Holothuria triterpene glycosides : a comprehensive guide for their structure elucidation and critical appraisal of reported compounds

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Abstract

Sea cucumbers or holothurians are marine invertebrates, belonging to the phylum Echinodermata (kingdom Animalia). In Asia, they are commonly used as food, while they are applied in traditional medicine as well. A wide range of secondary metabolites from these animals has been reported, but triterpene glycosides are the most predominant and most studied and are considered as valuable chemotaxonomic markers. With respect to this, the genus *Holothuria* is an important genus of sea cucumbers, given the high number and variety of triterpene glycosides previously reported. *Holothuria* triterpene glycosides are typically composed of a 3β-hydroxyholost-9(11)-ene aglycon and a glycosidic moiety comprised of one up to six sugar units, connected to position 3 of the sapogenin. In the past decade (2010-2020) as much as 63 saponins were identified for the first time in the *Holothuria* genus, of which 36 had not been reported before. Identification was mainly carried out by LC-MS (liquid chromatography – mass spectrometry) analysis, while also NMR (nuclear magnetic resonance) spectroscopy is often applied for structure elucidation. However, while carrying out a literature search on these compounds, various inconsistencies were observed. Therefore, this review intends to provide an overview of typical signals that can occur in NMR and MS data, accompanied with an extensive explanation of their interpretation, in order to facilitate any future research on identification/structure elucidation of *Holothuria* saponins. Moreover, the data published in the past decade were critically reviewed and various questionable results are discussed.

Keywords

Holothuria; Holothuriidae; sea cucumber; triterpene glycosides; saponins

Abbreviations

HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
LC-APCI-MS	Liquid chromatography – atmospheric pressure chemical ionization – mass spectrometry
LC-ESI-MS	Liquid chromatography – electrospray ionization – mass spectrometry
MALDI-FT-ICR MS	Matrix-assisted laser desorption ionization - fourier transform - ion cyclotron resonance mass spectrometry
MALDI-MS	Matrix-assisted laser desorption ionization mass spectrometry
MS	Mass spectrometry
MSI	Mass spectrometry imaging
NMR	Nuclear magnetic resonance
Q-ToF MS	Quadrupole time-of-flight mass spectrometry

Introduction

As opposed to what their name may imply, sea cucumbers do not belong to the plant kingdom, but they are sedentary, marine invertebrates, belonging to the phylum Echinodermata (kingdom Animalia) (Bondoc et al. 2013; Bahrami et al. 2016). They have a leathery skin and elongated body, varying in length from 2 up till 90 cm, and as their name infers, they are commonly shaped like soft-bodied cucumbers (Aminin et al. 2015; Bahrami et al. 2016). Over 1500 species of sea cucumbers (holothurians) have been described and they are found all around the world (Bahrami et al. 2016; Grauso et al. 2019). The genus *Holothuria* is one of the five genera belonging to the family Holothuriidae (order Holothuriida, formerly Aspidochirotida, class Holothuroidea) and contains hundreds of species (Bahrami and Franco 2015; Honey-Escandón et al. 2015; Miller et al. 2017; Bahrami et al. 2018; World Register of Marine Species (WORMS) 2021).

Particularly in Asia, sea cucumbers are used in traditional medicine, or are consumed as food (Grauso et al. 2019). The cooked holothuroids, also referred to as trepangs in Indonesia, are an example of marine functional food (Caulier et al. 2016). Their beneficial effects are accounted to their high content of nutrients, including vitamins A, B1, B2 and B3 and minerals, especially calcium, magnesium, iron and zinc. Apart from the predominant triterpene glycosides, they also contain glycosaminoglycans, chondroitin sulfates, phenols, flavonoids, alkaloids, cerebrosides, peptides, mucopolysaccharides and fatty acids (Mitu et al. 2017; Jattujan et al. 2018; Grauso et al. 2019; Nursid et al. 2019; Hal et al. 2020). A few examples of their use in traditional Asian medicine are diseases related to the joints, such as rheumatoid arthritis, and muscular disorders, hypertension, kidney deficiency, wounds and cancer (Bahrami et al. 2014a, 2018; Jattujan et al. 2018; Shahinozzaman et al. 2018a). Furthermore, they are believed to have an aphrodisiac effect (Bahrami et al. 2016; Grauso et al. 2019). They are a potential source of nutraceutical, pharmaceutical and cosmeceutical products (Kim and Himaya 2012; Bahrami et al. 2014a, b; Bahrami and Franco 2015; Grauso et al. 2019) and given the fact that they are a prolific source of bioactive compounds, they are also referred to as “marine ginseng” (Bahrami et al. 2018).

Saponins are naturally occurring surface-active glycosides produced by plants and marine organisms, and they are the predominant secondary metabolite class in sea cucumbers (Hostettmann and Marston 1995; Omran et al. 2020). *Holothuria* saponins are triterpenoid glycosides consisting of a hydrophobic 3β-hydroxyholost-9(11)-ene aglycon (sapogenin), derived from lanostane. Thus, they differ significantly from the saponin aglycons reported in the plant kingdom, which mostly contain either oleanane or dammarane skeletons, while ursanes, hopanes, lanostanes or lupanes are of secondary importance with respect to distribution (Hostettmann and Marston 1995).

Attached to the 3-position of the sea cucumber sapogenin is a hydrophilic oligosaccharide chain. The oligosaccharide chain consists of 1 to 6 sugar units, and is almost always comprised of xylose, quinovose, glucose and/or 3-*O*-methylglucose moieties. The oligosaccharide chain is linear, or can be branched once at the first sugar (Caulier et al. 2011, 2013b; Honey-Escandón et al. 2015). The first moiety is always xylose and can be either sulfated or non-sulfated, while a 3-*O*-methylglucose moiety can only occur as terminal unit (Bahrami and Franco 2015; Bahrami et al. 2016; Caulier et al. 2016; Kamyab et al. 2020). Several *Holothuria* species were reported to contain only sulfated saponins, some only non-sulfated and various species contain both sulfated and non-sulfated saponins (Caulier et al. 2011). The sulfated saponins are more hydrophilic and diffuse more easily in the surrounding sea water, which makes them more potent as toxins (Van Dyck et al. 2010b; Caulier et al. 2011; Omran et al. 2020). The presence of a sulfate group is another characteristic trait of saponins of marine origin and is not found in plant-derived saponins.

Moreover, sapogenins occurring in plants can be glycosylated in one, two or even three positions and the glycosidic moiety commonly contains rhamnose, which is not the case for marine saponins (Hostettmann and Marston 1995).

Due to their amphiphilic nature, saponins show strong membranolytic activity by interaction with 5,6-unsaturated sterols of the cellular membrane and a wide range of biological activities have been demonstrated for *Holothuria* saponins, including hemolytic, anti-fungal, anti-bacterial, anti-viral, immunomodulatory, anti-diabetic, ichthyotoxic, anti-fouling, cytotoxic, pro-apoptotic, anti-angiogenic and antineoplastic activities (Kalinin et al. 2008; Caulier et al. 2011, 2016; Kim and Himaya 2012; Bondoc et al. 2013; Bahrami et al. 2014a, b, 2016, 2018; Wang et al. 2014; Honey-Escandón et al. 2015; El Barky et al. 2016; Shahinozzaman et al. 2018a; Grauso et al. 2019; Kamyab et al. 2020). However, sea cucumbers are commonly consumed as food, and it is well-known that their consumption is devoid of toxic side effects, because saponins are not absorbed as such in the gastro-intestinal tract, but are subjected to microbial metabolism in the colon (Grauso et al. 2019).

The first report on purification of triterpene glycosides from *Holothuria* sea cucumbers dates back from 1942, when "holothurin" was obtained from *H. leucospilota* (Stonik et al. 1999) and since then, many reports were published about the isolation and structure elucidation of single saponins, or, more recently, about their tentative identification in mixtures. These saponins can be found in the body wall, the viscera, the gonads and/or in the Cuvierian tubules of the sea cucumber (Bondoc et al. 2013; Honey-Escandón et al. 2015). The latter are defensive organs consisting of multiple tubules, which can be expelled in case the sea cucumbers fears threat. The expelled tubules lengthen into sticky white threads which are capable of entangling a possible predator (Kalinin et al. 2008; Van Dyck et al. 2010a, b; Caulier et al. 2011, 2016; Honey-Escandón et al. 2015). Some, but not all *Holothuria* species possess Cuvierian tubules; for example, *H. leucospilota, H. forskali* and *H. sanctori* have Cuvierian tubules, while *H. tubulosa*, *H. atra* and *H. nobilis* have not (Kalinin et al. 2008; Van Dyck et al. 2010b; Honey-Escandón et al. 2015; Caulier et al. 2016). Another defense mechanism of sea cucumbers is called evisceration, during which the respiratory tree, digestive tract and gonads are expelled through the cloacum (Bahrami et al. 2014a; Honey-Escandón et al. 2015). During these processes, the surrounding environment will become exposed to the *Holothuria* saponins, which serve as a chemical defense, repelling predators (Kalinin et al. 2008; Caulier et al. 2013a, 2016; Bahrami et al. 2014a, b, 2018; Honey-Escandón et al. 2015). Indeed, various saponins were detected in the (conditioned) sea water surrounding sea cucumbers (Van Dyck et al. 2011; Caulier et al. 2016). Alternatively, the *Holothuria* saponins are thought to play a role in reproduction of these organisms, and they may have a function in interspecific chemical communication, for example to attract symbionts (kairomones) (Bondoc et al. 2013; Caulier et al. 2013a, 2016; Bahrami et al. 2014a, b, 2018). Although research on the occurrence of saponins in different organs of the sea cucumbers is rather limited, thus far it can be concluded that within one species many saponins are common to both body wall and Cuvierian tubules, while some congeners seem to be organ specific (Caulier et al. 2011).

Sea cucumber saponins are considered valuable chemotaxonomic markers, as closely related species contain saponins with the same molecular patterns (Bondoc et al. 2013; Omran et al. 2020). Some congeners are shared within the family Holothuriidae (e.g. holothurins A and B), while others are highly specific for certain species or genera, like hillasides A, B and C, which have only been reported in *H. hilla* (Wu et al. 2006a, 2007; Caulier et al. 2011; Bondoc et al. 2013; Mitu et al. 2017). For example, recently Omran et al. (2020) suggested to revise the taxonomic state of *H. polii*, and to consider assigning it to a

separate genus, based on the fact that only a single saponin (holothurin B1) was detected in this species, in very low levels, which is not typical for *Holothuria* species.

In the past ten years, a significant number of papers was published on the topic of triterpene glycosides from *Holothuria* species and numerous newly identified compounds were reported. In addition, also a high number of compounds, most probably saponins, were identified, but their structures could not be determined unequivocally, and a lot of research still remains to be done to reveal the complete saponin composition of sea cucumbers. Several review articles were also published, focusing on the biological activity of sea cucumbers, taxonomical implications of the saponin composition, and/or on the occurrence of saponins in the family Holothuriidae, or the class Holothuroidea (Stonik et al. 1999; Kalinin et al. 2008; Caulier et al. 2011; Kim and Himaya 2012; Aminin et al. 2015; Honey-Escandón et al. 2015; Bahrami et al. 2016). However, the most recent review, focusing on acetylated saponins, dates back from 2016. Whereas Honey-Escandón et al. (2015) highlighted the importance of the correct identification of sea cucumber species and confirmation by taxonomists in their review, we would like to emphasize in the present review the importance of a correct structural identification of saponins, since both factors may have implications on the classification of certain species of sea cucumbers. Furthermore, the importance of an unequivocal structure elucidation is obvious in case of structure-activity relationship studies. Several reviews already focused on structure-activity relationships of sea cucumber saponins, but none have focused on the actual structure elucidation. For example, Aminin et al. published a review on the anticancer activity of sea cucumber triterpene glycosides belonging to the class of Holothuroidea in 2015, and mentioned that in the years before, several reviews on the cytotoxic activity of triterpene glycosides had been published (Aminin et al. 2015). Of course, without decent characterization of extracts and identification of compounds, such studies cannot be performed in a reliable way. As Bahrami et al. (2016) already stated: "The notable abundance and diversity of sea cucumber saponins (> 50 in one species) makes the purification of saponins from their natural sources a challenge. The high complexity of the saponin mixtures makes their structure elucidation and the evaluation of their potential biological activity difficult"; thus, the correct identification of the saponin composition of sea cucumber is clearly challenging, but of great importance.

Therefore, in this review, an overview is given of the triterpene glycosides reported in the genus *Holothuria*, with particular attention for research papers published in the past ten years (2010-2020). To keep this review clear and concise it is limited to the genus *Holothuria*, which is one of the most studied genera of sea cucumbers. The structure elucidation of these compounds is mainly performed by MS (mass spectrometry) and NMR (Nuclear Magnetic Resonance) spectroscopy and we aim to provide a set of clear guidelines for the interpretation of data obtained by these two techniques, derived from previously published data. Typical signals and patterns will be discussed in relation to typical structural features. Moreover, when studying the published data, in several cases questions were raised regarding the soundness of the identified structures, and therefore, another aim is to address some possibly doubtful results published. As such, this review strives to serve as a guide for researchers studying the composition of triterpene glycosides from *Holothuria* sea cucumbers, and possibly also of other types of sea cucumbers, and may help to simplify and quicken the structure elucidation steps.

Overview of reported compounds

A SciFinder search was carried out on 6 July 2020, using the search term "Holothuria" in combination with "saponin" or "triterpene glycoside" and a selection of articles was made after reading the title and abstracts, omitting any publications in which no specific saponins were identified. Relevant articles were read thoroughly and sometimes led to the discovery of additional relevant references. In the genus *Holothuria* 135 saponins have been reported thus far. The saponins contain one, two, four, five or six sugar moieties, and 47 sulfated saponins and 86 non-sulfated saponins were listed, while for two saponins the reported structure and the presence or absence of a sulfate group was unclear. All saponins possessing a sulfate group contained one up till four sugar units. Almost half of the saponins (64) were reported for the first time in the genus *Holothuria* in the past ten years and 37 compounds had not at all been described before, thus showing the vast amount of progress that was made in this field of research in the past decade. Table 1 gives an overview of all reported compounds and their structural features and indicates whether the compound was newly identified and/or was reported for the first time in the genus *Holothuria*. Figure 1 shows the commonly occurring sapogenin structures, as well as the various possible side chain (R_1) substituents. Figure 2 shows the possible glycoside moieties, up to 5 monosaccharide units (R_2 substituents), while the R_2 substituents consisting of 6 monosaccharide units are shown in Figure 3, together with the R_3 substituents. Finally, Figure 4 shows structures of sapogenins not complying with the generally occurring sapogenins (indicated by "atypical" in Table 1) and the full structure of calcigeroside B (**19**), which is the only reported saponin in the *Holothuria* genus with two branching points in the glycosidic moiety. Seven of these "atypical" compounds do not comply with the regular 3β-hydroxyholost-9(11)-ene skeleton: hillaside A (**40**), holothurinogenin B (**56**), lefevreioside C (**94**), nobiliside A (or I) (**113**), nobiliside II (= ananaside C) (**116**), philinopside E (**130**) and calcigeroside B (**19**).

Mass spectrometry-based saponin research

Mass spectrometry, either via direct infusion or hyphenated with an LC-system, is a powerful technique for the tentative identification of saponins in mixtures. A majority of the papers published during the last decade were focused on the analysis of *Holothuria* saponins by means of (HR)MS and MS/MS. MALDI-MS (Van Dyck et al. 2009; Caulier et al. 2013b, 2016; Bahrami et al. 2014a, b; Sroyraya et al. 2018), MALDI-FT-ICR MS (Bondoc et al. 2013), MALDI-MSI (Mass Spectrometry Imaging) (Van Dyck et al. 2010a), LC-ESI-MS (Van Dyck et al. 2009; Bahrami et al. 2014a, b; Mitu et al. 2017; Grauso et al. 2019; Kamyab et al. 2020; Omran et al. 2020), or ESI-MS analysis after direct infusion (Sangpairoj et al. 2016), and rarely also LC-APCI-MS (Sangpairoj et al. 2016) and nano-HPLC-chip Q-ToF MS (Bondoc et al. 2013) were used for the MS analysis of *Holothuria* saponins.

The molecular weight (MW) of these saponins ranges from ≈ 600 to 1500 and by HRMS the most likely molecular formula can be deduced. Both positive and negative ion modes are suitable for analyzing *Holothuria* saponins. Since triterpene glycosides show a high affinity for alkaline metal ions, in positive ion mode $[M+Na]^+$ are the predominant adducts detected (Caulier et al. 2016; Sroyraya et al. 2018). In negative ion mode either $[M-H]^-$ or $[M-Na]^-$ adducts are observed for non-sulfated and sulfated compounds, respectively. However, given the fact that many isomeric saponins exist, MS/MS analysis is required to obtain additional information on the exact saponin structure. Both MALDI-MS and ESI-MS are soft ionization techniques, which will leave the core of the sapogenin intact upon analysis. Therefore, only limited information can be obtained about the sapogenin, and isomeric compounds cannot always be

distinguished, even though the lateral side chain can be cleaved in some cases (Bahrami et al. 2018). Typical fragments and neutral losses can be observed though, corresponding to certain structural features, in particular with regard to the oligosaccharide chain. In this part of the review, the most important findings of *Holothuria* sea cucumber saponins of the past 10 years, obtained by MS analysis, will be summarized, followed by an overview of typical MS signals, which can aid in the structure elucidation.

Van Dyck and coworkers studied the sea cucumber *H. forskali* by MALDI-MS, MALDI-MS/MS, and MALDI-MSI. This was one of the first research groups to focus on the distribution of saponins in different organs (body wall vs. Cuvierian tubules) and to assess the effect of stress of the sea cucumbers on the saponin composition (Van Dyck et al. 2009, 2010a, 2011). They revealed that the Cuvierian tubules contained a higher variety of saponins (26 vs. 12) and found that saponins in the high MW range (1400-1500) were more abundant in Cuvierian tubules of stressed holothuroids, while saponins with *m/z* 1125 and 1141 (both $[M+Na]^+$ adducts) seemed to be present in particular in relaxed specimens. A possible explanation is that during conditions of prolonged stress the latter are converted into the former by the addition of a disaccharide (Qui-Glc or MeGlc-Glc), making the saponin more hydrophilic and more membranolytic (Van Dyck et al. 2010a). Reports on various new saponins were already published by this team in the year 2009, and in 2011 they reported the discovery of two additional new saponins, namely holothurinosides L (**76**) and M (**77**). Both saponins contain the same glycosidic moiety, comprised of MeGlc-Glc-Qui-(MeGlc)-Xyl, and differ only in the presence or absence of a 17-OH group. Furthermore, they analyzed five sea cucumber species from the Indian ocean and determined their saponin composition qualitatively and quantitatively (Van Dyck et al. 2010b). This work led to the identification of one new saponin, holothurinoside K1 (**75**), in the body wall of *Holothuria leucospilota*.

