

GLYCOSIDES OF MARINE INVERTEBRATES—IV. A COMPARATIVE STUDY OF THE GLYCOSIDES FROM CUBAN SUBLITTORAL HOLOTHURIANS

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Abstract—1. Nine holothurian species were studied for triterpene glycosides precipitated by cholesterol; glycoside fractions were isolated from seven species.

2. The fractions obtained were separated into chromatographically individual components.

3. The hydrolysis products of the glycosides obtained were examined.

4. The previously-established relationships between the systematic position of the animals and their glycoside contents were corroborated.

INTRODUCTION

EARLIER, we described the isolation of glycoside fractions from 34 Pacific sea cucumber species (Elyakov *et al.*, 1973). It was shown that (1) triterpene saponins are widely distributed in Holothuroidea and (2) a dependency exists between the systematic position of the animals and the various glycosides contained therein. Thus, sea cucumbers of the families *Holothuriidae*, *Stichopodidae*, *Cucumariidae* and *Synaptidae* are characterized by different glycoside sets.

The present work is intended to specify our previous conclusions and deepen the available knowledge on holothurians as a new source of triterpene glycosides, which prove to be substances with anti-tumorous (Nigrelli, 1952; Sullivan *et al.*, 1955) and antifungal properties (Anisimov *et al.*, 1972) and irreversible neurotic effects (Friss *et al.*, 1960).

MATERIALS AND METHODS

Holothurian sources

The following species were collected in October through December 1972 along the Cuban coast: *Holothuria mexicana*, *Actinopyga agassizii* (near Havana); *Euapta lappa* (Artigosa Inlet); *Astichopus multifidus* (I. de Pinos); and *Holothuria cubana*, *H. arenicola*, *H. grisea*, *Isostichopus badiotus*, *H. surinamensis* (Manzanillo Islands).

Isolation of glycoside fractions and chromatographically individual glycosides

Glycoside fractions were isolated by a method described by us previously (Elyakov *et al.*, 1973). Individual glycosides were obtained by subjecting the glycoside fractions to chromatography on Silica Gel columns (Silicagel L. 40/100 μ , Praha, Czechoslovakia) in the system chloroform-methanol-water, 60:30:2 v/v (system A). Spots in chromatograms were detected by sulphuric acid vapours.

Acid hydrolysis and isolation of hydrolysis products

The acid hydrolysis procedure was described earlier (Elyakov *et al.*, 1973). The aglycons were separated by extraction with chloroform, and aglycon acetates were obtained in the usual way. Separation of the crude acetate

mixture to obtain individual substances was run with chromatography on Silica Gel columns (Silicagel L. 40/100 μ) in the system hexane-ethyl acetate, 5:1 v/v (system B).

The acid hydrolysates obtained after aglycon separation were neutralized with ion-exchange resin (Dowex-HCO³⁻). Monosaccharides were eluted from the resin with water. The monosaccharide mixtures were analysed in the form of aldononitril peracetates by means of gas-liquid chromatography (GLC) (Easterwood & Byron, 1969) in a Pye Unicam chromatograph (3% QF-1 on Gaschrom Q, 195-230°C, 2°/min).

Identification of glycosides and their hydrolysis products

The glycosides were identified with thin-layer chromatography (TLC) on a fixed Silica Gel layer (Silica Gel KCK, 280 mesh) in the presence of authentic samples of holothurin A from *A. agassizii* (Chanley *et al.*, 1959), holothurin B from *H. vagabunda* (Yasumoto *et al.*, 1967) and stichoposides A and C from *St. japonicus* (Elyakov *et al.*, 1968). Furthermore, the hydrolysis products of the isolated glycosides were examined to establish the nature of the glycoside components.

The non-acetylated aglycons were identified by TLC on plates with Silufol (Silufol, Kavalier, Czechoslovakia) in the system benzol-ethyl acetate, 1:1 v/v (system C). The system benzol-ethyl acetate, 5:1 v/v (system D), was used for TLC of aglycon acetates. Standard samples I, II, IV and their acetates were used for reference. In addition, the spectral characteristics of the isolated acetates were compared with the characteristics of previously known holothurinogenin acetates.

Spectrographic equipment

The mass-spectra (MS) were obtained on a mass-spectrometer (Model HX-1303) using a direct probe inlet system and a magnetic scan. The ion source temperature was 200°C, ionizing energy 70 eV. The u.v. spectra for solutions in ethanol were recorded on a "Specord UV VIS" instrument in the range of 200-360 nm, and the i.r. spectra on a "UR-20" instrument in chloroform.

