Elucidation of molecular diversity and body distribution of saponins in the sea cucumber *Holothuria forskali* (Echinodermata) by mass spectrometry

Séverine Van Dyck [a], Pascal Gerbaux [b,*], Patrick Flammang [a,*]

[a] *Marine Biology Laboratory, University of Mons-Hainaut, 6 Avenue du Champ de Mars, B-7000 Mons, Belgium*
[b] *Organic Chemistry Laboratory, Mass Spectrometry Center, University of Mons-Hainaut, 19 Avenue Maistriau, B-7000 Mons, Belgium*

ARTICLE INFO

ABSTRACT

Sea cucumbers contain triterpene glycosides called saponins. We investigated the complex saponin mixture extracted from the common Mediterranean species *Holothuria forskali*. Two different body components were analyzed separately: the body wall (which protects the animal and is moreover the most important organ in terms of surface and weight) and the Cuvierian tubules (a defensive organ that can be expelled on predators in response to an attack). MALDI/MS and MALDI/MS/MS were used to detect saponins and describe their molecular structures. As isomers have been found in the Cuvierian tubules, LC/MS and LC/MS/MS were performed to identify each saponin separately. Twelve saponins have been detected in the body wall and 26 in the Cuvierian tubules. All the saponins from the body wall are also present in the Cuvierian tubules but the latter also contain 14 specific saponins. The presence of isomeric saponins complicated structure elucidation for the whole set but 16 saponins have been described tentatively. Among these, 3 had already been reported in the literature as holothurinosides A and C, and desholothurin A. Molecular structures have been proposed for the 13 others which, in the present work, have been provisionally named holothurinosides E, F, G, H, I, A_1, C_1, E_1, F_1, G_1, H_1 and I_1 and desholothurin A_1. The diversity and organ specificity of the saponins described here are much higher than what had been reported to date in any sea cucumber species.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

Saponins are an important class of natural products first discovered in higher plants (Guo et al., 2006). A lot of studies have been conducted on these compounds which possess a variety of biological and pharmacological activities (Guo et al., 2006). In the marine environment, holothuroids (sea cucumbers) (Yamanouchi, 1955; Nigrelli, 1952), asteroids (sea stars) (Mackie et al., 1968; Mackie and Turner, 1970) and sponges (Pawlik, 1993; Kubanek et al., 2002) are the only organisms in which saponins have been hitherto found. Echinoderm saponins have been reported to possess a wide spectrum of pharmacological effects including hemolytic, antitumoral, anti-inflammatory, antifungal, antibacterial, antiviral, ichthyotoxic, cytostatic and antineoplastic activities (Kerr and Chen, 1995; Kalinin et al., 1995; Kalinin et al., 1996; Prokofieva et al., 2003). On the other hand, the biological roles of these molecules are still very speculative (Garneau et al., 1989; Kalinin et al., 1996). In sponges, it has been demonstrated that saponins have multiple defensive roles at natural volumetric concentration, acting as feeding-deterrent against predators and protecting the body surface from microbial attachment and fouling by invertebrates and algae (Kubanek et al., 2002). Similar roles have been proposed, but never demonstrated, for holothuroid saponins because, like sponges, these animals lack structural or behavioural defences (Hamel and Mercier, 2000). Indeed, their reduced skeleton and slow motion make sea cucumbers vulnerable to predation (Francour, 1997).

In addition to saponins, some sea cucumbers, all belonging to the family Holothuriidae, possess another defence system called the Cuvierian tubules (Hamel and Mercier, 2000). This system consists of multiple tubules located in the posterior part of the animal. When attacked, the sea cucumber undergoes a body contraction leading to the ejection of a few tubules via the anus. Expelled tubules lengthen into sticky white threads susceptible to entangle potential predators. In holothuriids, saponins appear to be particularly concentrated in the Cuvierian tubules (Matsuno and Ishida, 1969; Elyakov et al., 1973; Kobayashi et al., 1991). They are also quite abundant in the body wall which, in addition to its role as a physical barrier protecting the animal, is also the largest organ (Matsuno and Ishida, 1969; Kobayashi et al., 1991).

Holothuroid saponins are triterpene glycosides. The structure of the aglycone moiety, a holostane-3β-ol, is derived from the tetracyclic triterpene lanostane-3β-ol in which the D-ring contains a γ-18(20)-lactone (Habermehl and Volkwein, 1971; Stonik et al., 1999). The carbohydrate moiety is bound to the C3 of the aglycone and may include xylose, glucose, quinovose and 3-O-methylglucose residues, as well as a sulphate group (Stonik and Elyakov, 1988; Kalinin et al., 1994).

* Corresponding authors. Flammang is to be contacted at Tel.: +32 65 37 34 39; fax: +32 65 37 34 34. Gerbaux, Tel.: +32 65 37 33 42; fax: +32 65 37 30 54.
E-mail addresses: Pascal.Gerbaux@umh.ac.be (P. Gerbaux), Patrick.Flammang@umh.ac.be (P. Flammang).

Rodrigez et al. (1991) were the first to make an extensive study on the saponins of *Holothuria forskali*. They extracted saponins from a pool of 19 specimens and described five non-sulphated saponins: holothurinosides A, B, C, D and desholothurin A (**1**, **2**, **3**, **4** and **5**, respectively; Fig. 1). Structures were established by FAB/MS (Fast Atom Bombardment Mass Spectrometry), COSY (Correlation Spectroscopy), NOESY (Nuclear Overhauser Enhancement Spectroscopy) and long range and direct XHCORR experiments. Because saponins were extracted from whole specimens, no comparison could be made between different body components.

In the present report, we used MALDI/MS and MALDI/MS/MS (Matrix Assisted Laser Desorption/ionization Mass Spectrometry) methods to describe saponins from *H. forskali*. In addition, LC/MS and LC/MS/MS (Liquid Chromatography Mass Spectrometry) methodologies were necessary to differentiate all the saponins of the extracts. Saponins from the body wall and from Cuvierian tubules were extracted and analyzed separately, allowing comparisons to be made between these two body components.