Bondoc et al. (2013) investigated the saponin content of three *Holothuria* species, *H. scabra*, *H. impatiens* and *H. fuscocinerea,* by MALDI-FT-ICR MS and nano-HPLC-chip Q-ToF MS. They found 22 different *m/z* values typical for saponins, and numerous isomers were observed. Holothurin A (**43**) was found in the body wall of all three species with a relative abundance greater than 60%, and also holothurinosides C (**60**) and H (**70**) were detected in all three species, together with one non-sulfated and three sulfated unidentified saponins (Bondoc et al. 2013). They categorized the saponins into four distinct carbohydrate structural types: A) MeGlc-Glc-Qui-Xyl-Aglycone, B) MeGlc-Glc-Qui-(Qui-Glc)-Xyl-Aglycone, C) MeGlc-Glc-Qui-(MeGlc-Glc)-Xyl-Aglycone, D) MeGlc-Glc-Qui-sulfoXyl-Aglycone, each with its own characteristic MS/MS fragmentation pattern.

In the same year, Caulier et al. compared the saponin content of fresh *H. scabra* body wall and the trepang (the processed sea cucumber, which is eviscerated, salted and cooked for several hours, and which is edible), as well as its cooking water (Caulier et al. 2013b). Interestingly, at least six saponins were tentatively identified by MALDI-MS/MS in the fresh body walls, as well as in the trepangs (holothurinosides C (**60**) and G (**67**), scabrasides A (**131**) and B (= 17-hydroxyfuscocineroside B (**4**)), desholothurin A (**29**) and pervicoside C (**128**)), with varying relative intensities. No saponins were found in the cooking water. In addition, a paper was published regarding the function of sea cucumber saponins as putative kairomones that may attract the symbiotic Harlequin crab (Caulier et al. 2013a). *H. scabra*, as well as *H. lessoni* and *H. forskali* were studied and ten different saponins were tentatively identified by MALDI-MS in at least one of these species. However, no detailed description of the tentative identification is provided in either of the papers.

Bahrami and coworkers published two papers in 2014 and two more in 2015 and 2018, all about the study of saponins of *H. lessoni*, which is abundant in Australian waters (Bahrami et al. 2014a, b, 2018; Bahrami and Franco 2015). They analyzed the viscera of *H. lessoni* with MALDI-MS and ESI-MS and introduced sodium ions to the samples, to obtain predominantly $[M+Na]^+$ adducts (Bahrami et al. 2014a, b; Bahrami and Franco 2015). In their most recent paper also the saponin content in the body wall was analyzed and compared to that of the viscera (Bahrami et al. 2018). At least 75 saponins were detected in the viscera, of which 39 new, and over 89 saponins were found in the body wall, of which only 54 had been reported previously (Bahrami et al. 2014b, 2018). Considering these numbers, it can be concluded that they delivered a major contribution to the newly identified compounds during the past years.
It was found that the majority of saponins were common between the body wall and the viscera, while nine saponin congeners were found solely in the body wall, most of them having high molecular weights between 1400 and 1600 (Bahrami et al. 2018). The newly reported compounds were: holothurinosides O, P, Q, R, R1, S and T (**80**-**86**) (Bahrami et al. 2014b), holothurinosides X, Y, Z (**87**-**89**), holothurins D (**54**) and E (**55**) (Bahrami et al. 2014a) and lessoniosides A-G (**95**-**101**) (Bahrami and Franco 2015). Lessoniosides H, I, J and K (**102**-**105**) were four novel saponins isolated only from the body wall (Bahrami et al. 2018). Several of these saponins are acetylated, namely lessoniosides A-E (**95**-**99**) and H-K (**102**-**105**) (Bahrami and Franco 2015; Bahrami et al. 2018). Lessoniosides A (**95**), B (**96**), D (**98**) and H-K (**102**-**105**) bear an acetoxy-group in position 16, and lessoniosides C (**97**) and E (**99**) contain an acetoxy-group in position 25 of the side chain. Lessoniosides C (**97**), E, F and G (**99**-**101**) contain a 16-keto group, which is a rare feature among sea cucumber saponins. Remarkable is the composition of the oligosaccharide chain of lessonioside B (**96**), since it contains two quinovose units, which is rarely observed in sea cucumber saponins (Bahrami and Franco 2015). Furthermore, lessoniosides C, D and E (**97**-**99**) possess the same terminal saccharide moiety (3-*O*-MeXyl), which is also rare in *Holothuria* triterpene glycosides.

Two general fragmentation pathways were described: first, the (successive) loss of the sugar units (glycosidic bond cleavages) (Bahrami et al. 2014a, b) and secondly, the loss of the aglycone, followed by fragmentation of the sugar chain. One additional fragmentation pathway can occur in case of a sulfated molecule: loss of the sulfate group, followed by loss of the aglycone and sequential losses of sugar moieties (Bahrami et al. 2014a). In case of acetylated saponins, option 2 will involve loss of acetic acid and of the deacetylated aglycon, followed by fragmentation of the sugar chain (Bahrami and Franco 2015). Alternatively, after loss of the acetic acid moiety, fragmentation of the sugar chain can follow, finally resulting in the deacetylated aglycon ($[M\text{-sugar residue-AcOH+Na}]^+$) (Bahrami and Franco 2015). Some typical cleavage sites are shown in Figure 5.While Bondoc et al. categorized the saponins into four distinct categories, Bahrami et al. expanded this list to 7 distinct carbohydrate structural types and added the following three types: E) MeGlc-Glc-Glc-Xyl-Aglycone, F) MeGlc-Glc-Glc-(MeGlc-Glc)-Xyl-Aglycone and G) MeGlc-Glc-Glc-(Qui-Glc)-Xyl-Aglycone.

Caulier et al. (2016) studied the saponin composition of the Mediterranean *Holothuria (Platyperona) sanctori* and found that the body wall contained a higher diversity of saponins compared to the Cuvierian tubules (12 vs. 8), while the levels in the Cuvierian tubules were two to 2- to 3-fold higher than in the body wall (with an estimated concentration of 1 mg of saponins per g of body wall). Only two saponin signals were common to both tissues and only non-sulfated saponins were described for this species.
Two new compounds, i.e. holothurinosides M1 (**78**) and N1 (**79**) were reported, which are isomers of holothurinosides M (**77**) and N (= holothurinoside L (**76**)), respectively. Moreover, the names holothurinosides F1Ac (**66**) and G1Ac (**69**) were proposed for two new acetylated saponins with *m/z* 1493

and 1509 (both $[M+Na]^+$), since they were considered the acetylated counterparts of holothurinosides F1 (**65**) and G1 (**68**) (Caulier et al. 2016). However, attention should be paid, since the latter two compounds contain a 22,25-epoxy moiety, while the two acetylated saponins bear a linear side chain, and therefore, the acetyl group is not the only difference between holothurinosides F/G and F1/G1. Also their glycosidic part is not identical, since the acetylated compounds contain a glucose instead of a quinovose moiety.

Sangpairoj et al. (2016) studied the body wall of the sea cucumber *H. scabra* (sandfish) and its apoptic effect on human glioblastoma cell lines (Sangpairoj et al. 2016). This species is the most widely consumed sea cucumber in Asia. The compounds holothurin A (**43**), scabrasides A (**131**) and B (= 17-hydroxyfuscocineroside B (**4**) and holothurin A3 (**45**) were tentatively identified, and had been reported in this species before (Nguyen et al. 2007; Han et al. 2009a; Bondoc et al. 2013). In addition, holothurinosides D (**62**) and M (**77**) and holotoxin A1 (**90**) were tentatively identified in *H. scabra* for the first time. The same species was investigated by Mitu et al. (2017), who tried to unravel the biosynthetic pathway of saponins in the body wall. One part of their work involved LC-MS analysis of the conditioned water of *H. scabra*, in which holothurinoside C (**60**), desholothurin A (**29**) and pervicoside C (**128**) were identified. In addition, sixteen compounds were tentatively identified in the body wall of *H. scabra*, of which 13 triterpene glycosides (and one steroid glycoside: glycoside B2, previously only reported in starfish). Six of them were reported for the first time in the genus *Holothuria*. Jattujan et al. (2018) also studied *H. scabra* and analyzed different extracts from the whole body, body wall and viscera for their anti-oxidant and lifespan-extending properties in *Caenorhabditis elegans*. The most promising extracts, the butanolic extract of the whole body and the ethyl acetate extract of the body wall were analyzed by HPLC-ESI-MS and MS/MS in positive ionization mode. Several molecular ions were observed in the butanolic whole body extract, which could correspond to a total of 13 saponins. The LC-MS analysis of the ethyl acetate body wall extract showed 5 major and 4 minor peaks with *m/z* values ranging from 507 to 1522, which were also claimed to correspond to several known triterpene glycosides. A tentative identification of those compounds was not reported though.

Sroyraya et al. (2018) applied MALDI-MS and MALDI-MSI on the body wall of *H. leucospilota.* They reported 13 different *m/z* values that could correspond to saponins and names of corresponding compounds were proposed for ten of them. It was found that the epidermal layer was particularly rich in saponins, while no saponins could be detected in the dermis.

Grauso et al. (2019) were the first to use a molecular networking-based approach for the analysis of saponins from *H. atra*. The molecular network, showed one cluster with 12 nodes, presumably all saponins, and holothurins A (**43**), A5 (**47**), B (**49**) and D (**54**), 24-dehydroechinoside A (**6**), echinoside A (**35**) (= holothurin A2) and calcigeroside B (**19**) were tentatively identified. The identification of the latter compound is remarkable, since it does not contain a regular holostane skeleton and it has not been reported in any *Holothuria* species up till now. One major asset of their work was the combined use of MS screening and subsequent isolation and structure elucidation of the major compounds by means of NMR spectroscopy. Indeed, holothurins A (**43**), A5 (**47**), echinoside A (**35**) and 24-dehydroechinoside A (**6**) were purified and their structures confirmed by NMR analysis. In addition, two new compounds (compounds 5 (**133**) and 6 (**134**)) were identified, which, according to the authors, most probably are artifacts of holothurin A5 (**47**) formed by contact with water and/or methanol during the extraction and purification process, resulting in the addition of a hydroxy- or methoxygroup in position 24 of the saponin.

Kamyab et al. (2020) studied several Indo-Pacific sea cucumbers, including *H. whitmaei*, *H. hilla*, *H. atra* and *H. edulis* and investigated anti-fouling effects of the saponin containing extracts against the algae species *Cylindrotheca closterium* (Kamyab et al. 2020). UPLC-HRMS analysis was performed in ESI^+ MS^e mode and a high number of *m/z* values, most probably corresponding to saponin or sapogenin structures were observed. However, only a few compounds were putatively annotated (echinosides A (**35**) and B (**36**), desholothurin A (**29**), pervicoside B (**127**), bivittoside A-like compounds) and no detailed discussion of the interpretation of the MS-data is provided; instead, the paper mainly focuses on the anti-fouling activity.

Omran et al. (2020) analyzed several Egyptian sea-cucumbers, including body walls of *H. leucospilota*, *H. edulis*, *H. atra* and *H. polii* and holothurin B1 (= echinoside B (**36**), 24-dehydroechinoside A (**6**), bivittosides C (**17**) and D (**18**) were identified in certain *Holothuria* species. Fragment ions detected in positive ionization mode at either *m/z* 523 or *m/z* 507, indicative for a MeGlc-Glc-Glc or MeGlc-Glc-Qui glycosidic part, respectively, aided in the identification.

In summary, MS analysis of *Holothuria* saponins is feasible in both positive and negative ionization mode and HRMS can be valuable to deduce the most probable molecular formula of the *Holothuria* saponins. In case analysis is carried out in both ionization modes, the difference of the two monoisotopic ions can indicate whether the saponin bears a sulfate group. If the compound is not sulfated, in positive mode, the $[M+Na]^+$ ion will most probably be observed, and in negative mode the $[M-H]^-$ ions, thus showing a mass discrepancy of 24 mu. In case of a sulfated compound, in which the counterion of the sulfate group is usually a sodium ion, the $[M-Na]^-$ ion is most likely to be formed, resulting in a mass discrepancy of 46 mu (Bondoc et al. 2013; Bahrami et al. 2018).

Additional structural information can be obtained by MS/MS analysis. In almost all publications, this analysis has been carried out in the positive ionization mode and ESI and MALDI are the most commonly used ionization techniques. Throughout the years, typical fragmentation patterns were described and both the observed fragment ions and the neutral losses can provide valuable information about certain structural features. In Table 2, an overview is given of reported neutral loss values and the type of functional group/substructure which they indicate, while in Table 3 typical *m/z* values of fragment ions and their corresponding structures are listed. Using these soft ionization techniques, the fragmentation often occurs via cleavage of glycosidic bonds, while the sapogenin usually remains intact. One exception is the loss of the acetyl-group from the aglycon moiety, which is indicated by a neutral loss of 60 mu.

The sequential loss of single sugar units can give indications of the structure of the glycosidic part of the saponin, but also certain fragment ions of the glycosidic moiety are important to consider. The two most characteristic fragment ions in positive ionization mode are those with *m/z* 507 and 523, representing sodium-adducts of a MeGlc-Glc-Qui and a MeGlc-Glc-Glc moiety, respectively. However, the fragment ion at *m/z* 507 can also correspond to a sapogenin type B with a 22,25-epoxy side chain, or a sapogenin type A with a linear side chain and keto substitution. Another common fragment ion can be observed at *m/z* 491 and represents the sapogenin type A or type D (with a 12-OH and without a 17-OH, or without a 12-OH and with a 17-OH, respectively) bearing a 22,25-epoxy side chain. Isomers of the sapogenins can exist and especially in case of a linear side chain many variations can occur.

As can be deduced from Tables 1 and 2, MS analysis can provide a vast amount of information about the chemical structure of *Holothuria* saponins. Nevertheless, many papers published in the past years also report new saponins with *m/z* values not earlier reported, or which are isomers of reported saponins, and for which no exact structures are proposed (Bondoc et al. 2013; Bahrami et al. 2014a, b, 2018; Bahrami

and Franco 2015; Caulier et al. 2016). Indeed, in most cases it is not possible to distinguish isomers by MS analysis and it is difficult to elucidate the structure of unknown compounds. Therefore, purification of the compounds followed by NMR analysis, although more labor-intensive, is still required to determine the structure of these saponins unequivocally.

NMR spectroscopy-based saponin research

While mass spectrometry is highly informative for the tentative identification in mixtures, only NMR is capable of determining the molecular structures of the *Holothuria* saponins unequivocally. Various patterns can be deduced from the reported literature on the structure elucidation of these triterpene glycosides, linking certain structural properties with certain chemical shift values. Moreover, coupling constants and NOESY-spectra can aid in the determination of the relative conformation of these saponins. Various deuterated solvents were used for the NMR analysis of *Holothuria* triterpene glycosides, of which pyridine-d_5, or a mixture of pyridine-d_5 and D_2O (4:1) are by far the most common. Thus, the chemical shift values mentioned in this section were deduced mainly from NMR analyses in pyridine or in pyridine/water mixtures. It should be noted that slightly different chemical shift values could possibly be obtained when using other solvents, like methanol-d_4 or DMSO-d_6.

The saponin aglycon backbone

In the genus *Holothuria*, both saponins with or without a 17-hydroxy group (Figure 6) are reported; the latter are the presumptive precursors of the former (Stonik et al. 1999) and these two types of aglycons can easily be distinguished based on the ^{13}C-chemical shift value of C-17: in case of substitution with a hydroxy-group (sapogenin type B and D), the chemical shift is typically 89 ppm, while a value of 47 ppm is observed when the hydroxyl-group is absent (sapogenin type A and C). The more downfield shift in the hydroxylated analog is not limited to C-17, but also C-13, C-16, C-18 and C-20 are affected (Radhika et al. 2002; Silchenko et al. 2005; Zhang et al. 2006; Sun et al. 2008a). Typical chemical shift values of these carbon atoms in the presence or absence of the 17-OH group can be found in Table 4. An α-configuration is commonly assigned to the 17-OH group (Nguyen et al. 2007; Han et al. 2009a, 2012), although in most cases no clear proof for this can be obtained by NMR, nor by MS. The chemical shifts reported for C-18 and C-20 are typical for the 18,20 lactone, present in almost all *Holothuria* saponins.

In a majority of *Holothuria* saponins, also the 12-position is hydroxylated, with a chemical shift value of C-12 of 68-72 ppm. The 12α-OH-configuration is generally assigned (Han et al. 2008, 2009c, 2010), although proof for this relative configuration is often inconsistent and insufficient. Some of the *Holothuria* saponins do not contain a 12-OH group (sapogenin types C and D) and they are characterized by a signal with a chemical shift value of 28 ppm for C-12. Examples of such compounds are nobilisides A (**113**) and B (**117**) (Wu et al. 2006c).

Holothuria saponins are always monodesmosidic, with the glycosidic moiety bound to C-3. The C-3 NMR signal appears at 87-90 ppm and the 3β-configuration is generally reported (Wu et al. 2006; Nguyen et al. 2007; Han et al. 2007, 2010, 2012; Sun et al. 2008a; Grauso et al. 2019; Hoang et al. 2020).

Furthermore, a typical characteristic of the lanostane skeleton is the 9,11-double bond, corresponding to ^{13}C chemical shift values of 152-157 and 114-116 ppm, for the 9 and 11 positions, respectively (Nguyen et al. 2007; Han et al. 2009a; Yu et al. 2015). Occasionally also double bonds in other positions are reported, in particular in the linear side chain bound to C-20 (vide infra).

