**, **b4**, respectively). Chromatographic peaks at 33.6 and 34.2 min corresponds to $M + 2$ ions of compounds **57** and **59** at m/z 1,239

493 (the same elemental composition as in the group II), but unlike the group II there are no specific ion series $[M-100]$ of losses fragments of side chain. Since the loss of $C_6H_{12}O$ arises from retro aldol cleavage of C-20–C-22 bond with the participation hydroxyl group at C-20, it may be assumed, that aglycones of this group also have keto- and hydroxyl group in the side chain, but in different positions. According to literature data this fragmentation pattern is specific for asterosaponins having aglycones with $\Delta^{24(25)}$ -20,22-dihydroxy-cholestan side chain, 20-hydroxy-cholestan-22,23-epoxy side chain (Minale et al. 1993), 20-hydroxy-cholestan-22-one side chain (Stonik 2001) or 24-hydroxy-cholestan-23-one side chain as in aphelaketotriol isolated early from this starfish (Ivanchina et al. 2000). The LC–MS analysis of sample after hydrolysis has shown presence of two chromatographic peaks of aglycones of m/z 511. More intense peak was identified as aphelaketotriol on basis of comparison of its retention time with retention times of aphelaketotriols previously isolated from this starfish.

The group V of detected asterosaponins (**60**, **62**, **63**, and **65**) exhibited in $(-)$ MS/MS spectra product ions Y_0 and Z_0

at m/z 495 and 477 and did not show fragmentation of side chains. This is characteristic of asterosaponins with 3-*O*-sulfo-24,25-dihydromarthasterone aglycone having cholestan-23-one side chain isolated early from this starfish or aglycone with $\Delta^{24(25)}$ -20-hydroxy-cholestan side chain (Minale et al. 1993). The LC–MS analysis of sample after hydrolysis has shown presence of two chromatographic peaks of aglycones of m/z 495, one of them was identified as 3-*O*-sulfo-24,25-dihydromarthasterone on basis of comparison of their retention times with that of one previously isolated from this starfish (Kicha et al. 2001b).

The group VI (**55**, **57**, **59**, and **67**) produced Y_0 and Z_0 fragments at m/z 493 and 475 whereas group VII (**58**) had Y_0 and Z_0 at m/z 491 and 473 and no fragmentation of the side chains was observed in neither group. Thus, aglycones of these groups are differing in 2 and 4 Da versus aglycone of the group V. This indicates the presence of one and two double bonds in aglycones groups VI and VII, respectively. The analysis of literature data showed that these aglycones may be of marthasterone type with $\Delta^{24(25)}$ -cholestan-23-one side chain (Minale et al. 1993) or aglycone with