2. Materials and methods

2.1. Sampling

Individuals of *H. forskali* (Delle Chiaje, 1823) were collected at a depth of 20 to 30 m by SCUBA diving at the biological station of Banyuls-sur-Mer (France). They were transported to the Marine Biology Laboratory of the University of Mons-Hainaut, where they were kept in a marine aquarium with closed circulation (13 °C, 33 psu). Holothuroids were dissected and body wall and Cuvierian tubules were stored separately in 70% ethanol at −20 °C.

2.2. Extraction of saponins

The following extraction procedure is adapted from Campagnuolo et al. (2001) and Garneau et al. (1983). Body wall and Cuvierian tubules underwent the same extraction method, which was repeated on several individuals. The homogenized tissue was extracted twice with ethanol:water (70:30) followed by filtration. The extract was then evaporated at low pressure in a double boiler at 30 °C using a rotary evaporator (Laborota 4001 efficient, Heidolph). The dry extract was diluted in 90% methanol and partitioned against n-hexane (v/v). Then, the water content of the hydromethanolic phase was adjusted to 20% (v/v) and 40% (v/v) and the solutions partitioned against CCl_4 and $CHCl_3$, respectively. Finally, the hydromethanolic solution was evaporated and dissolved in water in order to undergo chromatographic purification.

2.3. Purification of the extract

The crude aqueous extract was placed on a column of Amberlite XAD-4 (Sigma-Aldrich St. Louis, MO, USA). Washing the column with water removed the inorganic salts and subsequent elution with methanol allowed to recover saponins. The methanolic phase was then evaporated and the dry extract was diluted in water in order to undergo a last partitioning against iso-butanol (v/v). The butanolic fraction contained the purified saponins.

2.4. Mass spectrometry

All mass spectrometry experiments were performed on a Waters QToF Premier mass spectrometer in the positive ion mode, either using the MALDI or the Electrospray ionization (ESI) sources.

The *MALDI source* was constituted by a nitrogen laser, operating at 337 nm with a maximum output of 500 mW delivered to the sample in 4 ns pulses at 20 Hz repeating rate. All samples were prepared using a 10 mg/mL solution of α-cyano-4-hydroxycinnamic acid (α-cyano) in acetone as the matrix. The matrix solution (1 μL) was spotted onto a stainless steel target and air dried. Then, 1 μL of each butanolic fraction was applied onto the spots of matrix crystals, and air dried. Finally, 1 μL droplets of a solution of NaI (2 mg/mL in acetonitrile) was added to the spots on the target plate.

Typical *ESI conditions* were: capillary voltage, 3.1 kV; cone voltage, 50 V; source temperature, 120 °C; desolvation temperature, 300 °C. Dry nitrogen was used as the ESI gas. For the recording of the *single-*
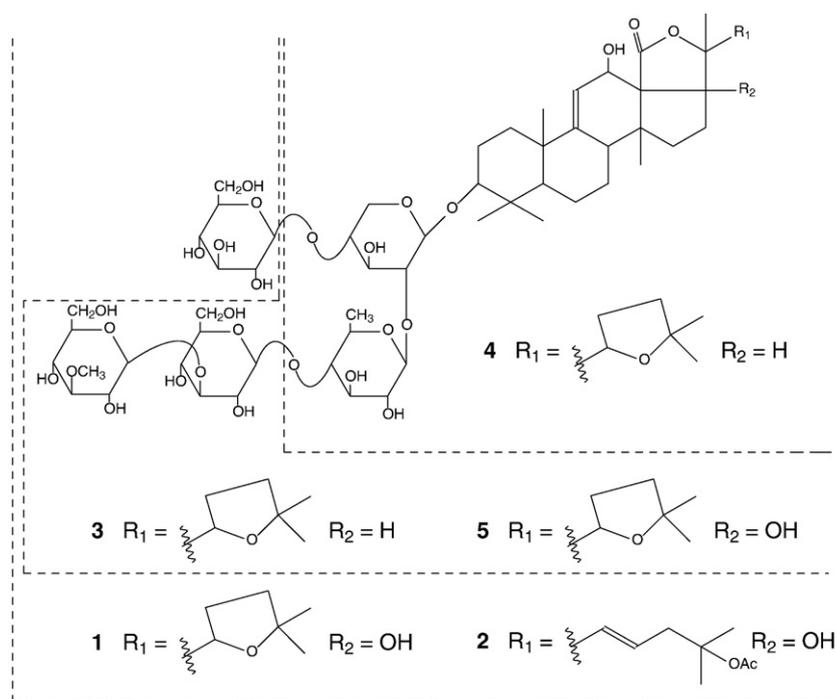
Fig. 1. Molecular structures of saponins isolated from the sea cucumber *Holothuria forskali* by Rodrigez et al. (1991): holothurinosides A, B, C, D and desholothurin A (respectively **1**, **2**, **3**, **4** and **5**).
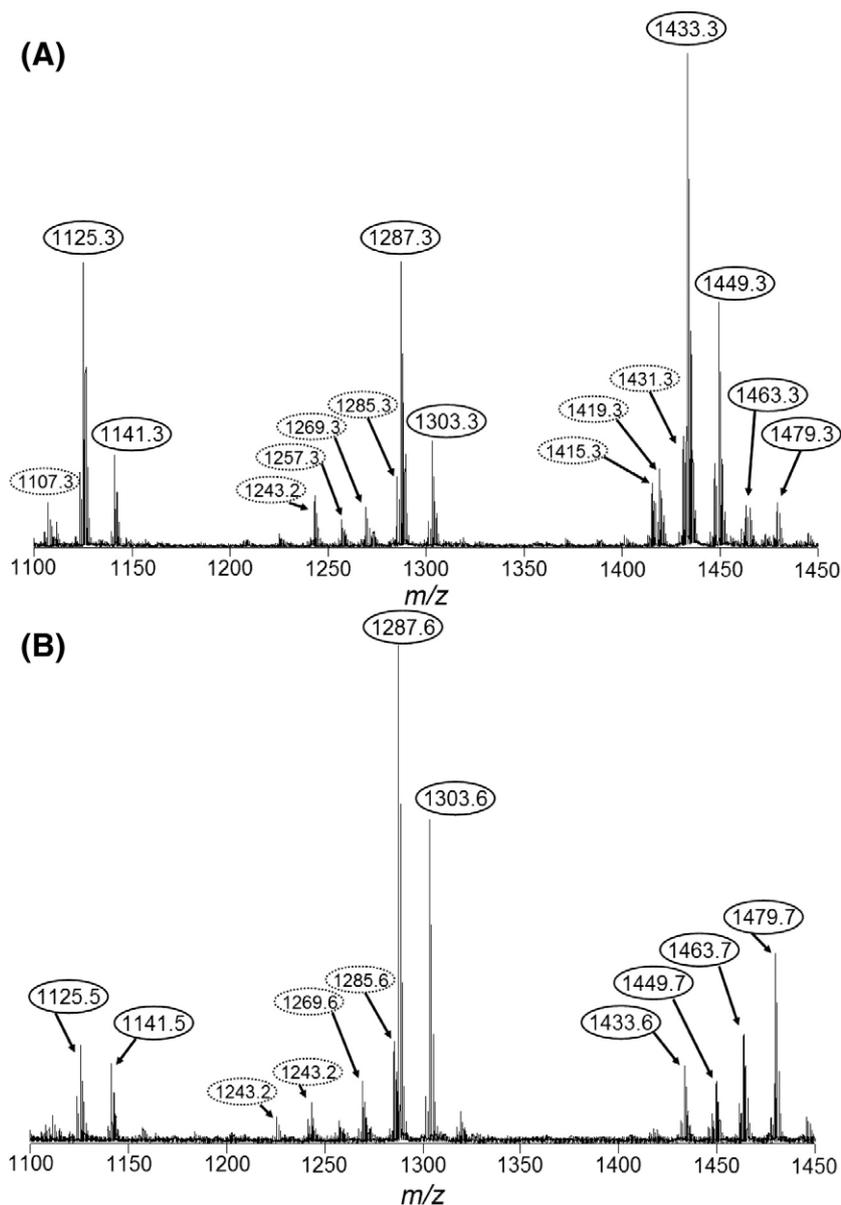
Fig. 2. Full-scan (+) MALDI-ToF mass spectrum analysis of the total saponin mixture extracted from the body wall (A) and the Cuvierian tubules (B) of *Holothuria forskali*. Saponins signals are highlighted by circles. Dotted circles show peaks corresponding to product ions (H_2O and/or CO_2 losses).