RESULTS

Nine species of holothurians were examined to determine the contents of triterpene glycoside frac-

Table 1. Composition of glycoside fraction from holothurians of cuban littoral

Family	Holothuriidae					Stichopodidae		Synaptidae	
Species	<i>H. cubana</i>	<i>H. arenicola</i>	<i>H. grisea</i>	<i>H. surinamensis</i>	<i>H. mexicana</i>	<i>Act. agassizii</i>	<i>Ast. multifidus</i>	<i>Isost. badionotus</i>	<i>Euapta lappa</i>
Glycosides									
Holothurin A	+++	+++	+	++*	+	+++	—	—	—
Holothurin B	traces	traces	+++	traces	+++	traces	—	—	—
Stichoposides A, C	—	—	—	—	—	—	—	—	—
Stichoposide B	—	—	—	—	—	—	+++	—	—
Other glycosides	+	+	++	++	++	+	++	+++†	+++†

* Glycosides closely related to holothurin A from *Actinopyga agassizii*, but differing in chromatographic behaviour.

† Glycosides unprecipitable by cholesterol.

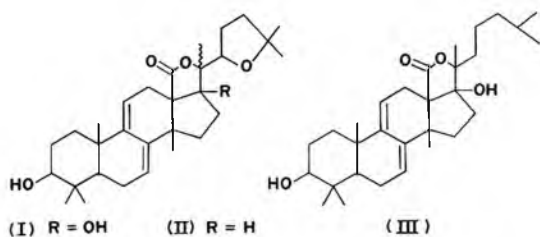
Table 2. Glycoside hydrolysis products

Species	Glycoside	Genins	Hydrolysis products
			Monosaccharides
<i>Holothuria cubana</i>	Holothurin A	I, II	Glucose, xylose, quinovose, 3-OMe-glucose
<i>Holothuria arenicola</i>	Holothurin A	I, II	Glucose, xylose, quinovose, 3-OMe-glucose
<i>Holothuria grisea</i>	Holothurin B	I, II	Xylose, quinovose
	Holothurin A	II, III, I	Glucose, xylose, quinovose, 3-OMe-glucose
<i>Holothuria surinamensis</i>	Holothurin A	I	Glucose, xylose, quinovose, 3-OMe-glucose, galactose
<i>Holothuria mexicana</i>	Holothurin B	I, II	Xylose, quinovose
	Holothurin A	III, I	Glucose, xylose, quinovose, 3-OMe-glucose
<i>Actinopyga agassizii</i>	Holothurin A	I, II	Glucose, xylose, quinovose, 3-OMe-glucose
<i>Astichopus multifidus</i>	Stichoposide B	IV, V	Glucose, xylose, quinovose, 3-OMe-glucose

tions. The main results are shown in Tables 1 and 2.

As is apparent from Table 1, the *Holothuriidae* species chiefly contain holothurin A or a mixture of holothurins A and B.

H. cubana. The main glycoside is holothurin A. When hydrolysed, it formed glucose, xylose, quinovose and 3-OMe-glucose. The chief component of the aglycon mixture obtained was 22,25-epoxy-7,9(11)-holostadien-3,17-diol (I). Its i.r. spectrum showed an absorption band typical of five-membered lactone (1760 cm^{-1}). The MS of the acetate was as follows: m/e 526 (M^+), 466 ($\text{M}^+ - \text{CH}_3\text{COOH}$), 451 ($\text{M}^+ - \text{CH}_3\text{COOH} - \text{CH}_3$), 397 ($\text{M} - \text{CH}_3\text{COOH}$ —cleavage of ring A) and 99 (side chain). The acetate u.v. spectrum showed a triplet absorption maximum (λ_{max} 236, 244 and 251 nm) characteristics of 7,9(11)-diene (Titov & Levina, 1967). The spectral characteristics of the acetate that we isolated coincided with those of the authentic acetate sample of I.



H. arenicola and *A. agassizii*. These animals contained glycoside sets, wherein holothurin A was predominant as in *H. cubana*.

H. surinamensis. The main glycoside was rather similar in chromatographic behaviour to holothurin A from *A. agassizii*. When hydrolysed, it yielded glucose, xylose, quinovose, 3-OMe-glucose and small amounts of galactose (identified by GLC). The dominant aglycon was holothurinogenin I, this being confirmed by the MS, i.r.- and u.v. spectra of its acetate.