Rarely, also in the 7,8 position a double bond can occur, for example in nobiliside A (**113**) and holothurinogenin B (**56**). Both compounds also contain the commonly found 9,11 double bond, but lack the 12-OH group, and thus, possess two conjugated double bonds in their aglycon backbone. Their NMR spectra are marked by upfield shifts of C-9 and C-11 from 152-155 to 148 ppm and from 114-116 to 112-113 ppm, respectively (Thanh et al. 2006; Wu et al. 2006c). However, it is claimed that genuine sapogenins with a 12α-hydroxy-Δ9(11)-ene moiety can be dehydrated in strong acidic conditions, resulting in the formation of artificial genins with a Δ7(8),9(11)-diene system, and thus, these compounds most probably are artifacts (Stonik et al. 1999; Honey-Escandón et al. 2015). Moreover, nobiliside A (**113**) contains two conjugated double bonds in its side chain; another rare structural feature among sea cucumber triterpene glycosides (Wu et al. 2006c). Also hillaside A (**40**) is atypical as it contains a 22E,24-diene and bears a 7,8 double bond (chemical shifts of 120 and 145 ppm for C-7 and C-8, respectively), while the characteristic 9,11 double bond is lacking (Wu et al. 2007).

Another type of functional group that can be present is an acetoxy-group in position 16 (sapogenin types E and F). In case this acetoxy group is present, like in arguside F (**14**), nobiliside C (**118**), hillaside B (**41**) and impatienside B (**92**), the C-16 signal can be observed between 73-76 ppm and signals of the acetoxy-group will appear at 170-171 ppm and 20-21 ppm for the quaternary carbon and the methyl-group, respectively (Wu et al. 2006c, 2007; Yuan et al. 2009a).

NOESY experiments are valuable to determine the relative configuration of the aglycon moiety, as shown by Sun et al. for impatienside A (**91**), or as mentioned by Han et al. for leucospilotasides A, B and C (**106**-**108**) (Sun et al. 2008a; Han et al. 2010). One specific NOESY correlation worth mentioning is the correlation between the CH_3-group in position 21 and the proton in position 12 (in case C-12 is hydroxylated). In case this correlation is observed, the β-orientation of the proton is inferred, which means the hydroxy-group in position 12 has the α-configuration (Zhang et al. 2006; Sun et al. 2008a; Yuan et al. 2009a). Another example is the determination of the relative configuration of the 16β-acetoxy group based on the NOESY correlation of H-16 with H-15α, for example in nobiliside C (**118**) and hillaside B (**41**) (Wu et al. 2006c, 2007). Also in arguside F (**14**), the 16β orientation of the acetoxy-group was determined by a NOESY experiment, as was the 17α-position of the hydroxy-group.

The saponin aglycon side chain

Either a linear side chain or a 22,25-epoxy moiety can be connected to C-20, as mentioned earlier, both consisting of 6 carbon atoms (Figure 7). The epoxy moiety can be distinguished from the linear chain by the observation of one CH-signal (C-22) and one quaternary carbon signal (C-25), both around 80-82 ppm (Han et al. 2007, 2008, 2009b; Sun et al. 2008a). In contrast, in case of a linear side chain with a 22-OH, for example in holothurin B2 (**50**) or griseaside A (**39**), C-22 is observed around 75 ppm (Silchenko et al. 2005; Sun et al. 2008a). In case of a 25-OH group, like in hillaside C (**42**), holothurins A5 (**47**) and B4 (**52**) and leucospilotaside A (**106**), C-25 can be found between 69 ppm and 81 ppm, (Silchenko et al. 2005; Wu et al. 2006a; Han et al. 2007; Grauso et al. 2019) i.e. chemical shifts are more upfield compared to the epoxy moiety.

Many variations can occur in case of a linear side chain. For example, hydroxy-groups, keto-groups, acetoxy-groups and double bonds have been reported as substitutions / variations of the isohexane side chain (Han et al. 2007, 2012). Holothurin A4 (**46**) and leucospilotaside B (**107**) both possess a 24-OH (δ_C 74-77 ppm); in both cases, the configuration of C-24 was not studied (Nguyen et al. 2007; Han et al. 2009b, 2010). In fact, the configuration of chiral centers in positions 22, 23 or 24 of *Holothuria* saponins with a linear side chain is usually not determined (Silchenko et al. 2005; Sun et al. 2008a; Grauso et al. 2019). Indeed, determination of the stereochemistry in these chiral centers is rather complex and NMR spectroscopy is not expected to provide conclusive proof.

In case of an unsaturated linear side chain, the double bond can occur in various positions and the chemical shift values vary accordingly. For example, chemical shift values of a 23,24 double bond can be 120 – 157 ppm, when conjugated with a carbonyl group at C-22 (Grauso et al. 2019); and chemical shift values of 145 – 110 ppm were reported for a 25,26-double bond in scabraside A (**131**) (Han et al. 2009a, c). Based on the coupling constant observed for the corresponding protons, usually the trans-orientation of the double bond is deduced (J ≈ 12-16 Hz) (Silchenko et al. 2005; Wu et al. 2007; Hoang et al., 2020).

Apart from the ^{13}C-chemical shift values indicative of a double bond and interpretation of the HMBC spectrum, determining the multiplicity of each carbon atom is valuable for the localization of the double bond. Related to this, a remark can be made concerning the identification of 24-dehydroechinoside A (**6**) by Shushizadeh et al. (2019). Chemical shift values of 120.6 ppm/6.13 ppm and 143.6 ppm/6.03 ppm were reported for the carbons and protons of positions 23 and 24, respectively, which is in line with the presence of a double bond in this position, as stated by the authors. However, the described 24-dehydroechinoside A (**6**) contains a 24,25 double bond, with C-25 as quaternary carbon, which is not in agreement with the reported data. Thus, the identification of this compound as 24-dehydroechinoside A does not seem justified.

Desulfated pervicoside A (DS-pervicoside A), obtained by Kitagawa et al. by removal of the sulfate group of pervicoside A (**126**), contains a 25-acetoxy group. This was deduced from the downfield shift of C-25 to 82 ppm and the observation of a quaternary carbon at 170 ppm and a primary carbon at 22 ppm (Kitagawa et al. 1989). Furthermore, Grauso et al. (2019) reported the new compound holothurin A5 (**47**), isolated from *H. atra*, bearing an electrophilic enone function in the side chain. Remarkably, Hoang et al. (2020) recently published a paper, with a sea cucumber saponin bearing a hydroperoxy group in the side chain and they also named their novel compound holothurin A5 (**48**). According to the authors, the downfield shift of C-25 to 81 ppm compared to 69 ppm reported for C-25 of leucospilotaside A (**106**) (Han et al. 2007) or 69.5 ppm for holothurin B4 (**52**) (Silchenko et al. 2005), the latter two both bearing a 25-OH group, indicated the presence of a hydroperoxy group (Hoang et al. 2020). However, a chemical shift of C-25 of 81 ppm was also reported for scabraside D (**132**), first reported by Han et al. (2012) and for nobiliside E (**119**), identified by Zhang et al. (2015) and these compounds contain a 25-hydroxy group as well (Han et al. 2012; Zhang et al. 2015). Thus, the combined interpretation of NMR and MS data is required to identify the correct structure.

Fuscocinerosides A and B (**37**,**38**) were the first reported sea cucumber saponins bearing a 22-keto-group (Zhang et al. 2006). Compared to the quaternary carbon of the 18,20-lactone, the quaternary carbon signal of a carbonyl-group in position 22 is much more downfield, and can be found around 208-211 ppm, or at 198 ppm in case a conjugated double bond is present in position 23,24 (Zhang et al. 2006; Han et al. 2007,

2009a, b; Yu et al. 2015; Grauso et al. 2019). The presence of this additional keto-group will also cause a downfield shift of the hydroxylated C-17 (from 89 to 91-93 ppm) and a slight upfield shift of C-18 (from 174-175 ppm to 172-173 ppm) (Nguyen et al. 2007; Han et al. 2009a).

An acetoxy-substitution is also reported in position 25 of the side chain, for example in the saponin pervicoside D (**129**) (Yuan et al. 2009a) and 3-*O*-[ß-D-quinovopyranosyl-(1→2)-4-sodium sulfato-ß-D-xylopyranosyl]-25-acetoxy-22-oxo-9(11)-holostene-3ß,12a,17a-triol, which corresponds to moebioside A (**112**) (Yu et al. 2015; Hoang et al. 2020). Chemical shift values of 170 ppm and 22 ppm are commonly reported for the acetoxy moiety, while C-25 appeared at 81-82 ppm (Zhang et al. 2006; Yuan et al. 2009a; Yu et al. 2015). Nevertheless, acetylated saponins are rare in the genus *Holothuria* and mainly occur in compounds with a linear side chain (Bahrami et al. 2016).

The saponin glycosidic moiety

As previously mentioned, in *Holothuria* saponins, the glycosidic moiety is always bound to the 3-position of the sapogenin. For the sugar moiety bound directly to the aglycon, the β-configuration is commonly reported. This can be deduced from the coupling constant of the anomeric proton of the D-xylose moiety directly connected to the aglycon, which is between 7 and 8 Hz (Silchenko et al. 2005; Han et al. 2012). Also the other glycoside moieties (most commonly D-glucose, D-quinovose, and/or 3-*O*-methyl-D-glucose) are usually present in the β-configuration, with *J*-values between 6 and 8 Hz (Han et al. 2009a; Yuan et al. 2009a; Yu et al. 2015; Zhang et al. 2015; Shushizadeh et al. 2019), while *J*-values of 1 to 2 Hz are typical for the α-configuration. Moreover, thus far all sugars were reported in their pyranose form and the positions of the interglycosidic linkages can be deduced from a downfield shift relative to the resonances expected for the corresponding methyl glycopyranosides (Han et al. 2008; Sun et al. 2008a; Yuan et al. 2009a; Shushizadeh et al. 2019). In addition, the order of the sugar sequence can be deduced from the HMBC-spectrum (Sun et al. 2007). However, as mentioned earlier, MS/MS analysis can be used to determine the sugar sequence as well, and the data can be interpreted more easily, especially in case of pentosides and hexosides.

As for the different types of sugars, a quinovose can be readily distinguished by the presence of a CH_3-group (δ_C 17-18 ppm) (Shushizadeh et al. 2019), and the corresponding ^{1}H-signal integrating for 3 protons and appearing as a doublet (Rodriguez et al. 1991). Also the methoxy-group of a 3-*O*-methylglucose, can be easily identified with a ^{13}C-chemical shift of 60-61 ppm and a proton signal between 3.5-3.9 ppm, integrating for 3 protons and appearing as singlet (Zhang et al. 2015; Shushizadeh et al. 2019; Hoang et al. 2020). The 3-position is commonly assumed for this methoxy-group, but may be confirmed by observation of an HMBC correlation between the proton signal representing the methoxy-group with C-3 of the respective methylglucose sugar, as reported by Hoang et al. (2020). Alternatively, the presence of a 3-*O*-methylglucose can be confirmed by acid hydrolysis followed by GC-MS analysis and comparison to reference compounds, as applied by Zhang et al. (2015). However, GC-MS analysis of the hydrolyzed saponin has mainly been used to determine the absolute configuration of the sugar moieties (D or L) (Nguyen et al. 2007; Wu et al. 2007; Sun et al. 2008a; Yuan et al. 2009a; Han et al. 2012; Yu et al. 2015; Zhang et al. 2015). D-sugars are most commonly encountered in sea cucumbers and can be expected to be present for biosynthetic reasons (Nguyen et al. 2007; Sun et al. 2008a; Han et al. 2009a, 2010; Hoang et al. 2020). Except for an α-L-rhamnose moiety reported for an unnamed *Holothuria* saponin by

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Anjaneyulu and Raju (1996) (vide infra), the images of structures in the current review thus are shown with D-sugar moieties, even though conclusive proof for this configuration was not always provided.

Amongst the *Holothuria* saponins, sulfated, as well as non-sulfated triterpene glycosides occur. In all sulfated *Holothuria* saponins, the sulfate group was found in position 4 of the xylose unit, resulting in a characteristic downfield shift of C-4 from 68-71 to 74-77 ppm (Kitagawa et al. 1979; Silchenko et al. 2005; Thanh et al. 2006; Wu et al. 2006c; Han et al. 2012; Shushizadeh et al. 2019). However, in case another glycoside moiety is bound to C-4 of the xylose unit, the chemical shift of C-4 will also be in the range of 77 ppm (Sun et al. 2007). Therefore, it is important to know the number of sugar moieties present and apart from the MS data, also the number of signals of anomeric protons in the ^{1}H-NMR spectrum (between 4.1 ppm and 5.3 ppm) and corresponding carbons (102-108 ppm in ^{13}C-NMR) can provide this information. Sulfated saponins in the genus *Holothuria* were only reported in compounds with 2 or 4 glycoside moieties; saponins with 5 or 6 sugar moieties have a branched sugar chain, and in these saponins C-4 of the xylose moiety is always substituted with another glycoside moiety, thus excluding the possibility of substitution with a sulfate group.

Reflections on reported data

When assembling this overview of *Holothuria* saponins and the characteristic signals that can be observed by MS and NMR, in several cases questions were raised regarding the validity of the identifications. Some of them will be discussed below.

Firstly, holothurins A (**43**) and A3 (**45**), scabrasides A (**131**) and B (= 17-hydroxyfuscocineroside B (**4**)), holothurinosides D (**62**) and M (**77**) and holotoxin A1 (**90**) were tentatively identified in *H. scabra* by Sangpairoj et al. in 2016, based on MS analysis in positive ionization mode (Sangpairoj et al. 2016). However, for holothurinoside D (**62**) and for holotoxin A1 (**90**), the detected *m/z*-values do not correspond to the $[M+H]^+$, nor the $[M+Na]^+$ adduct, which are expected to be observed in this ionization mode, while for the other identified compounds the reported *m/z* values seem to indicate the detection of sodium-adducts indeed. Also, no HRMS data were recorded, thus raising some concerns regarding the identification of these two compounds in particular.

One year later, thirteen saponins were reported from the body wall of *H. scabra* by Mitu et al. (2017). However, again some of the identifications can be questioned. For most of the detected ions, the type of adduct is not mentioned, but the *m/z* value does not seem to correspond to the $[M-H]^-$ or $[M-Na]^-$, which are most likely to be formed, without or with the loss of an H_2O molecule. In addition, also in this case no MS/MS data were reported. Moreover, six out of the 13 identified saponins had not been reported in any species of the *Holothuria* genus up till then and several bear a 7,8 double bond, instead of a 9,11 double bond, which is clearly divergent from all other *Holothuria* saponins previously reported. One example is the identification of lefevreioside D with *m/z* value 1199.5249, previously reported in *Cucumaria lefevrei* (Rodriguez and Riguera 1989). It has to be noted that in the initial report, the nomenclature of the identified lefevreisoides is not consistent and both lefevreioside C and lefevreioside D are mentioned as names for this compound. On Scifinder, the compound is reported with the name lefevreioside C (**94**) and therefore, this name is used in Table 1 and Figure 8. It is a sulfated compound with molecular formula $C_{55}H_{85}O_{25}SNa$, and a calculated *m/z* value for the $[M-H]^-$ ion of 1199.4920. Thus, the deviation with the experimental value is 27 ppm, which is higher than tolerated. Although not all required data are at our

disposal, in our opinion a more plausible tentative identification of the ion with *m/z* 1199.5249 is scabraside D (**132**) ([M-Na]$^-$, calculated: 1199.5160, Δ 7.4 ppm), previously reported in *H. scabra* and *H. lessoni* (Han et al. 2012; Bahrami et al. 2014b, 2018; Wang et al. 2014; Assawasuparerk et al. 2016). Nevertheless, the discrepancy of 7.4 ppm is still higher than the generally accepted accuracy of 5 ppm.

Another point of attention is the identification of nobiliside E (**119**). Mitu et al. (2017) refers to the publication of Zhang et al. (2015), who reported nobiliside E as a new compound, isolated from *H. nobilis* According to their paper, nobiliside E has a molecular formula of $C_{54}H_{88}O_{23}$ and nominal mass of 1104, which does not seem to fit with the reported *m/z* value of 1198.5034. However, according to Scifinder, nobiliside E was previously reported by another Chinese research group, who filed a patent regarding the "Anti-tumor compound nobiliside E separated from *Thelenota ananas*" (Wu et al. 2009). This compound differs from nobiliside E reported by Zhang et al. and has a molecular formula of $C_{55}H_{85}O_{26}SNa$ and nominal mass of 1216. Unfortunately, also this compound cannot be matched with the detected ion.

Also in 2017, Zhang and Zhu reported the novel compound nobiliside D, isolated from *Holothuria nobilis* (Zhang and Zhu 2017). However, neither the reported NMR data, nor the MS data are in agreement with the proposed chemical structure, and in fact, the reported structure was identified before, as hillaside C (**42**), by Wu et al. (2006a). The proposed structure bears a linear side chain with a 25-OH group (Figure 9), but the chemical shift observed for C-22 (80.7 ppm) is not in agreement with a CH_2-group. Instead, the chemical shifts of C-22 and C-25 of 80.7 and 81.4 ppm, respectively, are typical for a 22,25-epoxy moiety, as mentioned earlier. Furthermore, the reported MS data (*m/z* 891 [M+Na]$^+$ and *m/z* 845 [M-Na]$^-$ and molecular formula $C_{40}H_{61}O_{17}SNa$ show a discrepancy of two protons compared to the structure shown, but in case a 22,25-epoxy moiety would be present, these MS data would fit. Interestingly, the adapted structure that is proposed here has not been reported before.

Remarkably, nobilisides A-J (**114**, **118**, **117**, **42**, **120-125**, respectively) were already mentioned by Zhang et al. (2008), in a publication written in Chinese, entitled: "Screening of bioactive constituents from sea cucumber *Holothuria nobilis* using conidia of *Pyricularia oryzae*". The compound nobiliside D is the same as nobiliside D proposed as a novel compound in 2017 (Zhang and Zhu 2017), while its structure had previously been reported as hillaside C (**42**) (Wu et al. 2006a). Nobiliside A as reported by Wu et al. in 2006 (**113**) differs from nobiliside A reported by Zhang (**114**). Moreover, names of nobilisides B and C (**117**, **118**) seem to have been switched, compared to the publication of Wu et al. Furthermore, the chemical name of nobiliside E reported in 2008 (**120**) does not correspond with the structure reported by Zhang et al. in 2015 (**119**) (Wu et al. 2006c; Zhang et al. 2015). Since no data were provided supporting the structure elucidation of nobilisides A-J, no molecular formulas are mentioned and no images of the structures were provided, the actual structures of nobilisides E-J as reported in 2008 could not be confirmed with certainty.