stage MALDI- or ESI/MS spectra, the quadrupole (rf-only mode) was set to pass ions between m/z 50 and 1500, and all ions were transmitted into the pusher region of the time-of-flight analyzer where they were mass-analyzed with a 1 s integration time.

For the *MALDI- or ESI/MS/MS CID experiments*, the ions of interest were mass-selected by the quadrupole mass filter. The selected ions are then submitted to collision against argon in the T-wave collision cell (pressure estimated at 0.9–1 mbar) and the laboratory frame kinetic energy was selected to afford intense enough product ion signals. All the ions exiting the collision cell, either the product ions or the non dissociated precursor ions, were finally mass measured with

the oa-ToF analyzer. Time-of-flight mass analysis were performed in the reflectron mode at a resolution of about 10,000.

For the *on-line LC/MS(MS) analyses*, a Waters Alliance 2695 liquid chromatography apparatus was used. The HPLC device was coupled to the QToF Premier mass spectrometer (Waters) and consisted of a vacuum degasser, a quaternary pump and an autosampler. Sample volumes of 20 μL were injected. Chromatographic separation was performed on a non polar column (Symmetry C18, 4.6×150 mm, 5 μm, Waters) at 27 °C. The mobile phase (1 mL/min) was a nonlinear gradient programmed from methanol (eluant A) and water (eluant B). The gradient programmed was: 10% of eluant A at start, 0–6 min 10 to

Fig. 3. A. Collision-induced dissociation of m/z 1303 cations extracted from the body wall and Cuvierian tubules of *Holothuria forskali*: proposed fragmentation pattern for the ions presenting the m/z 507 signature. Glc: glucose, MeGlc: 3-O-methylglucose, Qui: quinovose, Xyl: xylose. B. Collision-induced dissociation of m/z 1303 cations extracted from the Cuvierian tubules of *Holothuria forskali*: proposed fragmentation pattern for the ions presenting the m/z 523 signature. Glc: glucose, MeGlc: 3-O-methylglucose, Qui: quinovose, Xyl: xylose.

(A)

(B)

Fig. 3 (*continued*).

60% A, 6–13 min 60 to 95% A and 13–15 min back to 10% of eluant A. The mobile phase flow (1 mL/min) was split prior injection in the Electrospray ionization source (200 μL/min).

3. Results and discussion

3.1. MALDI/MS analysis of body wall and Cuvierian tubules extracts

Because of the high affinity of alkali cations for triterpene glycosides (Fang et al., 1998), the mass spectra of all the saponins are dominated by $[M+Na]^+$ ions which makes them easier to analyze. The full-scan MALDI mass spectrum of the saponin extract obtained from the body wall of *H. forskali* is shown in Fig. 2A. This spectrum displays 8 intense peaks that could correspond to triterpene saponins. The 8 ions were detected at m/z 1125, 1141, 1287, 1303, 1433, 1449, 1463 and 1479. Other visible peaks seem to correspond to saponin product ions generated by losses of water and/or carbon dioxide from cationized saponins upon MALDI ionization (Fig. 2). The relative intensities of the 8 peaks were different suggesting that saponins are present in different proportions in the tissue (Supplementary Table S1). The saponin detected at m/z 1433 as a sodium cation adduct is always characterized by the most intense signal and consequently seems in first approximation to be the most abundant saponin in the body wall. On the other hand, the saponin detected at m/z 1479 generally presents the least intense signal (Fig. 2).

The MALDI mass spectrum obtained for the saponin extract of the Cuvierian tubules is quite similar to the body wall mass spectrum (Fig. 2B). However, although saponin ions were detected at the same m/z ratios, the relative intensities between both spectra are definitely different, with the peak showing the highest intensity in the Cuvierian tubules being always detected at m/z 1287 (Fig. 2; Supplementary Table S1). At first sight, this could indicate that saponins are identical in both tissues but that their relative proportions are different.