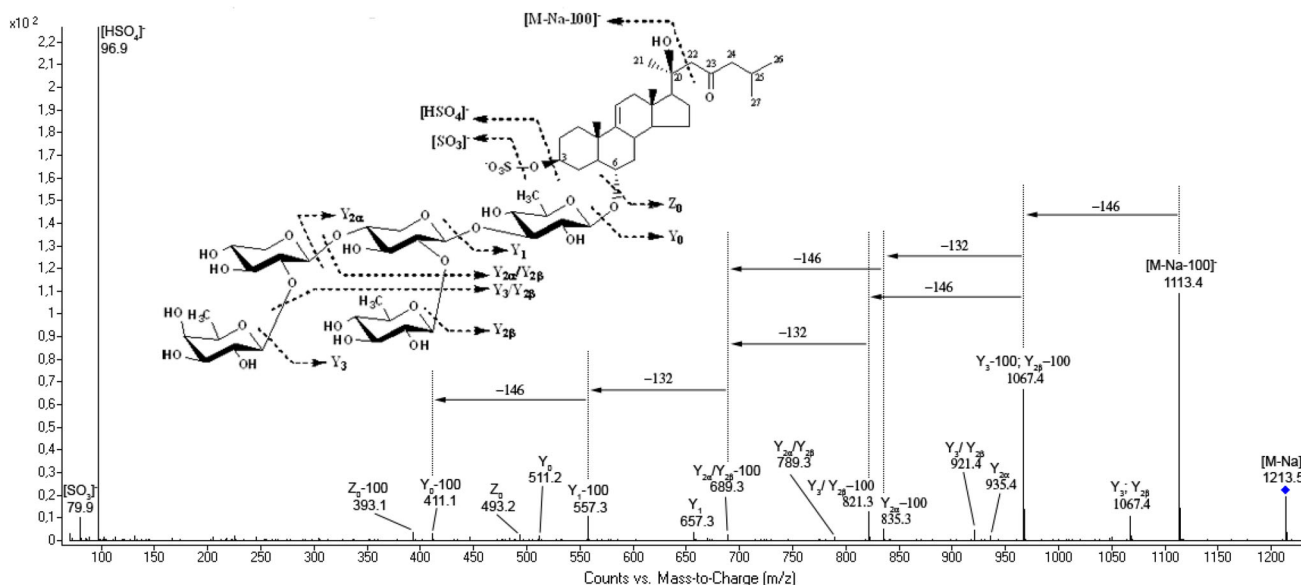


Fig. 3 ESI MS/MS spectrum of $[M-Na]^-$ precursor ion at m/z 1,213 (**42**) identified as ophidianside F

$\Delta^{20(22)}$ -cholestan-23-one side chain (Levina et al. 2010) for the group VI and aglycone with $\Delta^{17(20),24(25)}$ -cholestan-23-one side chain (Minale et al. 1993) for the group VII. The LC-MS analysis of the sample after hydrolysis has shown presence of two peaks of aglycones at m/z 493, one of them was identified as 3-*O*-sulfomarthasterone on basis of comparison of its retention times with that of one, previously isolated from starfish *Asterias rathbuni* (Ivanchina et al. 2001), and a peak of aglycone of asterosaponin **58** at m/z 491.

In addition, a number of asterosaponins with unusual monosaccharide units were found in analyzed sample. Tandem MS of asterosaponins **32**, **44**, **48**, **50**, **55**, **59**, **58**, and **63** provided intense product ion series $[M-H_2O]$, where M = molecular ions and Y-type ions, except Y_0 (as example, product ion spectrum of **63** is shown in Fig. 2-b4). Sequencing of carbohydrate chains of these asterosaponins showed neutral losses of four deoxyhexoses (except asterosaponin **55**, containing three deoxyhexose and one hexose residues) and fragment with mass 162 Da. According to literature data, neutral loss of 162 Da along with intense product ion series $[M-H_2O]$ in this case is due to the presence of rare deoxy-xylo-hexulose unit (DXU) in saccharide moieties (Sandvoss et al. 2001).

Fragmentation of oligosaccharide chains of compounds **54** and **56** displays similar product series of Y-type ions and neutral losses of four deoxyhexoses and fragment with mass 126 Da in the both cases. The exact mass of 126.0331 indicated the possible elemental composition of $C_6H_6O_3$ for this fragment, corresponding to a hexose that lost two H_2O molecules. The monosaccharides of this type were so far not identified from marine sources.

Asterosaponins (**61** and **67**) with even values of m/z of molecular ions, which indicate the possible presence of nitrogen in these compounds, were also found in analyzed sample mixture of secondary metabolites. The MS/MS spectra of both compounds produce unusual product ion series $[M-27.0116]$ (M = molecular ions and Y-type ions, except Y_0). Sequencing of oligosaccharide chains displays neutral losses of four deoxyhexoses and fragment with mass 171 Da. Moreover the positive product ion spectra of **61** and **67** gave the same series of B- and C-type ions, confirming structure of oligosaccharide chains: 796 $[4 \times dHex + 171 + Na + H_2O]^+$, 778 $[4 \times dHex + 171 + Na]^+$, 607 $[4 \times dHex + Na]^+$, 461 $[3 \times dHex + Na]^+$, 315 $[2 \times dHex + Na]^+$. The exact mass of 171.0527 indicated to the possible elemental composition $C_7H_9O_4N$ of the fragment. The monosaccharides of this type were also not being identified from marine sources so far. It should be noted that MS/MS spectra of asterosaponins with unusual monosaccharide units demonstrated Y_0 ions with higher intensities and $Y_{2\alpha}/Y_{2\beta}$ ions with lower intensities than same ions in MS/MS spectra of asterosaponins with usual monosaccharide units, while MS/MS spectra of compounds **54** and **56** had not consist of $Y_{2\alpha}/Y_{2\beta}$ fragment ions at all. All these data indicated the presence of new unusual asterosaponins in this starfish.