Furthermore, attention should also be paid to the difference between nobiliside I (with a capital letter i) (**124**) and nobiliside I (with the roman number 1) (Zhang et al. 2008; Zhang 2011). The latter compound is the same as holothurin A (**43**). Also nobiliside II (**116**) and ananaside C have the same chemical structure, and the same is true for nobiliside 2a and desholothurin A (DS-holothurin A (**29**)), which has already been highlighted in earlier review articles (Wu et al. 2006b; Caulier et al. 2011; Zhang 2011; Honey-Escandón et al. 2015; Bahrami et al. 2016).

A significant number of saponins first identified in the genus *Holothuria* and/or compounds reported for the first time, were tentatively identified by Bahrami and co-workers. However, not all of their data seem to support the proposed structures. One example is the case of holothurin E (**55**), as shown in Figure 10.

This saponin was first reported in 2014, and detected in the viscera of *H. lessoni* at *m/z* 1227.4 ([M+Na]$^+$) (Bahrami et al. 2014a). In the corresponding MS/MS spectrum, loss of the aglycon resulted in a signal with *m/z* 743.3 (neutral loss 484), followed by loss of the sulfate (*m/z* 623), xylose (*m/z* 491) and *O*-methylglucose (*m/z* 317). However, the neutral loss of the latter transition is 174, while a value of 176 is expected for a MeGlc moiety. Moreover, two different fragmentation patterns are reported for this compound; one in which the loss of the aglycon and the sulfate and xylose group is followed by a loss of MeGlc and Xyl moiety; and another starting with the loss of MeGlc, Glc, Xyl, sulfate, Xyl, and finally the aglycon, which indicates that the order of monosaccharides would be MeGlc-Glc-Xyl-sulfoXyl), while the reported structure contains a MeGlc-Xyl-Glc-sulfoXyl side chain, which is much less common. Finally, the nominal mass of the reported structure with molecular formula $C_{53}H_{83}O_{27}SNa$ is 1206, and a value of 1229 would be expected for the [M+Na]$^+$ adduct, again showing a difference of 2 mu. Therefore, more research is required in order to confirm the structure of the newly reported compound holothurin E (**55**).

In 2018, Bahrami et al. reported holothurin C (**53**) in the body wall of *H. lessoni*, with molecular formula $C_{67}H_{108}O_{33}$ (nominal mass 1440) (Bahrami et al. 2018). However, no structure of this compound was shown in this paper, nor could it be found in any earlier publications, although holothurin C (**53**) has been mentioned before by Elyakov et al. in 1973, and was isolated from the sea cucumber *Bohadschia argus* (Elyakov et al. 1973). They found that this compound contained a glycosidic chain comprised of four different monosaccharides, namely 3-*O*-MeGlc, Glc, Qui and Xyl, and although the ratio is not mentioned, most likely they are present in a 1:1:1:1 ratio. Nevertheless, only saponins with a glycosidic chain comprised of 6 monosaccharides can have a molecular weight in the range of 1400-1500 and therefore, the rationale behind the identification of holothurin C (**53**) in *H. lessoni* remains unclear.

The saponin holothurinoside K1 (**75**) was first reported in *Bohadschia subrubra* by Van Dyck et al. in 2010 (Van Dyck et al. 2010b), but unfortunately some inconsistency is observed in the values reported for this triterpene glycoside with a 6-sugar side chain: it was described that this compound was detected at *m/z* 1495 [M+Na]$^+$, but as molecular weight 1134 is given, while its nominal mass, derived from the reported structure (molecular formula $C_{67}H_{108}O_{35}$) is 1472. More recently, holothurinoside K1 (**75**) was reported for the first time in the genus *Holothuria*, as it was tentatively identified in *H. lessoni* and in *H. atra*, as reported by Bahrami and co-workers, and Shahinozzaman et al., respectively (Bahrami et al. 2014a, b, 2018; Shahinozzaman et al. 2018b). However, the identification in *H. lessoni* was based on the observation of a molecular ion with *m/z* 1495, while Shahinozzaman et al. observed an ion with *m/z* 1135.4, which they assumed was the [M+H]$^+$ adduct, and they wrongly assigned this peak to holothurinoside K1 (**75**). Since they did not record HRMS data or MS/MS data, their tentative identifications should be interpreted with caution. In 2011, Van Dyck et al. identified two new saponins in the body wall of *H. forskali*, both with a MeGlc-Glc-Qui-(MeGlc)Xyl sugar chain and a 22,25-epoxy moiety, and only differing in the presence or absence of a 17-OH group. These compounds were named holothurinosides L (**76**) and M (**77**), respectively (Van Dyck et al. 2011). Two years later, Caulier et al. analyzed the composition of several sea cucumbers, including *H. forskali,* with MALDI-MS. They reported the presence of six different saponins, including a compound named holothurinoside N (Caulier et al. 2013a). However, the elucidation of structures is not discussed in detail and while holothurinoside N was not previously mentioned in the literature, no specific explanation about its structure elucidation is provided. In fact, the structure reported for this compound is identical to that of holothurinoside L, and indeed, they were identified at the same *m/z* value. Thus, holothurinosides L and N (**76**) are the same compound and one of the two names should be selected to be used in future reports.

In line with these compounds, holothurinosides O, P, Q, R, R1 and S (**80**-**85**) were reported in 2014 as new compounds in the viscera of *H. lessoni* by Bahrami et al. (2014b). By interpretation of ESI+ MS/MS spectra, five isomers with *m/z* value 1287.6 ($[M+Na]^+$) were identified as 17-dehydroxyholothurinoside A (**3**), holothurinosides E (**3**) and E1 (**63**), and the new holothurinosides O (**80**) and P (**81**). However, 17-dehydroxyholothurinoside A is in fact a synonym of holothurinoside E (**3**), and these cannot be considered as two separate compounds. Furthermore, only two neutral losses, typical for the aglycons of 17-dehydroxyholothurinoside A / holothurinoside E (**3**), as well as holothurinoside O (**80**) (468 mu) and corresponding to the aglycon of holothurinoside P (**81**) (484 mu) were observed. Fragmentation of holothurinoside E1 (**63**) is expected to show a neutral loss of 452 when the aglycon is split off, but this was not observed in the mass spectrum, therefore making its identification less certain. Furthermore, the glycosidic chain of holothurinoside O (**80**) contains the same monosaccharide units as 17-dehydroxyholothurinoside A / holothurinoside E (**3**), and since all these compounds are isomeric, a difference must be present in the sapogenin part of the molecule, but the exact difference between this newly reported compound and these two already known compounds is not clear.

The two newly reported compounds holothurinosides R (**83**) and R1 (**84**) most probably contain the same aglycone moiety, which is characterized by a neutral loss of 500 mu. However, unfortunately the exact structures of the aglycone and of the entire saponin are not described. Moreover, the description of the glycosidic chain raises questions, as for both holothurinosides R (**83**) and R1 (**84**) MeGlc, Glc, Qui and Xyl are present, while the fifth monosaccharide in holothurinoside R (**83**) is a Qui and in holothurinoside R1 (**84**) a Glc. Obviously, quinovose and glucose differ in molecular weight, which is in contradiction with the fact that these two compounds are reported as isomers without any other structural differences. The same issue is observed for holothurinosides S (**85**) and A1 (**58**). Both are reported to contain an aglycon moiety with the same molecular weight, but a difference of 2 mu is found between the glycosidic chain of holothurinoside S (**85**) (MeGlc-Glc-Qui-(MeGlc)Xyl, $C_{31}H_{53}O_{24}$, 809 mu, Fig. 2, glycoside chain "n") and holothurinoside A1 (**58**) (MeGlc-Glc-Glc-(Glc)Xyl, $C_{30}H_{51}O_{25}$, 811 mu, Fig. 2, glycoside chain "p").

Finally, also for the newly reported compound holothurinoside T (**86**), detected in positive ion mode at *m/z* 1149 $[M+Na]^+$, no complete structure is proposed and no molecular formula is mentioned. For all of these compounds it is required to carry out additional analysis, preferably by purifying them and applying MS and/or NMR in order to determine or confirm their structures.

In another publication which is also focused on the structure elucidation of saponins in *H. lessoni*, five more new compounds were presented, namely holothurins D and E (**54**, **55**, discussed before) and holothurinosides X, Y and Z (**87**-**89**, figure 11) (Bahrami et al. 2014a). The latter three are claimed to be isomeric compounds, observed at *m/z* 1127.6 $[M+Na]^+$. Based on the images of the structures provided, the molecular formulas of holothurinosides X (**87**) and Y (**88**) are $C_{53}H_{84}O_{24}$ and $C_{54}H_{88}O_{23}$, respectively, both with a nominal mass of 1104 and thus in accordance to the observed molecular ion. However, for holothurinoside Z (**89**) a molecular formula of $C_{54}H_{88}O_{24}$ is found (nominal mass 1120) which cannot possibly result in an ion with the observed *m/z* value.

Another aspect that needs further investigation is the reported sequence of the monosaccharides in the glycosidic chain. For holothurinoside X (**87**), MeGlc-Xyl-Glc-Xyl and for holothurinosides Y (**88**) and Z (**89**) MeGlc-Qui-Glc-Xyl sequences are shown in the image of the publication (and the structures shown in this review are accordingly). When comparing with other previously reported triterpene glycosides, both oligosaccharide chains were not reported before in saponins of the genus *Holothuria*. Switching the

position of the second and third sugar moiety seems more likely in view of previously reported compounds, and seems to be implied in other parts of the respective publication as well. However, with the experimental data provided in the paper, in fact, the proof for either one of the two options does not seem conclusive. In addition, as mentioned earlier, the sapogenin is barely or not fragmented during the applied MS analysis and only limited information is available about its chemical structure. Indeed, Bahrami et al. (2014a). mentioned that isomeric compounds fuscoscineroside B (**38**) and C (= 17-dehydroxyholothurin A, (**2**)), which differ only in their side chain, cannot be distinguished by MS analysis. The former has a linear side chain with a carbonyl-group in position 22, while the latter bears a 22,25-epoxy moiety and indeed both side chains are isomeric (molecular formula $C_6H_{11}O$). However, in the same paper the novel compound holothurinoside X (**87**) was proposed, bearing the same linear side chain with 22-keto substitution as fuscoscineroside B (**38**), thereby contradicting their statement regarding the two fuscocineroside isomers. Thus, it must be concluded that the exact structures corresponding to holothurinosides X, Y and Z (**87**-**89)** cannot be determined by MALDI-MS and/or ESI-MS, but NMR is required to confirm the tentative structures.

In 2015 Bahrami published another paper on the saponin composition of the viscera from *H. lessoni* (Bahrami and Franco 2015). A total of seven new saponins were reported, and named lessoniosides A-G (**95**-**101**). All these saponins are isomers and were detected at *m/z* 1477.7 $[M+Na]^+$. Nevertheless, based on the images of the structures, lessoniosides A (**95**), B (**96**) and D (**98**) have a nominal mass of 1470 (molecular formula $C_{68}H_{110}O_{34}$) and the four other lessoniosides (C, E, F, G (**97**, **99**-**101**)) have a nominal mass of 1452 (molecular formula $C_{66}H_{100}O_{35}$). Thus, they do not comply with the reported monoisotopic ion. This discrepancy is corroborated by the reported fragmentation scheme. For example, the fragmentation of lessonioside A (**95**, Figure 12) can start with the loss of the acetoxy group, followed by loss of the aglycon, indicated by neutral losses of 60 u (acetic acid) and 470 u (uncharged aglycon), respectively, resulting in a sodium-adduct representing the glycosidic part (*m/z* 947.5). However, the first fragmentation would require two additional protons: one to form acetic acid from the acetoxy moiety, and another to be bound to the remaining fragment ion; otherwise, the second step, with a neutral loss of 470 mu for the aglycon is impossible. Thus, the actual difference between the monoisotopic ion and the ion representing the glycosidic part is 528 and not 530 mu. However, such fragmentation seems highly unlikely. More plausible seems the hydrolysis of the acetic ester bond, requiring one additional H_2O molecule, followed by a dehydration, resulting in formation of a double bond. This would result in a neutral loss of the deacetylated aglycon moiety of 468 mu. Furthermore, in the second step, when the glycosidic bond at C-3 is split, a H_2O molecule is released from the saponin and this is not accounted for in the schematic diagram either. Assuming a nominal mass of 1470 as deduced from the reported structure, a monoisotopic ion with *m/z* 1493 would be expected for the sodium adduct of lessonioside A (**95**). This indeed complies with the following calculation: fragment ion of the glycoside part (947 ($[M+Na]^+$)) + H_2O (18 mu) + sapogenin part (528 mu) = 1493. Also for lessoniosides F (**100**) and G (**101**) the neutral loss of the aglycon (496 mu) does not correspond to the neutral loss expected based on the reported structures, which is 494. Again, calculation of the expected monoisotopic ion can be carried out: fragment of the glycoside part (981 ($[M+Na]^+$)) + sapogenin part (494 mu) = 1475. This *m/z* value corresponds to the MW of 1452, obtained from the given structure. The same is true for lessonioside C (**97**).

Another remarkable point is the presence of a 3-*O*-methylxylose moiety in lessoniosides C, D and E (**97**-**99**). Although this type of sugar has been reported in sea cucumbers, it is a very rare feature among naturally occurring sea cucumber glycosides and none of the reports concerned species of the *Holothuria*

genus. In fact, *O*-methylxylose and quinovose are isomeric structures, but a clarification as to why the former moiety was identified, and not the latter, is lacking. This is particularly relevant with respect to lessoniosides B (**96**) and D (**98**), which only differ in the presence of a quinovose and a 3-*O*-methylxylose terminal sugar moiety, respectively. In our opinion, only purification and NMR analysis can provide conclusive proof to distinguish these two types of sugars.

Somewhat related to this is the identification of an unnamed saponin (**135**) by Anjaneyulu and Raju (1996). They reported that this saponin contained a glycosidic chain comprised of two saccharide moieties, one of them being α-L-rhamnose. This compound is the only saponin in the *Holothuria* genus reported to contain a rhamnose moiety, whereas quinovose (an isomer of rhamnose as well), is much more common. Rhamnose was identified by paper chromatography with "authentic samples". However, it was not mentioned if quinovose was included in this comparison. Therefore, the possibility that also in this case in fact quinovose is concerned cannot be excluded.

Finally, for some acetylated compounds, the fragmentation starts with the loss of the deacetylated aglycon, while only later on the acetoxy group is lost. This seems practically impossible.

In addition to the seven lessoniosides reported in 2015, four more acetylated saponins (lessoniosides H-K, **102**-**105**, Figure 13) were reported in 2018 (Bahrami et al. 2018). According to the authors, these four compounds were isomers detected with MS at *m/z* 1461 $[M+Na]^+$ and 1437 $[M-H]^-$, the latter ion only clearly described for lessoniosides H (**102**) and K (**105**). These data indicate a nominal mass of 1438. However, as was the case for lessoniosides A-G (**95**-**101**), also for these four compounds the molecular weights derived from the images of the structures do not comply with this *m/z*-value; for lessoniosides H (**102**) and K (**105**), a nominal mass of 1454 was found, and for lessoniosides I (**103**) and J (**104**) a value of 1456. Based on the proposed fragmentation pathways, the difference of 16 mu obtained for lessoniosides H (**102**) and K (**105**) again seems to be a consequence of not taking into account the hydrolysis and dehydration related to deacetylation and the release of a water molecule after cleavage of the glycosidic bond.

Also the compounds marmoratoside A (**109**) and B (**110**) were identified in the body wall of *H. lessoni* (Bahrami et al. 2018). It is the first report of these compounds in the genus *Holothuria*, as they were previously only isolated from genus *Bohadschia* (Yuan et al. 2009b; Elbandy et al. 2014). Marmoratoside A (**109**) is an isomer of impatienside A (**91**), only differing in the position of the double bond in the side chain, and therefore, correct identification of these compounds by MS is not straightforward. Huang et al. (2020) reported marmoroside C (**111**, = 17-hydroxyfuscocineroside A) in *H. edulis*, but no details on its identification are mentioned (Hoang et al. 2020). This compound too had previously only been identified in a *Bohadschia* species (*B. marmorata*) (Yuan et al. 2008b).

Thirteen saponins were tentatively identified by Jattujan et al. from the butanolic whole body extract of *H. scabra* in 2018 (Jattujan et al. 2018). Four of them were identified based on the detection of a monoisotopic ion at *m/z* 1302.7 $[M+Na]^+$, namely holothurinosides A (**57**), A1 (**58**), M (**77**) and M1 (**78**). However, these compounds are not isomers. For holothurinosides A (**57**) and A1 (**58**), with molecular formula $C_{60}H_{96}O_{29}$, a calculated monoisotopic mass of 1303.6 is expected for the sodium-adduct, while holothurinosides M (**77**) and M1 (**78**) have a molecular formula of $C_{61}H_{98}O_{28}$ and an ion with *m/z* 1301.6 would correspond to their sodium-adduct. Therefore, none of these four compounds seem to be in agreement with the experimental data.

Finally, Omran et al. (2020) recently identified holothurin B1 (= echinoside B, **36**), 24-dehydroechinoside A (**6**), bivittosides C (**17**) and D (**18**) in various *Holothuria* species. However, taking into account both the accurate *m/z* of the molecular ion and the glycosidic fragment, identification cannot always be carried out unequivocally. For example, 24-dehydroechinoside A (**6**), fuscocinerosides B (**38**) and C (= 17-dehydroxyholothurin A, **2**) and scabraside A (**131**) all are isomeric compounds with the same MeGlc-Glc-Qui glycosidic part and the same is true for bivittoside C (**17**) and holothurinoside F (**64**) and for bivittoside D (**18**) and holothurinoside G (**67**). The authors did not elaborate further on the tentative identification of the four reported compounds though.