Rodrigez et al. (1991), who studied saponins from *H. forskali*, described five saponins for this species (Fig. 1) but without making any differences between body components. The reported saponins were: holothurinoside A ([M+Na⁺]: m/z 1303), holothurinoside B ([M+Na⁺]: m/z 1345), holothurinoside C ([M+Na⁺]: m/z 1125), holothurinoside D ([M+Na⁺]: m/z 787) and desholothurin A ([M+Na⁺]: m/z 1141). These authors also described the molecular structures of these saponins which were taken as a starting point to identify saponins in the present work (Fig. 1). Considering only the m/z value of the ions detected in the first-order MALDI mass spectrum (Fig. 2), it appears that three out of the five saponins reported by Rodrigez et al. (1991) were also observed in our experiments at m/z 1125, 1141 and 1303. In order to correlate both studies, MALDI/MS/MS analysis were then undertaken to obtain more information on the compounds detected in MALDI/MS.

3.2. MALDI/MS/MS analysis of body wall and Cuvierian tubules extracts

To further validate that signals detected in the full-scan MALDI mass spectrum really correspond to saponin ions, MALDI/MS/MS analysis were undertaken for each ion. As a typical example, the MALDI/MS/MS mass spectrum for the ion detected at m/z 1303 in the body wall is depicted in Supplementary Fig. S1A. This MS/MS spectrum allows us to reconstruct the collision-induced fragmentation pattern of the parent ion (Fig. 3A) and consequently to confirm that ions detected at m/z 1303 correspond to the holothurinoside A described by Rodrigez et al. (1991). After collisional activation, m/z 1303 cations are submitted to two competitive dissociation pathways. First, as described in Fig. 3A, consecutive losses of 3-O-methylglucose (MeGlc), glucose (Glc), quinovose (Qui), glucose (Glc) and xylose (Xyl) residues afford product ions detected in Supplementary Fig. S1A at m/z 1127, 965, 819, 657 and 507, respectively. This sequence of decomposition confirms the proposed holothurinoside A structure. Secondly,

the fragmentation of the parent ions can also be initiated by the loss of the aglycone moiety, generating m/z 819 cations. Consecutive losses of Glc, Xyl, Qui and Glc residues, yielding signals detected at m/z 657, 507, 379 and 217, further confirm that the decomposing ions definitely originate from sodium-cationized holothurinoside A (m/z 1303).

Similar fragmentation patterns were obtained for all the ions detected upon MALDI ionization of the body wall extract (Fig. 2A). This methodology of molecular structure identification using fragmentation patterns derived from MS/MS measurements therefore indicated that ions detected at m/z 1125 and 1141 correspond to holothurinoside C and desholothurin A (Rodrigez et al., 1991). On the basis of the recorded MS/MS spectra, five new saponins provisionally named holothurinosides E, F, G, H and I and detected at m/z 1287, 1433, 1449, 1463 and 1479, respectively, were identified (Fig. 4A). All these holothurinosides are quite easily recognized upon MS/MS measurement since their CID spectra share the common m/z 507 key signal as a signature of the MeGlc-Glc-Qui oligosaccharide chain (actually [MeGlc-Glc-Qui+Na⁺], see Fig. 3A). It is important to emphasize that all the MS/MS spectra of the saponin ions detected in the body wall extract are characterized by the occurrence of this key signal at m/z 507 (Fig. 4A).

The same MS/MS method was applied for the eight major ions detected by MALDI analysis in the Cuvierian tubules extract (Supplementary Fig. S1B for m/z 1303 ion). Interestingly, the MS/MS spectrum obtained for the Cuvierian tubules m/z 1303 saponin ions was found to be different from the body wall saponin ion spectrum. Nevertheless, all the product ions identified in the body wall MS/MS spectrum were also detected in the corresponding Cuvierian tubules MS/MS spectrum. In particular, the presence of the signal at m/z 507 indicated the presence of holothurinoside A. The proposed explanation for the occurrence of additional product ions in the spectrum is that, in the case of the Cuvierian tubules extract, we are dealing with a mixture of isomeric saponins. In other words, a single peak in the MALDI full-scan spectrum would correspond to at least two different saponins. A closer inspection of the MS/MS spectrum of the m/z 1303 ions from the Cuvierian tubules extract (Supplementary Fig. S1B) reveals that, in addition to the m/z 507 signal, a more intense signal is now observed at m/z 523, corresponding presumably to [MeGlc-Glc-Glc+Na⁺] ions (Fig. 3B). The difference between this oligosaccharide sequence and the one associated with the m/z 507 ion (see above) is the replacement of a quinovose by a glucose residue, the two differing only by the presence of an additional oxygen atom in Glc compared to Qui. As a consequence and since both parent ions are isomers, the aglycone moiety associated with the m/z 523 cation must contain one hydroxyl less. Based on the study of Rodrigez et al. (1991) who identified three different aglycone groups (Fig. 1), we tentatively propose the structure presented in Fig. 3B for the new isomeric saponin and we propose to name this molecule holothurinoside A₁ (Fig. 4B).