Twenty-eight sulfated glycosides of polyhydroxysteroids and 7 sulfated polyhydroxysteroid compounds were also found among polar steroid metabolites of this species. The 29 of these compounds were characterized by tandem MS (Table 2). Because of small amount of presented polyhydroxysteroid compounds in analyzed mixture, not all of them were subjected of tandem MS. In other cases,

the (–)MS/MS spectra were uninformative and demonstrated only fragment ions of m/z 96.9 and 79.9 corresponding to sulfate group.

In MS/MS spectra of glycosides of polyhydroxysteroids, fragment ions connected with dissociation of monosaccharide units and sulfate group were detected. Some compounds showed fragment ions corresponding sulfated monosaccharide units: sulfated pentose (fragment ion at m/z 210.9 in MS/MS spectra of **11**, **14**, **21**, **25**, and **29**), sulfated hexose (fragment ion at m/z 225.0 in MS/MS spectra of **22**, **27**, and **35**) and sulfated deoxyhexose (fragment ion at m/z 241.0 in MS/MS spectra of **1** and **31**). Glycosides **1** and **31** with sulfated deoxyhexose unit have sulfate group in steroid nucleus additionally. These compounds showed intense double charged molecular ion peaks $[M-2Na]^{2-}$ at m/z 318 and 368, whereas intensities of single charged ion peaks $[M_{Na+H}-Na]^-$ and $[M_{2Na}-Na]^-$ were much lower. In some cases, in (–)MS/MS spectra of glycosides having sulfate monosaccharide units, the A-type product ions, formed by cross-ring cleavage of sulfated monosaccharide, were detected in a good agreement with earlier reported results (Minamisawa and Hirabayashi 2005; Gonçalves et al. 2010).

In (–)MS/MS spectra of other glycosides (**2**, **10**, **13**, **15**, **17**, **20**, **23**, **24**, **34**, **37**, **38**, and **47**), product ions Y_0 and Z_0 corresponding losses of one monosaccharide unit with charge located on aglycone were found. It should be noted that all those glycosides have pentose monosaccharide unit. One compound (**19**) with $[M-Na]^-$ at m/z 795 has two pentose monosaccharide units, one of which was sulfated, as was indicated by the ions 663 $[M-Na-Pent]^-$ and 210.9 $[PentSO_3]^-$ in (–)MS/MS spectra.

Among 14 previously known polyhydroxysteroid compounds from *A. japonica*, 3-*O*-sulfoasterone (**6**), forbeside E3 (**7**), aphelasteroside E (**10**), pycnopodioside C (**22**), 3-*O*-sulfothornasterol A (**46**), aphelaketotriol (**51**), 3-*O*-sulfo-24,25-dihydromarthasterone (**66**) were identified on basis of comparison of their retention times, MS spectra and elemental compositions with those of standard compounds, isolated earlier from this starfish. The cheliferoside L1 (**1**), aphelasteroside A (**15**), aphelasteroside B (**28**), 5 α -cholestane-3 β ,6 α ,8,15 α ,24-pentaol-24-sulfate (**16**), aphelasteroside C (**31**), and other compounds given on the Fig. 1 were identified on basis of their mass spectra, elemental compositions, and chromatographic behavior.

Analysis of structures of the compounds, isolated earlier from this species, as well as our previous analysis on more than 500 structures of similar compounds from 64 different starfishes (Kicha et al. 2009) shows that, whereas mosaic character of their biosynthesis, hydroxylations of these polar steroids during their biosynthesis from dietary sterols or sterol sulfates (Ivanchina et al. 2013) generally follows the next order: at the position 6, then at position 24 or 26

(for stigmastane derivatives at the position 29), then at position 15 or 8, then at position 16 and other positions. In some species retardation of hydroxylation at some positions was observed. In the case of *A. japonica*, it concerns mainly the position 8.