Conclusion

Sea cucumbers or holothurians are marine organisms that typically contain triterpene glycosides. These saponins, derived from a lanostane skeleton, have a protective function and can serve as chemotaxonomical markers. One of the most studied genera of sea cucumbers is the genus *Holothuria*. A review of the literature showed that up till now, 135 saponins have been identified within this genus (2 of them reported as possible artifacts), of which 63 compounds were reported in the *Holothuria* genus for the first time in the past decade (2010-2020), and 36 of these compounds were newly identified in the same period. Thus, it is clear that a lot of progress was made in this field of research in the last decade. Nevertheless, many possibly new *Holothuria* saponins were detected by MS, but their exact chemical structures could not be deduced. Hence, research focused on the identification of these compounds is still ongoing and it is expected that the number of newly identified compounds will keep rising in the upcoming years.

The structural elucidation of saponins is rather challenging and preferably NMR spectroscopy and MS are combined to determine structures unequivocally, especially in the case of novel compounds, although during the past ten years a tendency towards the sole use of MS analysis was observed, with less attention for isolation of single compounds followed by NMR analysis. When reviewing the literature, indeed it became very clear that MS alone in many cases does not suffice to unequivocally identify *Holothuria* saponins, even though often (tentatively) identified compounds are being reported. The identification of several compounds should be questioned and various examples were described in this review. In order to avoid such doubtful cases in upcoming publications and in order to simplify any future work on the identification of *Holothuria* saponins, an overview of typical signals and their interpretation was provided in this review, both for NMR and MS analysis. We hope that these guidelines may be useful to other researchers in the field and that they may facilitate the identification/structure elucidation of *Holothuria* saponins in particular, and perhaps also of saponins occurring in other genera of sea cucumbers in general.

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Conflicts of interest

The authors declare no conflict of interest.

References

Aminin DL, Menchinskaya ES, Pisliagin EA, et al (2015) Anticancer activity of sea cucumber triterpene glycosides. Mar Drugs 13:1202–1223. https://doi.org/10.3390/md13031202

Anjaneyulu ASR, Raju KVS (1996) A new triterpene glycoside from the sea cucumber Holothuria atra off Mandapam coast. Indian J Chem 35B:810–814.

Assawasuparerk K, Vanichviriyakit R, Chotwiwatthanakun C, et al (2016) Scabraside D extracted from Holothuria scabra induces apoptosis and inhibits growth of human cholangiocarcinoma xenografts in mice. Asian Pacific J Cancer Prev 17:511–517. https://doi.org/10.7314/APJCP.2016.17.2.511

Bahrami Y, Franco CMM (2015) Structure elucidation of new acetylated saponins, Lessoniosides A, B, C, D, and E, and non-acetylated saponins, Lessoniosides F and G, from the viscera of the sea cucumber holothuria lessoni. Mar Drugs 13:597–617. https://doi.org/10.3390/md13010597

Bahrami Y, Franco CMM, Benkendorff K (2016) Acetylated triterpene glycosides and their biological activity from holothuroidea reported in the past six decades. Mar Drugs 14:1–38. https://doi.org/10.3390/md14080147

Bahrami Y, Zhang W, Chataway T, Franco C (2014a) Structural elucidation of novel saponins in the sea cucumber Holothuria lessoni. Mar Drugs 12:4439–4473. https://doi.org/10.3390/md12084439

Bahrami Y, Zhang W, Franco C (2014b) Discovery of novel saponins from the viscera of the sea cucumber Holothuria lessoni. Mar Drugs 12:2633–2667. https://doi.org/10.3390/md12052633

Bahrami Y, Zhang W, Franco CMM (2018) Distribution of saponins in the sea cucumber holothuria lessoni; The body wall versus the viscera, and their biological activities. Mar Drugs 16(11):423. https://doi.org/10.3390/md16110423

Bondoc KG V., Lee H, Cruz LJ, et al (2013) Chemical fingerprinting and phylogenetic mapping of saponin congeners from three tropical holothurian sea cucumbers. Comp Biochem Physiol - B Biochem Mol Biol 166:182–193. https://doi.org/10.1016/j.cbpb.2013.09.002

Caulier G, Dyck S Van, Gerbaux P, et al (2011) Review of saponin diversity in sea cucumbers belonging to the family Holothuriidae. SPC Beche-de-mer Inf Bull #31 – January 2011 48–54

Caulier G, Flammang P, Gerbaux P, Eeckhaut I (2013a) When a repellent becomes an attractant: Harmful saponins are kairomones attracting the symbiotic Harlequin crab. Sci Rep 3:1–5. https://doi.org/10.1038/srep02639

Caulier G, Flammang P, Rakotorisoa P, et al (2013b) Preservation of the bioactive saponins of Holothuria scabra through the processing of trepang. Cah Biol Mar 54:685–690.

Caulier G, Mezali K, Soualili DL, et al (2016) Chemical characterization of saponins contained in the body wall and the Cuvierian tubules of the sea cucumber Holothuria (Platyperona) sanctori (Delle Chiaje,

1823). Biochem Syst Ecol 68:119–127. https://doi.org/10.1016/j.bse.2016.06.005

El Barky AR, Hussein SA, Alm-Eldeen AA, et al (2016) Anti-diabetic activity of Holothuria thomasi saponin. Biomed Pharmacother 84:1472–1487. https://doi.org/10.1016/j.biopha.2016.10.002

Elbandy M, Rho JR, Afifi R (2014) Analysis of saponins as bioactive zoochemicals from the marine functional food sea cucumber Bohadschia cousteaui. Eur Food Res Technol 238:937–955. https://doi.org/10.1007/s00217-014-2171-6

Elyakov GB, Kalinovskaya NI, Kalinovskii AI, et al (1982) Glycosides of marine invertebrates XIII. New holothurinogenins of holothurin B1 from Holothuria floridana. Chem Nat Compd 18:298–302. https://doi.org/10.1007/BF00580455

Elyakov GB, Kuznetsova TA, Stonik VA, et al (1975) Glycosides of marine invertebrates-IV. A comparative study of the glycosides from Cuban sublittoral holothurians. Comp Biochem Physiol - Part B Biochem 52:413–417. https://doi.org/10.1016/0305-0491(75)90154-6

Elyakov GB, Stonik VA, Levina E V., et al (1973) Glycosides of marine invertebrates-I. A comparative study of the glycoside fractions of pacific sea cucumbers. Comp Biochem Physiol - Part B Biochem 44:325–336. https://doi.org/10.1016/0305-0491(73)90005-9

Friess SL, Durant RC, Chanley JD (1968) Further studies on biological actions of steroidal saponins produced by poisonous echinoderms. Toxicon 6:81–92.

Grauso L, Yegdaneh A, Sharifi M, et al (2019) Molecular networking-based analysis of cytotoxic saponins from sea cucumber holothuria atra. Mar Drugs 17:1–13. https://doi.org/10.3390/md17020086

Hal DM, Eltamany EE, Abdelhameed RFA, et al (2020) Genus Holothuria an imminent source of diverse chemical entities : A review. Rec Pharm Biomed Sci 4:46–67. https://doi.org/10.21608/RPBS.2020.32514.1067

Han H, Li L, Yi Y, et al (2012) Triterpene glycosides from sea cucumber Holothuria scabra with cytotoxic activity. Chinese Herb Med 4:183–188. https://doi.org/10.3969/j.issn.1674-6384.2012.03.002

Han H, Yi Y, Xu Q, et al (2009a) Two new cytotoxic triterpene glycosides from the sea cucumber Holothuria scabra. Planta Med 75:1608–1612. https://doi.org/10.1055/s-0029-1185865

Han H, Yi YH, Li L, et al (2007) A new triterpene glycoside from sea cucumber Holothuria leucospilota. Chinese Chem Lett 18:161–164. https://doi.org/10.1016/j.cclet.2006.12.027

Han H, Yi YH, Li L, et al (2009b) Triterpene glycosides from sea cucumber Holothuria leucospilota. Chin J Nat Med 7:346–350. https://doi.org/10.3724/SP.J.1009.2009.00346

Han H, Yi YH, Li L, et al (2009c) Antifungal active triterpene glycosides from sea cucumber Holothuria scabra. Yao Xue Xue Bao 44(6):620-624.

Han H, Yi YH, Liu BS, et al (2008) Leucospilotaside C, a new sulfated triterpene glycoside from sea cucumber Holothuria leucospilota. Chinese Chem Lett 19:1462–1464. https://doi.org/10.1016/j.cclet.2008.09.051

Han H, Zhang W, Yi YH, et al (2010) A novel sulfated holostane glycoside from sea cucumber Holothuria leucospilota. Chem Biodivers 7:1764–1769. https://doi.org/10.1002/cbdv.200900094

Hoang L, Le Thi V, Tran Thi Hong H, et al (2020) Triterpene glycosides from the Vietnamese sea cucumber

Holothuria edulis. Nat Prod Res 34:1061–1067. https://doi.org/10.1080/14786419.2018.1548451

Honey-Escandón M, Arreguín-Espinosa R, Solís-Marín FA, Samyn Y (2015) Biological and taxonomic perspective of triterpenoid glycosides of sea cucumbers of the family Holothuriidae (Echinodermata, Holothuroidea). Comp Biochem Phys B 180:16–39. https://doi.org/10.1016/j.cbpb.2014.09.007

Hostettmann K, Marston A (1995) Occurrence and distribution. In: Saponins (Chemistry and Pharmacology of Natural Products. Cambridge University Press, Cambridge, pp 18–121.

Ivanova NS, Smetanina OF, Kuznetsova T a, Eastern F (1984) Glycosides of marine invertebrates. XXVI. Holothurin A from the Pacific Ocean holothurian, Holothuria squamifera. Isolation of native aglycon. Khimiya Prir Soedin 448–451.

Jattujan P, Chalorak P, Siangcham T, et al (2018) Holothuria scabra extracts possess anti-oxidant activity and promote stress resistance and lifespan extension in Caenorhabditis elegans. Exp Gerontol 110:158–171. https://doi.org/10.1016/j.exger.2018.06.006

Kalinin VI, Aminin DL, Avilov SA, et al (2008) Triterpene glycosides from sea cucucmbers (holothurioidea, echinodermata). Biological activities and functions. Stud Nat Prod Chem 35:135–196. https://doi.org/10.1016/S1572-5995(08)80006-3

Kalinin VI, Silchenko AS, Avilov SA, et al (2005) Sea cucumbers triterpene glycosides, the recent progress in structural elucidation and chemotaxonomy. Phytochem Rev 4:221–236. https://doi.org/10.1007/s11101-005-1354-y

Kalinin VI, V.A. S (1983) Glycosides of marine invertebrates. Structure of holothurin A2 from the holothurian Holothuria edulis. Khimiya Prir Soedin 2:215–219.

Kamyab E, Goebeler N, Kellermann MY, et al (2020) Anti-fouling effects of saponin-containing crude extracts from tropical Indo-Pacific sea cucumbers. Mar Drugs 18(4):181. https://doi.org/10.3390/md18040181

Kim SK, Himaya SWA (2012) Triterpene Glycosides from Sea Cucumbers and Their Biological Activities. Adv Food Nutr Res 65:297–319. https://doi.org/10.1016/B978-0-12-416003-3.00020-2

Kitagawa I, Kobayashi M, Son BW, et al (1989) Marine natural products. XIX. Pervicosides A, B, and C, lanostane-type triterpene-oligoglycoside sulfates from the sea cucumber Holothuria pervicax. Chem Pharm Bull 37:1230–1234.

Kitagawa I, Nishino T, Kobayashi M, Kyogoku Y (1981) Marine natural products. VIII. Bioactive triterpene-oligoglycosides from the sea cucumber Holothuria leucospilota Brandt (2). Structure of holothurin A. Chem Pharm Bull 29:1951–1956.

Kitagawa I, Nishino T, Kyogoku Y (1979) Structure of holothurin A a biologically active triterpene-oligoglycoside from the sea cucumber holothuria leucospilota brandt. Tetrahedron Lett 20(16):1419-1422. https://doi.org/10.1016/S0040-4039(01)86166-9

Kobayashi M, Hori M, Kan K, et al (1991) Marine Natural Products. XXVII. Distribution of lanostane-type triterpene oligoglycosides in ten kinds of Okinawan sea cucumbers. Chem Pharm Bull 39:2282–2287.

Kuznetsova TA, Anisimov MM, Popov AM, et al (1982a) A comparative study in vitro of physiological activity of triterpene glycosides of marine invertebrates of echinoderm type. Comp Biochem Physiol

Part C, Comp 73:41–43. https://doi.org/10.1016/0306-4492(82)90165-4

Kuznetsova TA, Kalinovskaya NI, Kalinovskii AI, et al (1982b) Glycosides of marine invertebrates. XIV. Structure of holoturin B1 from the holothurian Holothuria floridana. Khimiya Prir Soedin 482–484.

Li M, Miao ZH, Chen Z, et al (2010) Echinoside A, a new marine-derived anticancer saponin, targets topoisomerase2α by unique interference with its DNA binding and catalytic cycle. Ann Oncol 21:597–607. https://doi.org/10.1093/annonc/mdp335

Melek FR, Tadros MM, Yousif F, et al (2012) Screening of marine extracts for schistosomicidal activity in vitro. Isolation of the triterpene glycosides echinosides A and B with potential activity from the Sea Cucumbers Actinopyga echinites and Holothuria polii. Pharm Biol 50:490–496. https://doi.org/10.3109/13880209.2011.615842

Mert Ozupek N, Cavas L (2017) Triterpene glycosides associated antifouling activity from Holothuria tubulosa and H. polii. Reg Stud Mar Sci 13:32–41. https://doi.org/10.1016/j.rsma.2017.04.003

Miller AK, Kerr AM, Paulay G, et al (2017) Molecular phylogeny of extant Holothuroidea (Echinodermata). Mol Phylogenet Evol 111:110–131. https://doi.org/10.1016/j.ympev.2017.02.014

Minh C Van, Kiem P Van, Huong LM, et al (2005) Triterpene-glycosides of lanostane type with cytotoxic activity from Holothuria martensii. J Chem-NY 43:768–772.

Mitu SA, Bose U, Suwansa-ard S, et al (2017) Evidence for a saponin biosynthesis pathway in the body wall of the commercially significant sea cucumber Holothuria scabra. Mar Drugs 15(11):349. https://doi.org/10.3390/md15110349

Nguyen HD, Van Thanh N, Van Kiem P, et al (2007) Two new triterpene glycosides from the Vietnamese sea cucumber Holothuria scabra. Arch Pharm Res 30:1387-1391. https://doi.org/10.1007/bf02977361

Nursid M, Marraskuranto E, Chasanah E (2019) Cytotoxicity and apoptosis induction of sea cucumber Holothuria atra extracts. Pharmacognosy Res 11:41–46. https://doi.org/10.4103/pr.pr_3_18

Oleinikova GK, Kuznetsova TA (1986) Glycosides of the holothurian Holothuria atra. Chem Nat Compd 22:617. https://doi.org/10.1007/BF00599287

Oleinikova GK, Kuznetsova TA, Ivanova NS, et al (1982a) Glycosides of marine invertebrates XV. A new triterpene glycoside - holothurin A1 - from caribbean holothurians of the family holothuriidae. Khimiya Prir Soedin 464–469.

Oleinikova GK, Kuznetsova TA, Rovnykh N V, et al (1982b) Glycosides of marine invertebrates. XVIII. Holothurin A2 from the caribbean holothurian Holothuria floridana. Khimiya Prir Soedin 527–528.

Omran NE, Salem HK, Eissa SH, et al (2020) Chemotaxonomic study of the most abundant Egyptian sea-cucumbers using ultra-performance liquid chromatography (UPLC) coupled to high-resolution mass spectrometry (HRMS). Chemoecology 30:35–48. https://doi.org/10.1007/s00049-019-00296-y

Radhika P, Anjaneyulu V, Subba Rao PV, et al (2002) Chemical examination of the echinoderms of Indian Ocean: The triterpene glycosides of the sea cucumbers: Holothuria nobilis, Bohadschia aff. tenuissima and Actinopyga mauritana from Lakshadweep, Andaman and Nicobar Islands. Indian J Chem B 41:1276–1282.

Rodriguez J, Castro R, Riguera R (1991) Holothurinosides: new antitumour non sulphated triterpenoid

glycosides from the sea cucumber Holothuria forskalii. Tetrahedron 47:4753–4762. https://doi.org/10.1016/S0040-4020(01)86479-6

Rodriguez J, Riguera R (1989) Lefevreiosides: four novel triterpenoid glycosides from the sea cucumber Cucumaria lefevrei. J Chem Res 11:342–343.

Sangpairoj K, Chaithirayanon K, Vivithanaporn P, et al (2016) Extract of the sea cucumber, Holothuria scabra, induces apoptosis in human glioblastoma cell lines. Funct Foods Heal Dis 6:452. https://doi.org/10.31989/ffhd.v6i7.264

Shahinozzaman M, Ishii T, Takano R, et al (2018a) Cytotoxic desulfated saponin from Holothuria atra predicted to have high binding affinity to the oncogenic kinase PAK1: A combined in vitro and in silico study. Sci Pharm 86:1–13. https://doi.org/10.3390/scipharm86030032

Shahinozzaman M, Ishii T, Takano R, et al (2018b) Cytotoxic desulfated saponin from Holothuria atra predicted to have high binding affinity to the oncogenic kinase PAK1: A combined in vitro and in silico study. Sci Pharm 86:1–2. https://doi.org/10.3390/scipharm86030032

Shushizadeh M, Mohammadi Pour P, Mahdieh M, Yegdaneh A (2019) Phytochemical analysis of Holothuria leucospilota, a sea cucumber from Persian Gulf. Res Pharm Sci 14:432–440. https://doi.org/10.4103/1735-5362.268204

Silchenko AS, Stonik VA, Avilov SA, et al (2005) Holothurins B2, B3, and B4, new triterpene glycosides from Mediterranean sea cucumbers of the genus Holothuria. J Nat Prod 68:564–567. https://doi.org/10.1021/np049631n

Sroyraya M, Kaewphalug W, Anantachoke N, et al (2018) Saponins enriched in the epidermal layer of Holothuria leucospilota body wall. Microsc Res Tech 81:1182–1190. https://doi.org/10.1002/jemt.23115

Stonik VA (1986) Some terpenoid and steroid derivatives from echinoderms and sponges. Pure Appl Chem 58:423–436. https://doi.org/10.1351/pac198658030423

Stonik VA, Kalinin VI, Avilov SA (1999) Toxins from sea cucumber (Holothuroids): chemical structures, properties, taxonomic distribution, biosynthesis and evaluation. J Nat Toxins 8:235–248.