Similar fragmentation patterns were obtained for all the cations observed upon MALDI of the Cuvierian tubules extract, each presenting m/z 507 and m/z 523 ions as the key decomposition products. This reveals that we are dealing with 8 mixtures of isomers, one isomer at least being identical in both the body wall and the Cuvierian tubules extracts for each mixture. Mass spectrometry alone, however, is not sufficient to obtain more information about the isomer mixtures. Nevertheless, it allows a quick and straightforward characterisation of the body components by the presence of an ion at m/z 507 in the MS/MS spectrum of a body wall sample and of ions at m/z 507 and 523 in the MS/MS spectrum of a Cuvierian tubules sample. The identification of a second set of saponins possessing the m/z 523 signature in the Cuvierian tubules allows the addition of 8 new molecules to the list of saponins occurring in this body component. Similarly to what has been proposed for the body wall saponins, these new ones could be named provisionally holothurinosides A₁, C₁, E₁, F₁, G₁, H₁ and I₁ and desholothurin A₁ (Fig. 4B).
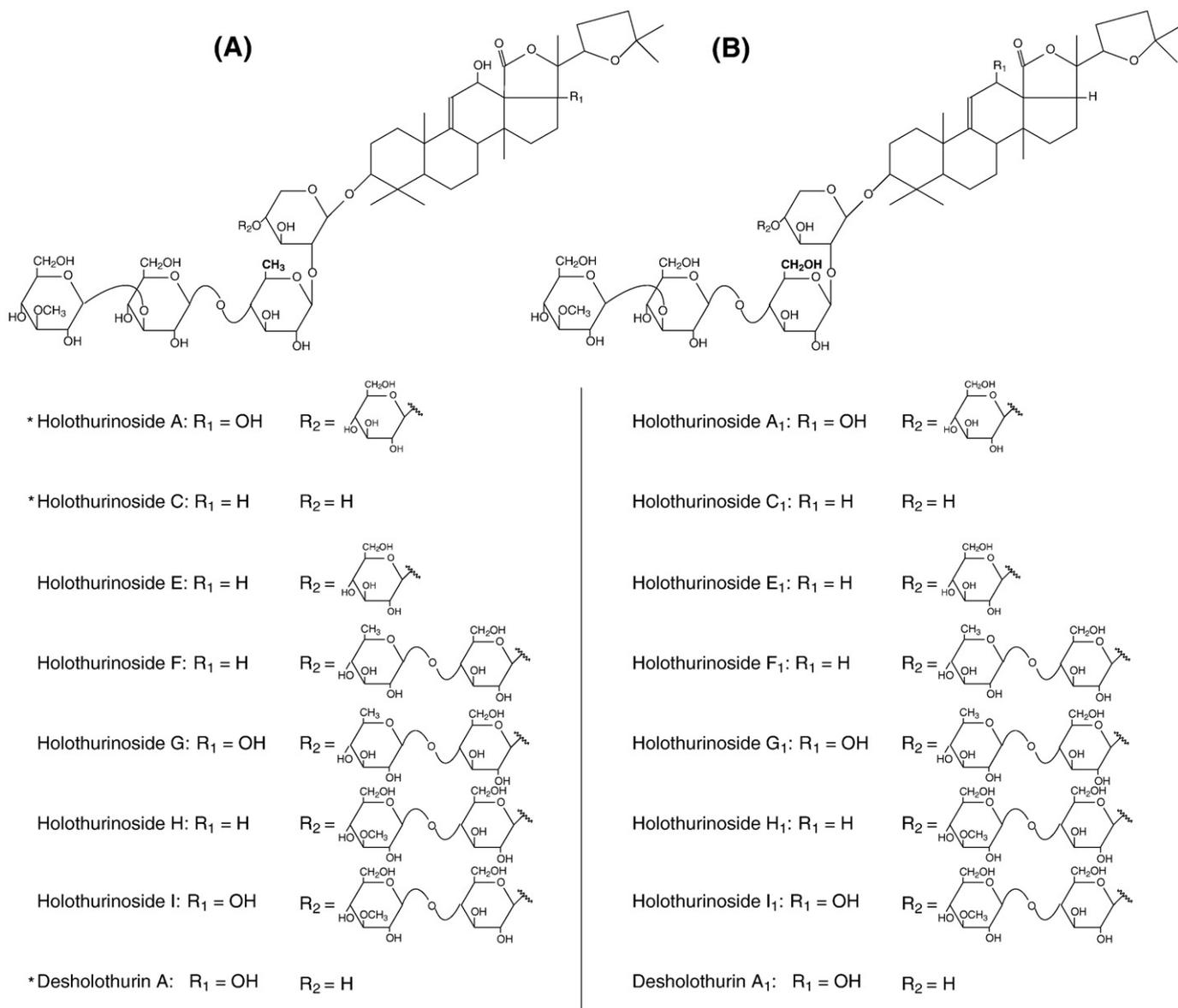
Fig. 4. Presumptive molecular structures of saponins detected in the body wall (A) and the Cuvierian tubules (A+B) extracts of *Holothuria forskali*. Asterisk labels indicate the saponins described by Rodrigez et al. (1991).

3.3. LC/MS and LC/MS/MS analysis of body wall and Cuvierian tubules extracts

To further validate the presence of isomers in Cuvierian tubules and their absence in the body wall, LC/MS experiments on the total saponin extracts were carried out. The chromatogram presented in Fig. 5A for the body wall corresponds to the m/z 1303 extracted ion chromatogram. Given the observation of a single signal, we can argue that we are dealing with only one molecule, which was moreover confirmed to be holothurinoside A by LC/MS/MS. This conclusion is in agreement with the data derived from the MALDI experiments. On the other hand, in the Cuvierian tubules extract, the presence of distinct signals characterized by different retention times in the chromatogram is an evidence for the occurrence of a mixture of isomers (Fig. 5B). Interestingly, the signal at 10.97 min is detected in both chromatograms. This observation confirms that holothurinoside A is also present in the Cuvierian tubules extract. Moreover, this experiment also reveals that the isomer mixture is even more complicated than expected, with more than two isomeric saponins present in the Cuvierian tubules

extract. As a consequence, LC/MS/MS experiments are required to obtain further information on the saponin contents in the Cuvierian tubules extract.