Another characteristic feature of biosynthesis in this species is early dihydroxylation of a part of metabolites at the positions 5 and 6 in combination with hydroxylation at the side chain. These regularities as well as chromatographic behavior of constituents from the analyzed mixture expedite their identification. Actually, the more polar constituents have smaller retention times, while the contributions of different fragments of their molecules into chromatographic behavior are quite different (Table 2). Sulfate group and xylosyl residue decreases retention times almost equally and much more than introduction of an additional hydroxyl group. Introduction of sulfate at C-26 (or at C-29 in stigmastane series) influence retention time more than that into C-3 or C-24 positions and that is much more than into other positions in tetracyclic system. Introduction of double bonds and especially elongation of side chain increases the retention times.

Absolute majority of metabolites belonging to polyhydroxylated derivatives from *A. japonica* contain sulfate groups. It means that sulfation terminates further biosynthetic transformations. Taking into account the termination of further transformations after sulfation, existence of many earlier unidentified constituents may be predicted, including the both additionally hydroxylated previously known metabolites or in the contrary less hydroxylated precursor compounds, whose further transformations were terminated after their sulfation. Some other metabolites are proved to be saturated or unsaturated analogs of other metabolites, with double bonds occupying either 22(23)- or 4(5)-position. Their structures must correspond to theoretical biosynthetic pathway of polyhydroxylated steroids with cholestane side chains in *A. japonica*, given on Fig. 4.

For example, one the most polar metabolite (**2**) of these series is most probably a 3 β -xylosyl-5 α -cholestane-3 β ,5,6 β ,8,15 α ,16 β ,26-heptaol 26-sulfate ($[M-Na]^-$ ion peak at m/z 695.3297 (calcd 695.3318)), which is a dihydroxylated analog of aphelasteroside E (**10**) ($[M-Na]^-$ ion at m/z 663.3416 (calcd 663.3420)). In fact, a sulfate group at C-26 along with two additional hydroxyl groups strongly increases polarity of this constituent and decreases its retention time.

In contrast with MS spectra of **2** and aphelasteroside E (**10**), there are two peaks at m/z 210.9 $[PentSO_3]^-$ and 192.9 $[PentSO_3-H_2O]^-$ in ESI MS spectra of **11**. It indicates that sulfate group is located on pentose residue. The compound **13** was preliminary identified as 22(23)-dehydroaphelasteroside E by $[M-Na]^-$ ion peak at m/z 661.3258 (calcd 661.3263).

The compound **15** with $[M-Na]^-$ ion peak at m/z 663.3408 (calcd 663.3420) was identified as aphelasteroside A. The compound **23** most probably is its 22(23)-unsaturated derivative ($[M-Na]^-$ ion peak at m/z 661.3239 (calcd 661.3263)). The compound **16** ($[M-Na]^-$ peak at m/z 531.2982 (calcd 531.2997)) was identified as 5 α -cholestan-3 β ,6 α ,8,15 α ,24-pentaol-24-sulfate, earlier isolated from the studied species (Finamore et al. 1992). The compound **19** ($[M-Na]^-$ ion peak at m/z 795.3844 (calcd 795.3842)) was found to be an analog of **15** containing an additional sulfated xylose residue instead of sulfate group. The compound **20** ($[M-Na]^-$ ion peak at m/z 647.3479 (calcd 647.3471)) and **24** ($[M-Na]^-$ ion peak at m/z 645.3326 (calcd 645.3314)) are probably 8-dehydroxy aphelasteroside A (**15**) and its unsaturated derivative. The compounds **21** ($[M-Na]^-$ ion peak at m/z 663.3407 (calcd 663.3420)) and **25** ($[M-Na]^-$ ion peak at m/z 663.3409 (calcd 663.3420)) are isomers of aphelasteroside A, differing in the presence of sulfate group in xylose residue. ESI MS spectra of these metabolites contain ion peaks at m/z 210.9 $[PentSO_3]^-$ and 150.9 $[C_3H_3O_5S]^-$ and others showing this fragment. The compound **29** most probably is 22(23)-unsaturated derivative of **25** ($[M-Na]^-$ ion peak at m/z 661.3271 (calcd 661.3263)).