Sun G, Li L, Yi Y, et al (2008a) Two new cytotoxic nonsulfated pentasaccharide holostane (= 20-hydroxylanostan-18-oic acid -gamma-lactone) glycosides from the sea cucumber Holothuria grisea. Helv Chim Acta 91:1453–1460.

Sun P, Liu BS, Yi YH, et al (2007) A new cytotoxic lanostane-type triterpene glycoside from the sea cucumber Holothuria impatiens. Chem Biodivers 4:450–457. https://doi.org/10.1002/cbdv.200790037

Sun P, Yi YH, Li L, et al (2006) 17-deydroxyholothurin A, a new sulfated triterpene glycoside from sea cucumber Holothuria impatie. Chinese Chem Lett 17:1454–1456.

Sun P, Yi YH, Li L, Tang HF (2008b) Studies on chemical constituents from the sea cucumber Holothuria impatiens. Zhongguo Haiyang Yaowu 27:1–7.

Thanh N Van, Dang NH, Kiem P Van, et al (2006) A New Triterpene Glycoside from the Sea Cucumber Holothuria Scabra Collected in Vietnam. ASEAN J Sci Technol Dev 23:253–259. https://doi.org/10.3125/asean.v23i4.986

Van Dyck S, Caulier G, Todesco M, et al (2011) The triterpene glycosides of Holothuria forskali: Usefulness and efficiency as a chemical defense mechanism against predatory fish. J Exp Biol 214:1347–1356. https://doi.org/10.1242/jeb.050930

Van Dyck S, Flammang P, Meriaux C, et al (2010a) Localization of secondary metabolites in marine invertebrates: Contribution of MALDI MSI for the study of saponins in Cuvierian tubules of H. forskali. PLoS One 5(11):e13923:. https://doi.org/10.1371/journal.pone.0013923

Van Dyck S, Gerbaux P, Flammang P (2010b) Qualitative and quantitative saponin contents in five sea cucumbers from the Indian ocean. Mar Drugs 8:173–189. https://doi.org/10.3390/md8010173

Van Dyck S, Gerbaux P, Flammang P (2009) Elucidation of molecular diversity and body distribution of saponins in the sea cucumber Holothuria forskali (Echinodermata) by mass spectrometry. Comp Biochem Physiol - B Biochem Mol Biol 152:124–134. https://doi.org/10.1016/j.cbpb.2008.10.011

Wang J, Han H, Chen X, et al (2014) Cytotoxic and apoptosis-inducing activity of triterpene glycosides from Holothuria scabra and Cucumaria frondosa against HepG2 Cells. Mar Drugs 12:4274–4290. https://doi.org/10.3390/md12084274

Weng Y, Yi Y, Li L, et al (2007) Three triterpene glycosides from Holothuria arenicola. Zhongguo Tianran Yaowu 5:96–100.

World Register of Marine Species (WORMS) (2021) Holothuriidae Burmeister, 1837. http://www.marinespecies.org/aphia.php?p=taxdetails&id=731943. Accessed 29 Jan 2021.

Wu J, Yi Y, Tang H, et al (2006a) Structure and Cytotoxicity of a New Lanostane-Type Triterpene Glycoside from the Sea Cucumber Holothuria hilla. Chem Biodivers 3:1249–1254.

Wu J, Yi Y, Zou ZR, et al (2006b) Two new triterpene glycosides from sea cucumber Holothuria nobilis. Zhongcaoyao 37:497–500.

Wu J, Yi YH, Tang HF, et al (2007) Hillasides A and B, two new cytotoxic triterpene glycosides from the sea cucumber Holothuria hilla Lesson. J Asian Nat Prod Res 9:609–615. https://doi.org/10.1080/10286020600882676

Wu J, Yi YH, Tang HF, et al (2006c) Nobilisides A-C, three new triterpene glycosides from the sea cucumber Holothuria nobilis. Planta Med 72:932–935. https://doi.org/10.1055/s-2006-931603

Wu J, Zhang JJ, Zhu KQ, et al (2009) Anti-tumor compound nobiliside E separated from Thelenota ananas (patent CN 101463066, Faming Zhuanli Shenqing).

Yu S, Ye X, Huang H, et al (2015) Bioactive sulfated saponins from sea cucumber Holothuria moebii. Planta Med 81:152–159. https://doi.org/10.1055/s-0034-1383404

Yuan W-H, Yi Y-H, Xue M, et al (2008a) Two Antifungal Active Triterpene Glycosides from Sea Cucumber Holothuria (Microthele) axiloga. Chin J Nat Med 6:105–108. https://doi.org/10.1016/s1875-5364(09)60010-8

Yuan WH, Yi YH, Li L, et al (2008b) Two triterpene glycosides from the sea cucumber Bohadschia marmorata Jaeger. Chinese Chem Lett 19:457–460. https://doi.org/10.1016/j.cclet.2008.01.026

Yuan WH, Yi YH, Tan RX, et al (2009a) Antifungal triterpene glycosides from the sea cucumber Holothuria (Microthele) axiloga. Planta Med 75:647–653. https://doi.org/10.1055/s-0029-1185381

Yuan WH, Yi YH, Tang HF, et al (2009b) Antifungal triterpene glycosides from the sea cucumber Bohadschia marmorata. Planta Med 75:168–173. https://doi.org/10.1055/s-0028-1088348

Zhang J jia, Dai J bo, Chen L li, et al (2008) Screening of bioactive constituents from sea cucumber Holothuria nobilis using conidia of Pyricularia oryzae. J Chinese Med Mater 31:1001–1003.

Zhang JJ (2011) Extraction, isolation, and structure elucidation of two new triterpene glycosides from sea cucumber Holothuria nobilis. Zhong Cao Yao 8:1467–1472.

Zhang JJ, Zhu KQ (2017) A novel antitumor compound nobiliside D isolated from sea cucumber (Holothuria nobilis Selenka). Exp Ther Med 14:1653–1658. https://doi.org/10.3892/etm.2017.4656

Zhang JJ, Zhu QK, Wu J, Zhang HW (2015) New Cytotoxic Triterpene Glycoside from the East China Sea Cucumber Holothuria nobilis. Nat Prod Commun 10:247–248. https://doi.org/10.1177/1934578x1501000207

Zhang SY, Yi YH, Tang HF (2006) Bioactive triterpene glycosides from the sea cucumber Holothuria fuscocinerea. J Nat Prod 69:1492–1495. https://doi.org/10.1021/np060106t

Table 1 Overview of saponins reported in the genus Holothuria, their structural properties, and occurrence in certain species and/or organs of Holothuria sea cucumbers. Compounds underlined were first reported in the genus Holothuria in the period 2010-2020. Compounds underlined and in bold were newly identified between 2010-2020. DS: desulfated. *: Part of the structure of the respective saponin is unclear. The letters and numbers in the columns "sapogenin", "R_1", "R_2" and "R_3" correspond to the substructures as shown in Figures 1-3. Structures of atypical sapogenins/saponins can be found in Figure 4. $^{\Delta}$Molecular formula derived from the structure provided in the reference does not correspond to the reported molecular formula and/or MS data. Organ: BW = Body Wall, V = Viscera, W = Whole body.

No.	Compound name	Sapo-genin	R1	R2	R3	Molecular Formula	Nominal mass	Species	Organ	Remarks	Reference
1	17α-hydroxyimpatienside A	B	4	*f*	III	$C_{67}H_{108}O_{33}$	1440	*lessoni*	BW		(Bahrami et al. 2018)
2	17-dehydroxyholothurin A (=fuscocineroside C)	A	1	*f*	II	$C_{54}H_{85}O_{26}SNa$	1204	*impatiens*	W		(Sun et al. 2006)
3	17-dehydroxyholothurinoside A (= holothurinoside E)	A	1	*m*		$C_{60}H_{96}O_{28}$	1264	*grisea, lessoni*	W, V, BW		(Sun et al. 2008a; Bahrami et al. 2014a, b, 2018)
4	17-hydroxyfuscocineroside B (= scabraside B)	B	11	*f*	II	$C_{54}H_{85}O_{27}SNa$	1220	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
5	22-acetoxyechinoside A	B	16	*f*	II	$C_{56}H_{89}O_{28}SNa$	1264	*lessoni*	V, BW		(Bahrami et al. 2018)
6	24-dehydroechinoside A	B	4	*f*	II	$C_{54}H_{85}O_{26}SNa$	1204	*atra, edulis, lessoni, leucospilota, scabra*	W, BW	MS data of Omran et al. (2020) not conclusive	(Kobayashi et al. 1991; Han et al. 2012; Bondoc et al. 2013; Bahrami et al. 2014a, b; Grauso et al. 2019; Omran et al. 2020)
7	24-dehydroechinoside B	B	4	*c*	II	$C_{41}H_{63}O_{16}SNa$	866	*moebii*	W		(Yu et al. 2015)
8	25-acetoxy-bivittoside D	A	18	*f*	III	$C_{69}H_{112}O_{34}$	1484	*lessoni*	V, BW	Acetylated	(Bahrami et al. 2018)
9	25-hydroxyfuscocineroside B	A	12	*f*	II	$C_{54}H_{85}O_{27}SNa$	1220	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
	Arenicolaside A (= holothurin A)	B	1	*f*	II	$C_{54}H_{86}O_{27}S$	1198	*arenicola*		Holothurin A, but with H instead of Na bound to the sulfate	(Weng et al. 2007)
10	Arguside A	E	4	*g*	I	$C_{56}H_{88}O_{24}$	1144	*lessoni*	V		(Bahrami et al. 2018)
11	Arguside B	B	2	*f*	III	$C_{67}H_{110}O_{33}$	1442	*lessoni*	V		(Bahrami et al. 2018)
12	Arguside C	A	2	*g*	III	$C_{67}H_{110}O_{33}$	1442	*lessoni*	V		(Bahrami et al. 2018)
13	Arguside D	A	19	*g*	I	$C_{56}H_{88}O_{26}$	1176	*lessoni, scabra*	V, BW	Acetylated	(Mitu et al. 2017; Bahrami et al. 2018)
14	Arguside F	E	4	*m*		$C_{62}H_{98}O_{29}$	1306	*axiloga, lessoni*	W, V		(Yuan et al. 2009a; Bahrami et al. 2018)
	Axilogoside A (= holothurin B)	B	1	*c*	I	$C_{41}H_{64}O_{17}S$	860	*axiloga*	W	Holothurin B, but with H instead of Na bound to the sulfate	(Yuan et al. 2008a)
15	Bivittoside A	A	2	*c*	I	$C_{41}H_{66}O_{12}$	750	*scabra*	BW		(Mitu et al. 2017)
16	Bivittoside B	A	2	*f*	I	$C_{54}H_{88}O_{22}$	1088	*lessoni*	V, BW		(Bahrami et al. 2018)
17	Bivittoside C (= bohadschioside B)	C	2	*f*	III	$C_{67}H_{110}O_{31}$	1410	*edulis*	BW	MS data of Omran et al. (2020) not conclusive	(Omran et al. 2020)
18	Bivittoside D (=bohadschioside A)	A	2	*f*	III	$C_{67}H_{110}O_{32}$	1426	*fuscocinerea, impatiens, lessoni;*	W, V, BW, CT	MS data of Omran et al. (2020) not conclusive	(Sun et al. 2007; Van Dyck et al. 2010b; Bondoc et al. 2013; Bahrami et al. 2018; Omran et al. 2020)

								leucospilota, scabra			
19	Calcigeroside B	Atypical				$C_{54}H_{83}O_{27}SNa$	1218	*atra*	W		(Grauso et al. 2019)
20	Cladoloside A2	Atypical	22	*j*	I	$C_{57}H_{88}O_{24}$	1156	*scabra*	BW	Acetylated	(Mitu et al. 2017)
21	Cousteside A	A	4	*g*	III	$C_{67}H_{108}O_{33}$	1440	*lessoni*	BW		(Bahrami et al. 2018)
22	Cousteside C	Atypical	4	*m*		$C_{60}H_{96}O_{29}$	1280	*lessoni*	V, BW	16-OH	(Bahrami et al. 2018)
23	Cousteside E	A	4	*p*		$C_{60}H_{96}O_{28}$	1264	*lessoni, scabra*	V, BW		(Mitu et al. 2017; Bahrami et al. 2018)
24	Cousteside F	B	4	*m*		$C_{60}H_{96}O_{28}$	1264	*lessoni*	V, BW		(Bahrami et al. 2018)
25	Cousteside G	A	7	*m*		$C_{60}H_{94}O_{27}$	1246	*lessoni*	V		(Bahrami et al. 2018)
26	Cousteside H	A	5	*m*		$C_{60}H_{96}O_{27}$	1248	*lessoni*	V, BW		(Bahrami et al. 2018)
27	Cousteside I	A	2	*p*		$C_{60}H_{98}O_{28}$	1266	*lessoni, scabra*	V, BW		(Mitu et al. 2017; Bahrami et al. 2018)
28	Cousteside J	A	2	*m*		$C_{60}H_{98}O_{27}$	1250	*lessoni*	V, BW		(Bahrami et al. 2018)
29	Desholothurin A (= nobiliside 2A)	B	1	*f*	I	$C_{54}H_{86}O_{24}$	1118	*forskali, fuscocinerea, lessoni, leucospilota, nobilis, scabra,*	W, V, BW, CT, SW		(Rodriguez et al. 1991; Wu et al. 2006b; Van Dyck et al. 2009, 2010a, b, 2011; Bondoc et al. 2013; Caulier et al. 2013a, b; Bahrami et al. 2014a, b, 2018; Mitu et al. 2017; Sroyraya et al. 2018)
30	Desholothurin A1 (= arguside E)	A	1	*g*	I	$C_{54}H_{86}O_{24}$	1118	*atra, forskali, lessoni*	V, BW, CT		(Kobayashi et al. 1991; Van Dyck et al. 2009, 2010a; Caulier et al. 2013a; Bahrami et al. 2014a, b, 2018; Sroyraya et al. 2018)
31	**DS-echinoside B**	B	2	*c*	I	$C_{41}H_{66}O_{13}$	766	*atra*	BW	Kobayashi, 1991: obtained by desulfation of echinoside B	(Shahinozzaman et al. 2018a)
32	DS-holothurin B = Des-holothurin B	B	1	*c*	I	$C_{41}H_{64}O_{14}$	780	*atra*	W		(Oleinikova and Kuznetsova 1986)
33	DS-patagonicoside A	Atypical	2	*f*	I	$C_{54}H_{88}O_{23}$	1104	*scabra*	BW		(Mitu et al. 2017)
34	**DS-pervicoside B**	A	4	*f*	I	$C_{54}H_{86}O_{22}$	1086	*lessoni*	V, BW	Kitagawa, 1989: obtained by desulfation of pervicoside B	(Bahrami et al. 2018)
35	Echinoside A (= holothurin A2)	B	2	*f*	II	$C_{54}H_{87}O_{26}SNa$	1206	*atra, axiologa, edulis, floridana, lessoni, nobilis, scabra, polii*	W, V, BW		(Kalinin and V.A. 1983; Kobayashi et al. 1991; Li et al. 2010; Melek et al. 2012; Bahrami et al. 2014b, 2018; Wang et al. 2014; Grauso et al. 2019)
36	Echinoside B (= holothurin B1)	B	2	*c*	II	$C_{41}H_{65}O_{16}SNa$	868	*atra, floridana, leucospilota, polii*	W, BW		(Elyakov et al. 1982; Kobayashi et al. 1991; Han et al. 2009b; Melek et al. 2012)