The LC/MS/MS analysis of the Cuvierian tubules extract was then performed on m/z 1303 cations as the parent ions. The LC/MS/MS m/z 1303 total ion current (TIC) chromatogram is presented in Fig. 6B and is totally similar to the LC/MS m/z 1303 extracted ion chromatogram presented in Fig. 5B. Corresponding LC/MS/MS m/z 507 and 523 extracted ion chromatograms are shown in Fig. 6A and C, respectively. Amongst the m/z 1303 parent ions, at least three isomeric ions are present which, in their dissociation products, possess the m/z 507 product ions (Fig. 6A). Again, holothurinoside A is unambiguously identified at a retention time of 10.97 min. As suggested by the MALDI/MS/MS experiments, other isomeric parent ions that decompose after collisional activation by generating m/z 523 product ions are also detected. Nevertheless, from the LC/MS/MS experiments, it is obvious that more than one such molecule is present (Fig. 6C). Further investigations have to be done to separate those different saponins and characterize their molecular structures. At this state of the study,
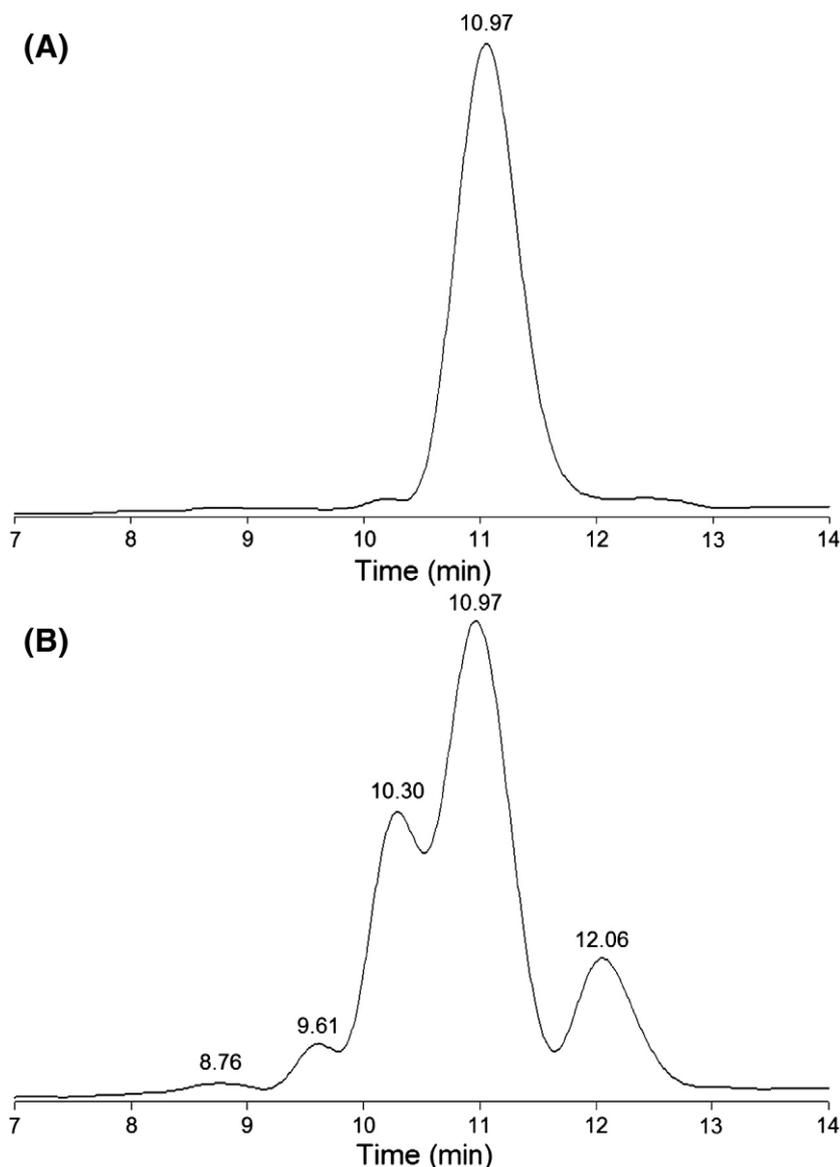
Fig. 5. (+) LC/MS *m/z* 1303 extracted ion chromatogram of the body wall (A) and the Cuvierian tubules (B) of *Holothuria forskali*. Retention times (min) are written on the top of each peak.

the different saponins are only distinguished by their retention time (Table 1).

This complete LC/MS/MS analysis has been carried out for all the signals detected in the full scan spectrum of the body wall and Cuvierian tubules of *H. forskali* (Fig. 2). Results of the whole analysis are summarized in Table 1. They show that at least 16 saponins have been detected (13 new ones) and their structure proposed, but also that other congeners are present and need to be investigated with other techniques. So far, retention time is the only feature permitting to distinguish some of these isomeric saponins. The results also confirm that saponins with the "*m/z* 523" signature are only present in the Cuvierian tubules while saponins having the "*m/z* 507" signature are always present the extracts from both body components.

4. Conclusions and future work

Secondary metabolites of the common Mediterranean sea cucumber *H. forskali* have been investigated and the variety of saponins detected proved to be much more diverse than previously reported (Rodrigez et al., 1991), with about 38 saponins highlighted by MS-based methodol-ogies. MALDI/MS, MALDI/MS/MS, LC/MS and LC/MS/MS techniques have been complementarily used and are considered as strong tools to detect these molecules in complex mixtures. MALDI techniques were used for direct analysis of saponin mixtures, while LC/MS techniques were used to achieve chromatographic separation of isomers. This presence of numerous isomers (up to five for some compounds) explains why only 16 saponins have been described. Structure elucidation of each isomer is indeed impossible with the techniques used.