The compound **22** ($[M-Na]^-$ peak at m/z 693.3511 (calcd 693.3525)) was identified as pycnopodioside C, earlier isolated from this starfish (Kicha et al. 2001b). The compound **28** ($[M-Na]^-$ ion peak at m/z 675.3076 (calcd 675.3056)) was identified as aphelasteroside B, earlier found in this species (Finamore et al. 1992). The compound **35** ($[M-Na]^-$ ion peak at m/z 721.3832 (calcd 721.3838)) was proposed to be 8-dehydroxy derivative of aphelasteroside D on the basis of chromatographic behavior and the order of hydroxylation stages of polar steroids in this starfish with retardation of hydroxylation of the position 8 (Kicha et al. 2001b). In ESI MS spectrum of **35**, a peak at m/z 241.0 $[HexSO_3]^-$ and a series of other ion peaks confirmed the presence of sulfate glucose residue in this metabolite.

The compound **37** ($[M-Na]^-$ ion peak at m/z 647.3461 (calcd 647.3471)) and **38** ($[M-Na]^-$ ion peak at m/z 645.3324 (calcd 645.3314)) are significantly less polar analogs of **20** and **24**. It may be explained by the presence tertiary hydroxyl instead of secondary ones in their polycyclic moieties.

The compound **66** ($[M-Na]^-$ peak at m/z 495.2792 (calcd 495.2786)) was identified as 3-*O*-sulfo-24,25-dihydromarthasterone on basis of comparison of their retention times with those previously isolated from this starfish (Kicha et al. 2001b). The compound **64** ($[M-Na]^-$ ion peak at m/z 493.2636 (calcd 493.2629)) was identified as 3-*O*-sulfomarthasterone on basis of comparison of its retention times with those previously isolated from starfish

A. rathbuni (Ivanchina et al. 2001). The compound **68** ($[M-Na]^-$ peak at m/z 511.3101 (calcd 511.3099)) is the least polar one from all metabolites. Taking into account elemental composition and that elongation of side chain increases retention time, the compound **68** was indicated as containing a hydroxy-ergostane side chain (probably 20-hydroxy-ergostane side chain).

Structures of these preliminary identified metabolites are given on the Fig. 1 and Fig. 4. These structures confirm theoretical biosynthetic pathway of polar polyhydroxylated steroids in *A. japonica* via the consecutive introduction of hydroxyl groups into sterol precursors followed by glycosylation and sulfation.

From the 17 steroid compounds previously isolated from this starfish only 15 compound were found in analyzed sample. The aphelasteroside D isolated early from the Kuril population and (23*S*)-5 α -cholest-9(11)-en-3 β ,6 α ,23-triol 3,6-disulfate isolated early from the Japanese population of this starfish were not found in the analyzed mixture. This fact confirms the assumption that steroid compositions of starfish depends on ecological factors such as environmental conditions, diet and season (Ivanchina et al. 2000; Kicha et al. 2004).

Quantitative analysis of polar steroids from *A. japonica* was also performed. We used sulfated polyhydroxysteroid glycoside asterosaponin P₁ isolated from starfish *Patiria pectinifera* as a reference standard (Ivanchina et al. 2013). Asterosaponin P₁ at concentrations of 1.0, 2.5, 5.0, and 10.0 μ g/ml was used for building calibration curve. All the experiments were carried out at least three times, experimental conditions are identical to those described above. As a result, a rough evaluation of amount of detected compounds was calculated from calibration curve ($R^2 = 0.9982$). Results are shown in Tables 1 and 2.

4 Conclusions

Profiling of steroid glycosides and related compounds from *A. japonica* was performed by LC-ESI MS. LC-ESI MS/MS allowed the preassignment and identification of the constituents to be performed. Structural information on the different steroid glycosides, their aglycones and the saccharide moieties was obtained from the MS and MS/MS spectra. The MS and MS/MS spectra of 33 asterosaponins including 3 previously isolated compounds and of 35 polyhydroxysteroids compounds including 13 previously isolated compounds were described. Although in many cases stereochemistry and some details of exact structures cannot be deduced from mass spectra, reasonable proposals for new glycosides structures can be given as it was demonstrated for 33 asterosaponins. A big series of polyhydroxylated steroids compounds were detected and their

exact or preliminary structures were also proposed. A theoretical scheme of biogenesis of this type of polar steroids in the studied species was given. It was shown that LC–ESI MS and LC–ESI MS/MS are quite applicable for the profiling of steroid compounds in such complex mixtures as starfish extracts and useful for comparing steroid metabolomic profiles of different starfish species and populations for ecological, dietary and biosynthesis studies.

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