37	Fuscocineroside A	A	19	*f*	II	$C_{56}H_{87}O_{28}SNa$	1262	*fuscocinerea, lessoni*	W, V, BW		(Zhang et al. 2006; Bondoc et al. 2013; Bahrami et al. 2018)
38	Fuscocineroside B	A	11	*f*	II	$C_{54}H_{85}O_{26}SNa$	1204	*fuscocinerea, lessoni*	W, V		(Zhang et al. 2006; Bahrami et al. 2014a, b)
	Fuscocineroside C (=17-dehydroxyholothurin A)	A	1	*f*	II	$C_{54}H_{85}O_{26}SNa$	1204	*arenicola, fuscocinerea, lessoni, scabra*	W, V, BW		(Zhang et al. 2006; Han et al. 2012; Bondoc et al. 2013; Bahrami et al. 2014a, b; Sroyraya et al. 2018)
39	Griseaside A	A	8	*m*	I	$C_{60}H_{98}O_{28}$	1266	*grisea, lessoni*	W, V, BW		(Sun et al. 2008a; Bahrami et al. 2014b, 2018; Sroyraya et al. 2018)
40	Hillaside A	Atypical	6	*a*	I	$C_{35}H_{52}O_8$	600	*hilla*	W	7,8 double bond	(Wu et al. 2007)
41	Hillaside B	F	10	*a*	I	$C_{37}H_{58}O_{12}$	694	*hilla*	W	Acetylated	(Wu et al. 2007)
42	Hillaside C (= nobiliside D)	B	10	*d*	II	$C_{40}H_{63}O_{17}SNa$	870	*hilla*	W		(Wu et al. 2006a)
43	Holothurin A	B	1	*f*	II	$C_{54}H_{85}O_{27}SNa$	1220	*atra, arenicola, axiologa, cinerascens, coluber, cubana, difficilis, edulis, fuscocinerea, gracilis, grisea, hilla, impatiens, lessoni, leucospilota, martensii, mexicana, moebii, nobilis, pervicax, polii, pulla, scabra, surinamensis, squamifera, tubulosa*	W, V, BW, CT		(Elyakov et al. 1973, 1975; Kitagawa et al. 1981; Kuznetsova et al. 1982a; Ivanova et al. 1984; Stonik 1986; Kobayashi et al. 1991; Minh et al. 2005; Silchenko et al. 2005; Thanh et al. 2006; Weng et al. 2007; Sun et al. 2008b; Han et al. 2009b; Van Dyck et al. 2010b; Bondoc et al. 2013; Bahrami et al. 2014b, 2018; Wang et al. 2014; Yu et al. 2015; Sangpairoj et al. 2016; Mert Ozupek and Cavas 2017; Hoang et al. 2020)
44	Holothurin A1	B	8	*f*	II	$C_{54}H_{87}O_{27}SNa$	1222	*floridana, grisea, impatiens lessoni, scabra*	W, V, BW		(Oleinikova et al. 1982a; Stonik 1986; Sun et al. 2008b; Bahrami et al. 2014b, 2018; Wang et al. 2014)
	Holothurin A2 (= Echinoside A)	B	2	*f*	II	$C_{54}H_{87}O_{26}SNa$	1206	*edulis, floridana,*	W, SW		(Oleinikova et al. 1982b; Stonik 1986; Thanh et al. 2006; Bondoc et al. 2013;

								lessoni, scabra			Caulier et al. 2013a; Hoang et al. 2020)
45	Holothurin A3	B	11	*f*	II	$C_{54}H_{85}O_{28}SNa$	1236	*lessoni, scabra*	W, V, BW		(Nguyen et al. 2007; Bondoc et al. 2013; Bahrami et al. 2014b, 2018; Sangpairoj et al. 2016)
46	Holothurin A4	B	9	*f*	II	$C_{54}H_{87}O_{27}SNa$	1222	*lessoni, scabra*	W, V, BW		(Nguyen et al. 2007; Bahrami et al. 2014b, 2018)
47	**Holothurin A5 (I)**	B	14	*f*	II	$C_{54}H_{83}O_{28}SNa$	1234	*atra*	W		(Grauso et al. 2019)
48	**Holothurin A5 (II)**	B	15	*f*	II	$C_{54}H_{85}O_{28}SNa$	1236	*edulis*	W		(Hoang et al. 2020)
49	Holothurin B	B	1	*c*	II	$C_{41}H_{63}O_{17}SNa$	882	*arenicola, atra, axiloga, coluber, cubana, edulis, fuscocinerea, gracilis, grisea, hilla, lessoni, leucospilota, lubrica, martensii, mexicana, moebii, nobilis, pervicax, polii, pulla, scabra, surinamensis, tubulosa, vagabunda,*	W, V, BW		(Friess et al. 1968; Kuznetsova et al. 1982a; Kobayashi et al. 1991; Radhika et al. 2002; Minh et al. 2005; Kalinin et al. 2005; Han et al. 2007, 2009b, 2010; Bahrami et al. 2014b, 2018; Wang et al. 2014; Yu et al. 2015; Sroyraya et al. 2018; Grauso et al. 2019; Hoang et al. 2020)
	Holothurin B1 (= Echinoside B)	B	2	*c*	II	$C_{41}H_{65}O_{16}SNa$	868	*atra, floridana, leucospilota, polii*	W, BW	MS data of Omran et al. (2020) not conclusive	(Elyakov et al. 1982; Kuznetsova et al. 1982b; Oleinikova et al. 1982a; Stonik 1986; Van Dyck et al. 2010b; Omran et al. 2020)
50	Holothurin B2	B	8	*c*	II	$C_{41}H_{65}O_{17}SNa$	884	*atra, lessoni, leucospilota, polii*	W, V, BW		(Silchenko et al. 2005; Han et al. 2009b, 2010; Van Dyck et al. 2010b; Bahrami et al. 2014b, 2018)
51	Holothurin B3	A	1	*c*	II	$C_{41}H_{63}O_{16}SNa$	866	*atra, lessoni, leucospilota, polii*	V, BW, CT		(Silchenko et al. 2005; Van Dyck et al. 2010b; Bahrami et al. 2014b, 2018; Sroyraya et al. 2018)
52	Holothurin B4	B	13	*c*	II	$C_{41}H_{63}O_{17}SNa$	882	*atra, lessoni, leucospilota, polii, scabra,*	V, BW, CT		(Silchenko et al. 2005; Van Dyck et al. 2010b; Bahrami et al. 2014b, 2018; Sroyraya et al. 2018)
53	Holothurin C*	Unclear			No	Unclear	Unclear	*lessoni*	BW	Molecular formula according to Bahrami et	(Bahrami et al. 2018)

										al. (2018): $C_{67}H_{108}O_{33}$ (nominal mass 1440)	
54	**Holothurin D**	B	1	*g*	II	$C_{54}H_{85}O_{28}SNa$	1236	*lessoni*	V, BW		(Bahrami et al. 2014a, 2018)
55	**Holothurin E**	B	1	*k*	II	$C_{53}H_{83}O_{27}SNa^{\Delta}$	1206	*lessoni*	V	According to Bahrami et al. (2014): $[M+Na]^+$ at *m/z* 1227.4 → nominal mass 1204	(Bahrami et al. 2014a)
56	Holothurinogenin B	Atypical	1	*c*	I	$C_{41}H_{62}O_{13}$	762	*atra, scabra*	W, BW	7,8 and 9,11 double bond	(Thanh et al. 2006; Shahinozzaman et al. 2018b)
57	Holothurinoside A	B	1	*m*		$C_{60}H_{96}O_{29}$	1280	*forskali, lessoni, sanctori, scabra*	V, BW, CT	Identified in *H. scabra* by Jattujan et al. (2018), but MS data not in agreement.	(Rodriguez et al. 1991; Van Dyck et al. 2009, 2010a, 2011; Bahrami et al. 2014a, b, 2018; Caulier et al. 2016; Jattujan et al. 2018)
58	Holothurinoside A1	A	1	*p*		$C_{60}H_{96}O_{29}$	1280	*forskali, lessoni, sanctori, scabra*	V, BW, CT	Identified in *H. scabra* by Jattujan et al. (2018), but MS data not in agreement.	(Van Dyck et al. 2009, 2010a; Bahrami et al. 2014a, b, 2018; Jattujan et al. 2018)
59	Holothurinoside B	A	20	*m*		$C_{62}H_{98}O_{30}$	1322	forskalii	BW	Acetylated	(Rodriguez et al. 1991)
60	Holothurinoside C	A	1	*f*	I	$C_{54}H_{86}O_{23}$	1102	*forskali, fuscocinerea, impatiens, lessoni, sanctori, scabra*	W, V, BW, CT, SW		(Rodriguez et al. 1991; Van Dyck et al. 2010a, 2011, 2009; Bondoc et al. 2013; Caulier et al. 2013a, b, 2016; Bahrami et al. 2014a, b, 2018; Mitu et al. 2017)
61	Holothurinoside C1	C	1	*g*	I	$C_{54}H_{86}O_{23}$	1102	*forskali, lessoni, sanctori*	V, BW, CT		(Van Dyck et al. 2009, 2010a; Bahrami et al. 2014a, b, 2018)
62	Holothurinoside D	A	1	*c*	I	$C_{41}H_{64}O_{13}$	764	*forskali, scabra*	BW	Identified in *H. scabra* by Sangpairoj et al. (2016), but MS data not in agreement.	(Rodriguez et al. 1991; Sangpairoj et al. 2016)
	Holothurinoside E (= 17-dehydroxyholothurinoside A)	A	1	*m*		$C_{60}H_{96}O_{28}$	1264	*forskali, lessoni, leucospilota, sanctori*	V, BW, CT		(Rodriguez et al. 1991; Van Dyck et al. 2009, 2010a, b, 2011; Bahrami et al. 2014b, 2018; Caulier et al. 2016)
63	Holothurinoside E1	C	1	*p*	I	$C_{60}H_{96}O_{28}$	1264	*forskali, lessoni*	V, BW, CT		(Van Dyck et al. 2009, 2010a; Bahrami et al. 2014a, b, 2018)
64	Holothurinoside F	A	1	*f*	V	$C_{66}H_{106}O_{32}$	1410	*forskali*	BW, CT, SW		(Van Dyck et al. 2009, 2010a, 2011; Caulier et al. 2013a)
65	Holothurinoside F1	C	1	*g*	V	$C_{66}H_{106}O_{32}$	1410	*forskali, sanctori*	BW, CT		(Van Dyck et al. 2009, 2010a; Caulier et al. 2016)
66	**Holothurinoside F1Ac**	Atypical	2	*g*	IV	$C_{68}H_{110}O_{34}$	1470	*sanctori*	BW	Acetylated Lacks 12-OH, 16-OAc	(Caulier et al. 2016)
67	Holothurinoside G	B	1	*f*	V	$C_{66}H_{106}O_{33}$	1426	*forskali, scabra*	BW, CT, SW		(Van Dyck et al. 2009, 2010a, 2011; Caulier et al. 2013a, b)

68	Holothurinoside G1	A	1	*g*	V	$C_{66}H_{106}O_{33}$	1426	*forskali, sanctori*	BW, CT		(Van Dyck et al. 2009, 2010a; Caulier et al. 2016)
69	**Holothurinoside G1Ac**	E	2	*g*	IV	$C_{68}H_{110}O_{35}$	1486	*sanctori*	BW	Acetylated	(Caulier et al. 2016)
70	Holothurinoside H	C	1	*f*	III	$C_{67}H_{108}O_{33}$	1440	*forskali, fuscocinerea, impatiens, lessoni, scabra*	W, BW, CT, SW		(Van Dyck et al. 2009, 2010a, 2011; Bondoc et al. 2013; Caulier et al. 2013a; Bahrami et al. 2018)
71	Holothurinoside H1	C	1	*g*	III	$C_{67}H_{108}O_{33}$	1440	*forskali, lessoni*	BW, CT		(Van Dyck et al. 2009, 2010a; Bahrami et al. 2018)
72	Holothurinoside I	B	1	*f*	III	$C_{67}H_{108}O_{34}$	1456	*forskali, lessoni*	V, BW, CT		(Van Dyck et al. 2009, 2010a, 2011; Bahrami et al. 2014a, 2018)
73	Holothurinoside I1	B	1	*g*	III	$C_{67}H_{108}O_{34}$	1456	*forskali, lessoni, sanctori*	V, BW, CT		(Van Dyck et al. 2009, 2010a; Caulier et al. 2016; Bahrami et al. 2018)
74	**Holothurinoside J1**	B	1	*g*	I	$C_{54}H_{86}O_{25}$	1134	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
75	Holothurinoside K1	B	1	*g*	III	$C_{67}H_{108}O_{35}$	1472	*atra, lessoni*	V, BW	Molecular weight 1134 reported by Van Dyck et al. (2010) and referred to by Shahinozzaman et al. (2018)	(Bahrami et al. 2014a, 2018; Shahinozzaman et al. 2018b)
76	**Holothurinoside L (=holothurinoside N)**	B	1	*n*		$C_{61}H_{98}O_{29}$	1294	*lessoni*	V, BW		(Van Dyck et al. 2011; Bahrami et al. 2018)
77	**Holothurinoside M**	A	1	*n*		$C_{61}H_{98}O_{28}$	1278	*lessoni, sanctori, scabra*	V, BW, CT,SW	Identified in *H. scabra* by Jattujan et al. (2018), but MS data not in agreement.	(Caulier et al. 2013a, 2016; Bahrami et al. 2014a, b, 2018; Sangpairoj et al. 2016; Jattujan et al. 2018)
78	**Holothurinoside M1**	C	1	*q*		$C_{61}H_{98}O_{28}$	1278	*sanctori, scabra*	CT	Identified in *H. scabra* by Jattujan et al. (2018), but MS data not in agreement.	(Caulier et al. 2016; Jattujan et al. 2018)
	Holothurinoside N (= holothurinoside L)	B	1	*n*		$C_{61}H_{98}O_{29}$	1294	*lessoni, sanctori*	V, CT, SW		(Caulier et al. 2013a, 2016; Bahrami et al. 2014a)
79	**Holothurinoside N1**	C	1	*q*		$C_{61}H_{98}O_{29}$	1294	*sanctori*	CT		(Caulier et al. 2016)
80	**Holothurinoside O***	Unclear	1	*m*		$C_{60}H_{96}O_{28}$	1264	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
81	**Holothurinoside P**	B	1	*o*		$C_{60}H_{96}O_{28}$	1264	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
82	**Holothurinoside Q**	B	1	*p*		$C_{60}H_{96}O_{29}$	1280	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
83	**Holothurinoside R***	Unclear		MeGlc, Glc, Qui, (Qui) Xyl		$C_{60}H_{96}O_{29}$	1280	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)

84	**Holothurinoside R1***	Unclear		MeGlc, Glc, Qui, (Qui) Xyl		$C_{60}H_{96}O_{29}$	1280	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
85	**Holothurinoside S***	Unclear		*n*		$C_{60}H_{96}O_{29}$	1280	*lessoni*	V, BW		(Bahrami et al. 2014a, b, 2018)
86	**Holothurinoside T***	Unclear		*f*	I	Not reported	1126	*lessoni*	V		(Bahrami et al. 2014a, b)
87	**Holothurinoside X**	B	11	*k*	I	$C_{53}H_{84}O_{24}$	1104	*lessoni*	V, BW	MS data not sufficient to determine sapogenin structure unequivocally	(Bahrami et al. 2014a, 2018)
88	**Holothurinoside Y**	B	2	*h*	I	$C_{54}H_{88}O_{23}$	1104	*lessoni*	V, BW		(Bahrami et al. 2014a, 2018)
89	**Holothurinoside Z**	B	8	*h*	I	$C_{54}H_{88}O_{24}$ $^{\Delta}$	1121 (1104)	*lessoni, scabra*	V, BW	According to reference: $[M+Na]^+$ at *m/z* 1127.6 → nominal mass 1104	(Bahrami et al. 2014a, 2018; Mitu et al. 2017)
90	Holotoxin A1	Atypical	4	*j*	III	$C_{66}H_{104}O_{31}$	1392	*scabra*	BW	16-keto Identified in *H. scabra* by Sangpairoj et al. (2016), but MS data not in agreement.	(Sangpairoj et al. 2016)
91	Impatienside A	A	4	*f*	III	$C_{67}H_{108}O_{32}$	1424	*impatiens, lessoni*	W, V, BW		(Sun et al. 2007; Bondoc et al. 2013; Bahrami et al. 2018)
92	Impatienside B	A	4	*m*		$C_{60}H_{96}O_{27}$	1248	*axiloga, lessoni, leucospilota*	W, V, BW		(Yuan et al. 2009a; Bahrami et al. 2014b, 2018; Sroyraya et al. 2018)
93	Intercedenside A	E	6	*j*	II	$C_{55}H_{83}O_{25}SNa$	1198	*lessoni*	V, BW		(Bahrami et al. 2018)
94	Lefevreioside C	Atypical	5	*j*	II	$C_{55}H_{85}O_{25}SNa$	1200	*scabra*	BW	Acetylated Identified in *H. scabra* by Mitu et al. (2017), but MS data not in agreement.	(Mitu et al. 2017)
95	**Lessonioside A**	F	2	*j*	III	$C_{68}H_{110}O_{34}$ $^{\Delta}$	1470	*lessoni*	V, BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015; Bahrami et al. 2018)
96	**Lessonioside B**	F	2	*r*		$C_{68}H_{110}O_{34}$ $^{\Delta}$	1470	*lessoni*	V, BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015; Bahrami et al. 2018)
97	**Lessonioside C**	Atypical	21	*k*	VII	$C_{66}H_{100}O_{35}$ $^{\Delta}$	1452	*lessoni*	V, BW	Acetylated, 16-keto, According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015; Bahrami et al. 2018)
98	**Lessonioside D**	F	2	*t*		$C_{68}H_{110}O_{34}$ $^{\Delta}$	1470	*lessoni*	V, BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015; Bahrami et al. 2018)

99	**Lessonioside E**	Atypical	21	*l*	VII	$C_{66}H_{100}O_{35}$ Δ	1452	*lessoni*	V, BW	Acetylated, 16-keto According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015; Bahrami et al. 2018)
100	**Lessonioside F**	Atypical	23	*l*	III	$C_{66}H_{100}O_{35}$ Δ	1452	*lessoni*	V	16-keto According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015)
101	**Lessonioside G**	Atypical	23	*k*	III	$C_{66}H_{100}O_{35}$ Δ	1452	*lessoni*	V	16-keto According to reference: $[M+Na]^+$ at *m/z* 1477.7 → nominal mass 1454	(Bahrami and Franco 2015)
102	**Lessonioside H**	A	17	*j*	III	$C_{68}H_{110}O_{33}$ Δ	1454	*lessoni*	V and/or BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1461.7 and $[M-H]^-$ at *m/z* 1437 → nominal mass 1438	(Bahrami et al. 2018)
103	**Lessonioside I**	A	17	*s*		$C_{67}H_{108}O_{34}$ Δ	1456	*lessoni*	V and/or BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1461.7 → nominal mass 1438	(Bahrami et al. 2018)
104	**Lessonioside J**	A	17	*k*	IV	$C_{67}H_{108}O_{34}$ Δ	1456	*lessoni*	V and/or BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1461.7 → nominal mass 1438	(Bahrami et al. 2018)
105	**Lessonioside K**	A	17	*r*		$C_{68}H_{110}O_{33}$ Δ	1454	*lessoni*	V and/or BW	Acetylated According to reference: $[M+Na]^+$ at *m/z* 1461.7 and $[M-H]^-$ at *m/z* 1437 → nominal mass 1438	(Bahrami et al. 2018)
106	Leucospilotaside A	B	13	*c*	II	$C_{41}H_{63}O_{18}SNa$	898	edulis, lessoni, leucospilota	W, V, BW		(Han et al. 2007, 2009b; Bahrami et al. 2014b; Sroyraya et al. 2018; Hoang et al. 2020)
107	Leucospilotaside B	B	9	*c*	II	$C_{41}H_{65}O_{17}SNa$	884	*lessoni, leucospilota*	W, V		(Han et al. 2009b, 2010; Bahrami et al. 2014b, 2018)
108	Leucospilotaside C	B	1	*a*	II	$C_{35}H_{53}O_{13}SNa$	736	*edulis, leucospilota*	W		(Han et al. 2008, 2009b; Hoang et al. 2020)
109	Marmoratoside A	A	5	*f*	III	$C_{67}H_{108}O_{32}$	1424	*lessoni*	V, BW	MS data not sufficient to determine sapogenin structure unequivocally	(Bahrami et al. 2018)
110	Marmoratoside B	A	13	*f*	III	$C_{67}H_{108}O_{33}$	1440	*lessoni*	BW		(Bahrami et al. 2018)
111	Marmoroside C (= 17-hydroxyfuscocineroside A)	B	19	*f*	II	$C_{56}H_{87}O_{29}SNa$	1278	*edulis*	W	Acetylated	(Hoang et al. 2020)