Body wall and Cuvierian tubules of *H. forskali* have been treated separately allowing the comparison between body components. Although MALDI/MS only suggested differences in the relative proportions of the saponins, the complete analysis of the two extracts revealed important differences in composition too, with more than twice as many saponins in the Cuvierian tubules as compared to the body wall. Such differences in saponin distribution have rarely been demonstrated in sea cucumbers, Kobayashi et al. (1991) being the only authors to investigate differences in saponins content between the body wall and the Cuvierian tubules. They reported quantitative but also qualitative differences in the species *Bohadschia argus*, *Holothuria leucospilota*, *H. pervicax* and *Pearsonothuria graffei*. In all these species,
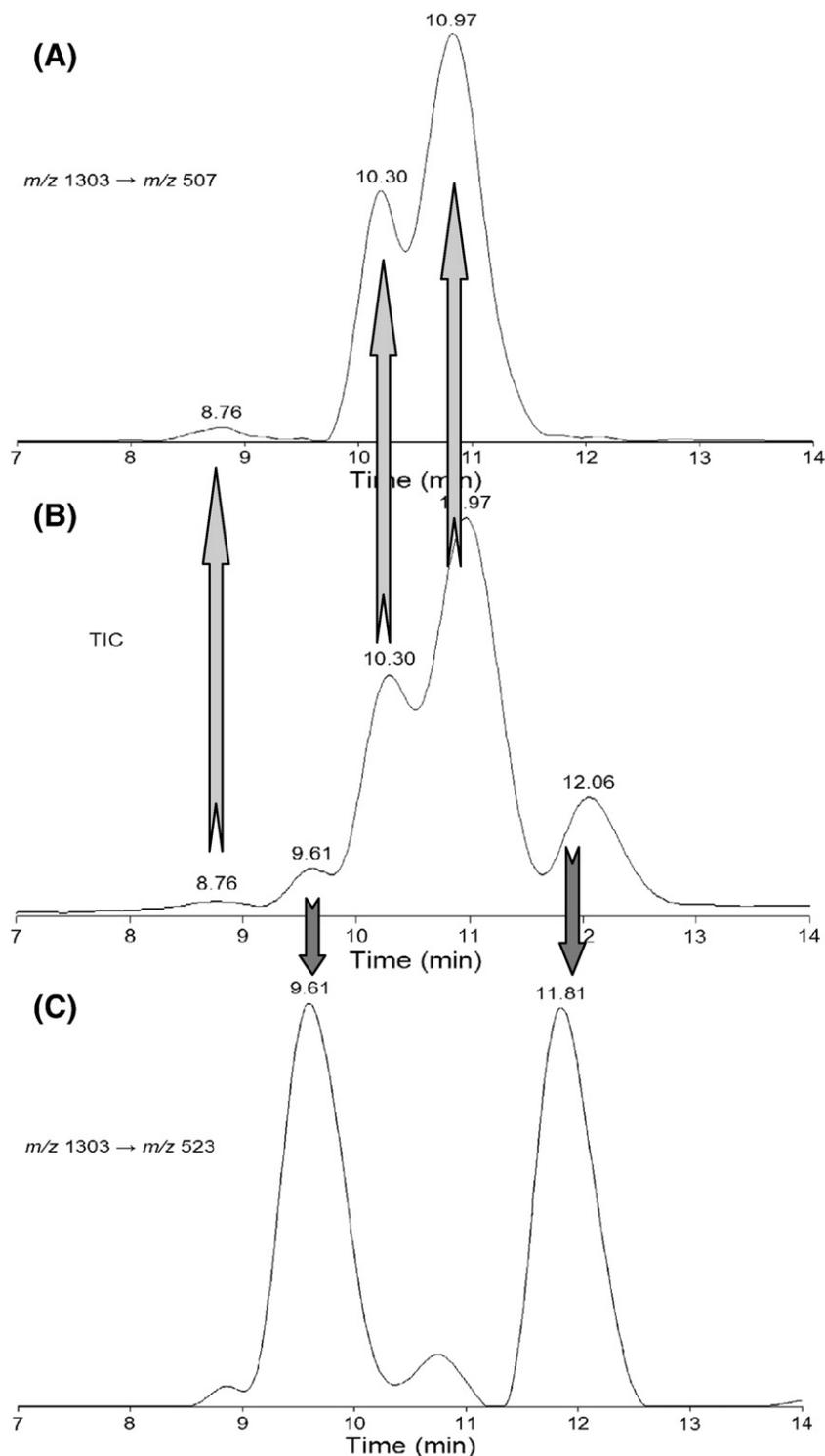
Fig. 6. (+) LC/MS/MS analysis of isomeric ions detected at *m/z* 1303 in the Cuvierian tubules extract of *Holothuria forskali*. (A) shows the MS/MS *m/z* 507 extracted ion chromatogram; (B) shows the *m/z* 1303 total ion current (TIC) chromatogram and (C) shows the MS/MS *m/z* 523 extracted ions chromatogram. Retention times are written on the top of each peak.

the saponin mixtures were simple (maximum of 3 different saponins) with usually only one saponin differing from one body compartment to the other. In sea stars, a single study investigated the distribution of saponins between the body components of *Leptasterias polaris* (Garneau et al., 1989): saponin mixtures were much more complex (up to 19 saponins) and each body component presented a distinctive mixture. The selective distribution of saponins within the body of *H.*

forskali could also explain why some of the saponins described by Rodrigez et al. (1991) were not found in our study. These saponins could indeed be restricted to an organ we have not investigated like, for example, the gonads.

In the future, the methodologies presented in this report will be used to identify saponins in other sea cucumber species. The morphological and behavioural characteristics of the species chosen and

Table 1
Complete list of the saponins found in the body wall and Cuvierian tubules of *Holothuria forskali*, with their LC/MS/MS retention times and fragment signatures

Molecular formula	Molecular weight	$[M+Na]^+$	Retention time (min)	Fragment signature		Names
				507	523	
Body wall						
$C_{54}H_{86}O_{23}$	1102	1125	11.56	x		Holothurinoside C*
$C_{54}H_{86}O_{24}$	1118	1141	11.46	x		Desholothurin A*
$C_{60}H_{96}O_{28}$	1264	1287	10.38	x		Unidentified
			11.14	x		Holothurinoside E
			12.38	x		Unidentified
$C_{60}H_{96}O_{29}$	1280	1303	10.97	x		Holothurinoside A*
$C_{66}H_{106}O_{32}$	1410	1433	11.24	x		Holothurinoside F
			12.50	x		Unidentified
$C_{66}H_{106}O_{33}$	1426	1449	11.02	x		Holothurinoside G
$C_{67}H_{108}O_{33}$	1440	1463	11.20	x		Holothurinoside H
			12.36	x		Unidentified
$C_{67}H_{108}O_{34}$	1456	1479	11.13	x		Holothurinoside I
Cuvierian tubules						
$C_{54}H_{86}O_{23}$	1102	1125	11.54	x		Holothurinoside C*
			12.45		x	Holothurinoside C_1
$C_{54}H_{86}O_{24}$	1118	1141	9.00	x		Unidentified
			10.00		x	Desholothurin A_1
			11.47	x		Desholothurin A*
$C_{60}H_{96}O_{28}$	1264	1287	10.27	x		Unidentified
			11.03	x		Holothurinoside E
			12.00		x	Holothurinoside E_1
			12.30	x		Unidentified
$C_{60}H_{96}O_{29}$	1280	1303	8.78	x		Unidentified
			9.61		x	Unidentified
			10.30	x		Unidentified
			10.97	x		Holothurinoside A*
			12.06		x	Holothurinoside A_1
$C_{66}H_{106}O_{32}$	1410	1433	11.22	x		Holothurinoside F
			12.01		x	Holothurinoside F_1
			12.44	x		Unidentified
$C_{66}H_{106}O_{33}$	1426	1449	10.97	x		Holothurinoside G
			11.81		x	Holothurinoside G_1
			12.73	x		Unidentified
$C_{67}H_{108}O_{33}$	1440	1463	11.28	x		Holothurinoside H
			11.93		x	Holothurinoside H_1
			12.31	x		Unidentified
$C_{67}H_{108}O_{34}$	1456	1479	10.63	x		Holothurinoside I
			10.70	x		Unelucidated
			11.85		x	Holothurinoside I_1

* Saponins described by Rodrigez et al. (1991).

the distribution of saponins in their organs should permit to clarify the biological role of these molecules.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpb.2008.10.011.

References

Campagnuolo, C., Fattorusso, E., Taglialatela-Scafati, O., 2001. Feroxosides A–B, two norlanostane tetraglycosides from the Caribbean sponge *Ectyoplasia ferox*. Tetrahedron 57, 4049–4055.

Elyakov, G.B., Stonik, V.A., Levina, E.V., Slanke, V.P., Kuznetsova, T.A., Levin, V.S., 1973. Glycosides of marine invertebrates-I. A comparative study of the glycosides fraction of Pacific sea cucumbers. Comp. Biochem. Physiol. B 44, 325–336.

Fang, S., Hao, C., Sun, W., Liu, Z., Liu, S., 1998. Rapid analysis of steroidal saponin mixture using electrospray ionization mass spectrometry combined with sequential tandem mass spectrometry. Rapid Commun. Mass Spectrom. 12, 589–594.

Francour, P., 1997. Predation on holothurians: a literature review. Invert. Biol., 116, 52–60.

Garneau, F.X., Simard, J.L., Harvey, O., Apsimon, J.W., Girard, M., 1983. The structure of psoluthurin A, the major triterpene glycoside of the sea cucumber *Psolus fabricii*. Can. J. Chem. 61, 1465–1471.

Garneau, F.X., Harvey, O., Simard, J.L., Apsimon, J.W., Burnell, D.J., Himmelman, J.H., 1989. The distribution of asterosaponins in various body components of the starfish *Leptasterias polaris*. Comp. Biochem. Physiol. B 92, 411–416.

Guo, M., Song, F., Liu, Z., Liu, S., 2006. Characterization of triterpenoidic saponin mixture in crude extracts from leaves of *Acanthopanax senticosus* harms by saponin structural correlation and mass spectrometry. Anal. Chim. Acta. 557, 198–203.

Habermehl, G., Volkwein, G., 1971. Aglycones of the toxins from the Cuvierian organs of *Holothuria forskali* and a new nomenclature for the aglycones form Holothurioideae. Toxicon 9, 319–326.

Hamel, J.F., Mercier, A., 2000. Cuvierian tubules in tropical holothurian: usefulness and efficiency as a defence mechanism. Mar. Freshw. Behav. Physiol. 33, 115–139.

Kalinin, V.I., Levin, V.S., Stonik, V.A., 1994. Chemical Morphology: Triterpene Glycosides from Sea Cucumber (Holothuroidea, Echinodermata). Dalnauka, Vladivostok. p. 284.

Kalinin, V.I., Anisimov, M.M., Prokofieva, N.G., Avilov, S.A., Afiyatullov, SH.SH., Stonik, V.A., 1995. Biological activities and biological role of triterpene glycosides from holothuroids (Echinodermata). In: Jangoux, M., Lawrence, L.M. (Eds.), Echinoderm Studies, vol. 5. Balkema, Rotterdam, pp. 139–181.

Kalinin, V.I., Prokofieva, N.G., Likhatskaya, G.N., Schentsova, E.B., Agafonova, I.G., Avilov, S.A., Drozdova, O.A., 1996. Hemolytic activities of triterpene glycosides from the holothurian order Dendrochirotida: some trends in the evolution of this group of toxins. Toxicon. 34, 475–483.

Kerr, R.G., Chen, Z., 1995. In vivo and in vitro biosynthesis of saponins in sea cucumbers. J. Nat. Prod. 58, 172–176.

Kubanek, J., Whalen, K.E., Engel, S., Kelly, S.R., Henkel, T.P., Fenical, W., Pawlik, J.R., 2002. Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 131, 125–136.

Kobayashi, M., Hori, M., Kan, K., Yasuzawa, T., Matsui, M., Suzuki, S., Kitagawa, I., 1991. Marine Natural Product. XXVII. Distribution of lanostane-type triterpene oligoglycosides in ten kinds of Okinawan sea cucumbers. Chem. Pharm. Bull. 39, 2282–2287.

Mackie, A.M., Turner, A.B., 1970. Partial characterization of biologically active steroid glycoside isolated from the starfish *Marthasterias glacialis*. Biochem. J. 117, 543–550.

Mackie, A.M., Lasker, R., Grant, P.T., 1968. Avoidance reactions of a mollusc *Buccinum undatum* to saponin-like surface-active substances in extracts of the starfish *Asterias rubens* and *Marthasterias glacialis*. Comp. Biochem. Physiol. 26, 415–428.

Matsuno, T., Ishida, T., 1969. Distribution and seasonal variation of toxic principles of sea cucumber (*Holothuria leucospilota* Brandt). Experientia 25, 1261.

Nigrelli, R.F., 1952. The effect of holothurin on fish, and mice with sarcoma 180. Zoologica 37, 89–90.

Pawlik, J.R., 1993. Marine invertebrate chemical defences. Chem. Rev. 93, 1911–1922.

Prokofieva, N.G., Chaikina, E.L., Kicha, A.A., Ivanchina, N.V., 2003. Biological activities of steroid glycosides from starfish. Comp. Bioch. Physiol. B 134, 695–701.

Rodrigez, J., Castro, R., Riguera, R., 1991. Holothurinosides: new antitumour non sulphated triterpenoid glycosides from the sea cucumber *Holothuria forskali*. Tetrahedron 47, 4753–4762.

Stonik, V.A., Elyakov, G.B., 1988. Secondary metabolites from Echinoderms as chemotaxonomic markers. In: Scheuer, P.J. (Ed.), Bioorganic Marine Chemistry, vol. 2. Springer-Verlag, Berlin, p. 43.

Stonik, V.A., Kalinin, V.I., Avilov, S.A., 1999. Toxins from sea cucumbers (Holothuroids): chemical structures, properties, taxonomic distribution, biosynthesis and evolution. J. Nat. Toxins 8, 235–248.

Yamanouchi, T., 1955. On the poisonous substance contained in holothurians. Publ. Seto Mar. Biol. Lab. 4, 183–203.