112	**Moebioside A (= 22-oxo-25-acetoxy-echinoside B)**	B	19	*c*	II	$C_{43}H_{65}O_{19}SNa$	940	*edulis, moebii*	W	Acetylated Trivial name not mentioned by Yu et al. (2015)	(Yu et al. 2015; Hoang et al. 2020)
113	Nobiliside A (I)	Atypical	6	*a*	I	$C_{35}H_{50}O_8$	598	*nobilis*	W		(Wu et al. 2006c)
114	Nobiliside A (II)	B	10	*f*	II	$C_{54}H_{87}O_{27}SNa$	1222	*nobilis*	W		(Zhang et al. 2008)
115	Nobiliside 1a	B	10	*i*	I	$C_{54}H_{88}O_{23}$	1104	*nobilis*	W		(Wu et al. 2006b)
	Nobiliside I (= holothurin A)	B	1	*f*	II	$C_{54}H_{85}O_{27}SNa$	1220	*nobilis*	W		(Zhang 2011)
116	Nobiliside II (= ananaside C)	Atypical	1	*c*	II	$C_{41}H_{63}O_{17}SNa$	882	*nobilis*	W		(Zhang 2011)
	Nobiliside 2A (=desholothurin A)	B	1	*f*	I	$C_{54}H_{86}O_{24}$	1118	*forskali, fuscocinerea, lessoni, leucospilota, nobilis, scabra*	W, V, BW, CT		(Rodriguez et al. 1991; Wu et al. 2006b; Van Dyck et al. 2010a, 2011, 2009, 2010b; Bondoc et al. 2013; Caulier et al. 2013a; Bahrami et al. 2014a, b, 2018; Mitu et al. 2017; Sroyraya et al. 2018)
117	Nobiliside B (I)	D	1	*b*	II	$C_{41}H_{63}O_{17}SNa$	882	*lessoni, leucospilota, nobilis*	W, V, BW		(Wu et al. 2006c; Bahrami et al. 2014b, 2018; Sroyraya et al. 2018)
	Nobiliside B (II) (= nobiliside C (I))	F	1	*a*	I	$C_{35}H_{54}O_{10}$	634	*nobilis*		Acetylated	(Zhang et al. 2008)
118	Nobiliside C (I)	F	1	*a*	I	$C_{37}H_{56}O_{12}$	692	*nobilis*	W	Acetylated	(Wu et al. 2006c)
	Nobiliside C (II) (=nobiliside B (I))	D	1	*b*	II	$C_{41}H_{63}O_{17}SNa$	882	*nobilis*			(Zhang et al. 2008)
	Nobiliside D (= hillaside C)	B	10	*d*	I	$C_{40}H_{63}O_{17}SNa$	870	*nobilis*	W	Identified in *H. nobilis* by Zhang and Zhu (2017), but NMR and MS data not in agreement.	(Zhang et al. 2008; Zhang and Zhu 2017)
119	Nobiliside E (I)	B	3	*i*	I	$C_{54}H_{88}O_{23}$	1104	*nobilis, scabra*	W	Identified in *H. scabra* by Mitu et al. (2017), but MS data not in agreement.	(Zhang et al. 2015; Mitu et al. 2017)
120	Nobiliside E (II)*	Unclear		*j*	II			*nobilis*			(Zhang et al. 2008)
121	Nobiliside F*	Unclear		*j*	II			*nobilis*	W	7,8-ene, 16-Oac, lacks 12-OH	(Zhang et al. 2008)
122	Nobiliside G*	Unclear		*c*	II			*nobilis*	W		(Zhang et al. 2008)
123	Nobiliside H*	Unclear		*c*	II			*nobilis*	W		(Zhang et al. 2008)
124	Nobiliside I*	Unclear		OMeGlc, Glc, Xyl, 4-*O*-sulfo-Xyl				*nobilis*	W	7,8-ene, 16-Oac, lacks 12-OH	(Zhang et al. 2008)
125	Nobiliside J*	Unclear		6-*O*-sulfo-OMeGlc, Qui, Xyl, 4-*O*-sulfo-Xyl				nobilis	W		(Zhang et al. 2008)

126	Pervicoside A (= neothyoside A)	A	18	*x*	II	$C_{56}H_{89}O_{27}SNa$	1248	*pervicax*	BW	Acetylated	(Kitagawa et al. 1989; Kobayashi et al. 1991)
127	Pervicoside B	A	4	*f*	II	$C_{54}H_{85}O_{25}SNa$	1188	*lessoni, pervicax*	V, BW		(Kitagawa et al. 1989; Kobayashi et al. 1991; Bahrami et al. 2018)
128	Pervicoside C	A	2	*f*	II	$C_{54}H_{87}O_{25}SNa$	1190	*fuscocinerea, impatiens, pervicax, scabra*	W, BW		(Kitagawa et al. 1989; Kobayashi et al. 1991; Sun et al. 2008b; Bondoc et al. 2013; Caulier et al. 2013b; Mitu et al. 2017)
129	Pervicoside D	A	18	*m*		$C_{62}H_{100}O_{29}$	1308	*axiloga*	W	Acetylated	(Yuan et al. 2009a)
130	Philinopside E	Atypical	5	*j*	II	$C_{53}H_{81}O_{24}SNa$	1156	*scabra*	BW	16-keto	(Mitu et al. 2017)
131	Scabraside A	B	5	*f*	II	$C_{54}H_{85}O_{26}SNa$	1204	*lessoni, scabra*	W, V, BW, SW		(Han et al. 2009a; Caulier et al. 2013a, b; Bahrami et al. 2014a, b; Sangpairoj et al. 2016)
	Scabraside B (= 17-hydroxyfuscocineroside B)	B	11	*f*	II	$C_{54}H_{85}O_{27}SNa$	1220	*lessoni, scabra*	W, V, BW, SW		(Han et al. 2009a; Caulier et al. 2013a, b; Bahrami et al. 2014b, 2018; Wang et al. 2014; Sangpairoj et al. 2016)
132	**scabraside D**	B	10	*g*	II	$C_{54}H_{87}O_{27}SNa$	1222	*lessoni, scabra*	W, V, BW		(Han et al. 2012; Bahrami et al. 2014b, 2018; Wang et al. 2014; Assawasuparerk et al. 2016)
133	"Compound 5"	B	24	*f*	II	$C_{54}H_{85}O_{29}SNa$	1252		W	Most probably an artifact	(Grauso et al. 2019)
134	"Compound 6"	B	25	*f*	II	$C_{55}H_{87}O_{29}SNa$	1266		W	Most probably an artifact	(Grauso et al. 2019)
135	"Compound 7" 3-*O*-[α-L-rhamnopyranosyl (1→2)-β-D-xylopyranosyl]-3β,12α,17α,20(S)-tetrahydroxylanost-9(11)-en-18,20-olide	B	2	*e*	I	$C_{41}H_{66}O_{13}$	766	*atra*	V+BW	Rhamnose sugar moiety, identified based on paper chromatography and comparison to authentic samples	(Anjaneyulu and Raju 1996)

Table 2 Neutral loss values and corresponding fragments as reported for Holothuria saponins in the literature, in case of MALDI-MS or ESI-MS analysis.

Neutral loss (mu)	Fragment lost	Remark	References
18	H_2O	Can result from cleavage at glycosidic linkages	(Bondoc et al. 2013; Bahrami et al. 2014a, b; Bahrami and Franco 2015)
44	CO_2		(Bondoc et al. 2013; Bahrami et al. 2014a, b; Bahrami and Franco 2015)
30	CH_2O	Cross-ring cleavage of glycoside moiety	(Bahrami and Franco 2015)
60	$C_2H_4O_2$	Cross-ring cleavage of glycoside moiety (α-1,4-linked disaccharide)	(Bahrami et al. 2014b; Bahrami and Franco 2015)
60 (59) (42)	CH_3COOH ($C_2H_3O_2$) (CH_2CO)	Acetyl group	(Wu et al. 2006c; Zhang et al. 2006; Yuan et al. 2009a; Bahrami and Franco 2015)
62	$H_2O + CO_2$		(Bondoc et al. 2013)
100	$C_6H_{12}O$	Side chain with 23-oxo substitution, loss of 4-methylpent-1-en-ol	(Bahrami et al. 2018)
120	$NaHSO_4$	Sulfate group	(Sun et al. 2006; Zhang et al. 2006; Han et al. 2007, 2008, 2009a, b, c, 2010; Van Dyck et al. 2010b; Han et al. 2012; Bondoc et al. 2013; Bahrami et al. 2014b)
132 or 150	$C_5H_8O_4$ or $C_5H_{10}O_5$	Xylose (with or without loss of H_2O)	(Sun et al. 2007; Van Dyck et al. 2009, 2010b; Bondoc et al. 2013; Bahrami et al. 2014b; Bahrami and Franco 2015; Bahrami et al. 2018)
146	$C_6H_{10}O_4$	Quinovose	(Sun et al. 2007, 2008b; Van Dyck et al. 2009, 2010b; Bondoc et al. 2013; Bahrami et al. 2014b; Bahrami and Franco 2015; Bahrami et al. 2018; Hoang et al. 2020)
162	$C_6H_{10}O_5$	Glucose	(Rodriguez et al. 1991; Sun et al. 2007, 2008b; Van Dyck et al. 2009, 2010b; Bondoc et al. 2013; Bahrami et al. 2014b; Bahrami and Franco 2015; Bahrami et al. 2018; Hoang et al. 2020)
176	$C_7H_{12}O_5$	Methylglucose	(Rodriguez et al. 1991; Sun et al. 2007, 2008b; Van Dyck et al. 2009, 2010b; Bondoc et al. 2013; Bahrami et al. 2014b; Bahrami and Franco 2015; Bahrami et al. 2018; Hoang et al. 2020)
308	$C_{12}H_{20}O_9$	MeGlc + Xyl	(Bahrami and Franco 2015)
338	$C_{13}H_{22}O_{10}$	MeGlc + Glc	(Rodriguez et al. 1991; Bondoc et al. 2013)
468	$C_{30}H_{44}O_4$	Sapogenin type A with 22,25-epoxy side chain or sapogenin type D with 22,25-epoxy side chain (or other sapogenins)	(Van Dyck et al. 2009; Bahrami et al. 2014b)
470	$C_{30}H_{46}O_4$	Sapogenin type B with saturated linear side chain (or other sapogenins)	(Bahrami et al. 2014a, b)
484	$C_{30}H_{44}O_5$	Sapogenin type B with 22,25-epoxy side chain or sapogenin type A with linear side chain and keto substitution (or other sapogenins)	(Van Dyck et al. 2009, 2010b; Bahrami et al. 2014a, b)
500	$C_{19}H_{32}O_{15}$	MeGlc + Glc + Glc or sapogenin (structure not reported)	(Rodriguez et al. 1991; Bahrami et al. 2014b)
646	$C_{25}H_{42}O_{19}$	MeGlc + Glc + Glc + Qui	(Rodriguez et al. 1991)

Table 3 Substructures of Holothuria saponins and m/z values of the corresponding ions, detected MALDI-MS/MS or ESI-MS/MS analysis in the positive ionization mode as reported in the literature. Key fragments are shown in bold.

***m/z* value**	**Detected ion**	**Substructure**	**References**
204	$C_{10}H_{20}O_4$	Saturated lateral chain	(Bahrami et al. 2018)
230	$C_{12}H_{22}O_4$	Saturated lateral chain	(Bahrami et al. 2018)
331	[MeGlc-Xyl +Na]$^+$	MeGlc-Xyl	(Bahrami and Franco 2015)
361	[MeGlc-Glc +Na]$^+$	MeGlc-Glc	(Bahrami and Franco 2015)
451	[MeXyl-Xyl-Xyl +Na]$^+$ (or [Qui-Xyl-Xyl +Na]$^+$)	MeXyl-Xyl-Xyl (or Qui-Xyl-Xyl)	(Bahrami and Franco 2015)
491	[Sapogenin +Na]$^+$	Sapogenin type A with 22,25-epoxy side chain or sapogenin type D with 22,25-epoxy side chain (other sapogenins possible too!)	(Van Dyck et al. 2009; Bahrami et al. 2014b)
493	[MeGlc-Glc-Xyl +Na]$^+$ or [Sapogenin +Na]$^+$	MeGlc-Glc-Xyl or sapogenin type B with saturated linear side chain (other sapogenins possible too!)	(Bahrami et al. 2014a, b; Caulier et al. 2016)
507	[MeGlc-Glc-Qui +Na]$^+$ or [Sapogenin +Na]$^+$	MeGlc-Glc-Qui or Sapogenin type B with 22,25-epoxy side chain or sapogenin type A with linear side chain and keto substitution (other sapogenins possible too!)	(Van Dyck et al. 2009, 2010b; Bondoc et al. 2013; Bahrami et al. 2014a, b; Caulier et al. 2016; Sangpairoj et al. 2016; Bahrami et al. 2018; Sroyraya et al. 2018; Kamyab et al. 2020; Omran et al. 2020)
523	[MeGlc-Glc-Glc +Na]$^+$	MeGlc-Glc-Glc	(Van Dyck et al. 2009, 2010a; Bahrami et al. 2014a, b, 2018; Caulier et al. 2016; Omran et al. 2020)
593	?	$^{1,5}A_4$ cross-ring cleavage of the Xyl moiety	(Bondoc et al. 2013; Bahrami et al. 2014a, b)
625	[MeGlc-Xyl-Glc-Xyl +Na]$^+$ or [deacetylated aglycon-Xyl +Na]$^+$	MeGlc-Xyl-Glc-Xyl or sapogenin type F with linear side chain -Xyl	(Bahrami et al. 2014a; Bahrami and Franco 2015)
639	[MeGlc-Glc-Qui-Xyl +Na]$^+$	MeGlc-Glc-Qui-Xyl	(Bondoc et al. 2013; Bahrami et al. 2014a, b, 2018)
643	[MeGlc-Xyl-Glc-Xyl +H_2O+Na]$^+$ or [MeGlc--Glc-Xyl-Xyl +H_2O+Na]$^+$	MeGlc-Xyl-Glc-Xyl or MeGlc-Glc-Xyl-Xyl	(Bahrami et al. 2014a; Bahrami and Franco 2015)
655	[MeGlc-Glc-Glc-Xyl +Na]$^+$	MeGlc-Glc-Glc-Xyl	(Bahrami et al. 2014a)
657	[MeGlc-Glc-Qui-Xyl +H_2O+Na]$^+$	MeGlc-Glc-Qui-Xyl	(Bahrami et al. 2014a, 2018; Bahrami and Franco 2015)
673	[MeGlc-Glc-Glc-Xyl +H_2O+Na]$^+$	MeGlc-Glc-Glc-Xyl	(Bahrami et al. 2018)
771	[MeGlc-Xyl-Qui-(Glc)Xyl +Na]$^+$ or [Xyl-Qui-(MeGlc-Glc)Xyl +Na]$^+$ or [deacetylated aglycon-Xyl-Qui +Na]$^+$	MeGlc-Xyl-Qui-(Glc)Xyl or Xyl-Qui-(MeGlc-Glc)Xyl or sapogenin type F with linear side chain-Xyl-Qui	(Bahrami and Franco 2015)
801	[MeGlc-Glc-Qui-(Glc)Xyl +Na]$^+$	MeGlc-Glc-Qui-(Glc)Xyl	(Rodriguez et al. 1991)
803	[MeGlc-Glc-Qui-(Qui)Xyl +H_2O+Na]$^+$	MeGlc-Glc-Qui-(Qui)Xyl	(Bahrami et al. 2014b)
819	[MeGlc-Glc-Qui-(Glc)Xyl +H_2O+Na]$^+$	MeGlc-Glc-Qui-(Glc)Xyl	(Van Dyck et al. 2009; Bahrami et al. 2014b)
835	[MeGlc-Glc-Glc-(Glc)Xyl +H_2O+Na]$^+$	MeGlc-Glc-Glc-(Glc)Xyl	(Van Dyck et al. 2009)
947	[MeGlc-Xyl-Qui-(MeGlc-Glc)Xyl +Na]$^+$ or [deacetylated aglycon-Xyl-Qui-MeGlc +Na]$^+$	MeGlc-Xyl-Qui-(MeGlc-Glc)Xyl or sapogenin type F with linear side chain-Xyl-Qui-MeGlc	(Bahrami and Franco 2015)

Table 4 Effect of the presence or absence of a 17-OH group in the lanostane skeleton on the chemical shift values of neighboring carbon atoms.

Atom	17-OH saponin δC (ppm)	17-H saponin δC (ppm)
13	57-59	63-65
16	34-36	23-25
17	88-90	47
18	173-175	177-180
20	86-87	83-85

Figure captions

Fig. 1 Commonly occurring sapogenin backbones and side chains (R_1 substituents), as reported for saponins occurring in the genus *Holothuria*

Fig. 2 Glycoside moieties (R_2 substituents) of *Holothuria* saponins bearing up till 5 monosaccharide units

Fig. 3 Glycoside moieties (R_2 substituents) of *Holothuria* saponins bearing 6 monosaccharide units and possible R_3 substituents

Fig. 4 Atypical sapogenins and one atypical triterpene glycoside reported in the genus *Holothuria*

Fig. 5 Typical cleavage sites (indicated by the dotted lines) during MS/MS analysis of a hypothetical *Holothuria* triterpene glycoside

Fig. 6 General sapogenin aglycon backbone with numbering of atoms where varying substitutions can occur

Fig. 7 Two common sapogenin aglycon substituents. Left: 22,25-epoxy moiety, right: linear side chain.

Fig. 8 Chemical structures of lefevreioside C (**94**) and scabraside D (**132**)

Fig. 9 Chemical structure of nobiliside D (= hillaside C (**42**)) (left) and possible alternative structure fitting the spectral data of Zhang and Zhu (2017) (right)

Fig. 10 Chemical structure of holothurin E (**55**), as reported by Bahrami et al. (2014a)

Fig. 11 Chemical structures of holothurinosides X, Y and Z (**87**-**89)** as reported by Bahrami et al. (2014a)

Fig. 12 Chemical structure of lessonioside A (**95**, left) and supposed aglycon moiety as reported by Bahrami et al. (2015) (top right), as well as expected aglycon moiety (bottom right)

Fig. 13 Chemical structures of lessoniosides H, I, J, K (**102**-**105**), as reported by Bahrami et al. (2018)