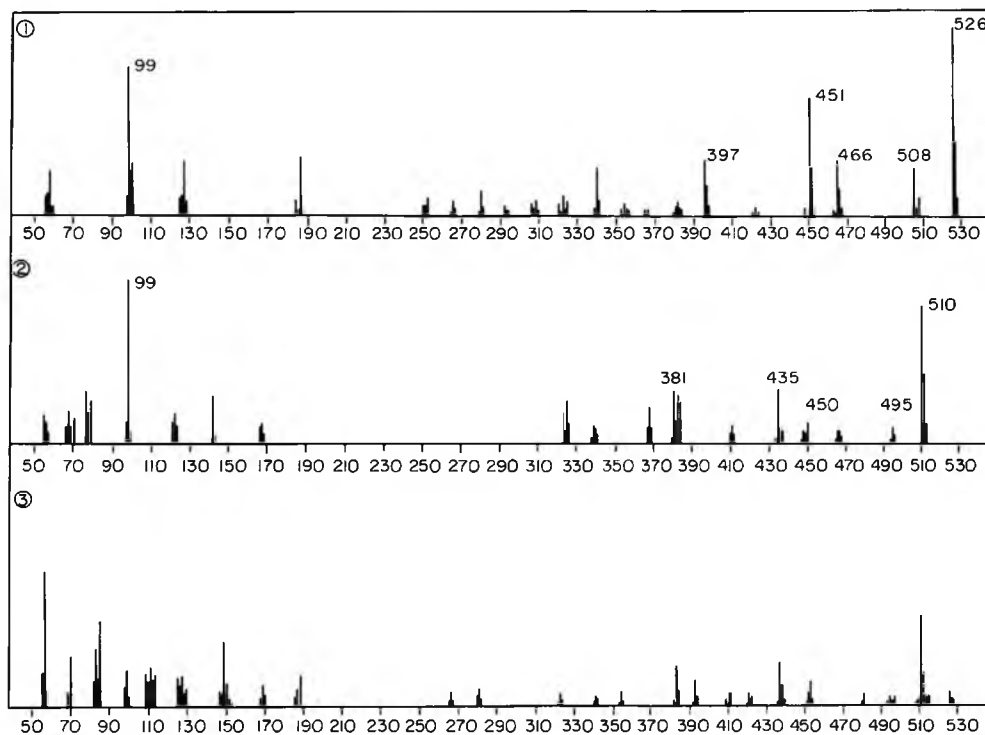
H. mexicana. The glycoside fraction contained holothurins A and B, the latter being the main component. When hydrolysed, holothurin B yielded xylose and quinovose, and also a mixture of the known

holothurinogenins I and II, whose spectra corresponded to those of the authentic samples (see Fig. 1).

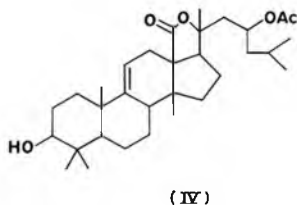
Holothurin A from *A. mexicana* contained 4 monosaccharides, namely glucose, quinovose, 3-OMe-glucose and xylose. When subjected to acid hydrolysis, it yielded holothurinogenin III, isolated earlier by Habermehl (Habermehl & Volkwein, 1970) from *H. polii*. The i.r. spectrum of the acetate of holothurinogenin III from *H. mexicana* showed absorption bands at 1760 cm^{-1} (five-membered lactone) and 1725 cm^{-1} (acetate). The u.v. spectrum revealed absorption maximum at 236, 244 and 252 nm. The MS of the acetate of III was as follows: m/e 512 (M^+), 494 ($\text{M}^+ - \text{H}_2\text{O}$), 452 ($\text{M}^+ - \text{CH}_3\text{COOH}$), 437 ($\text{M}^+ - \text{CH}_3 - \text{CH}_3 - \text{COOH}$) and 383 (M^+ —cleavage of ring A— CH_3CO). Besides III, acid hydrolysis of the glycoside also produced holothurinogenin I, though in lesser amounts.

H. grisea. The glycoside fraction contained holothurins A and B, the latter being predominant. With acid hydrolysis, the glycoside produced xylose, quinovose and holothurinogenin I, the main component of the genin mixture. Acid hydrolysis of holothurin A from *H. grisea* yielded glucose, xylose, quinovose, 3-OMe-glucose and holothurinogenins II (main product) and III. The MS for these substances are shown in Fig. 1.

Of the two Stichopodidae species studied, only *A. multifidus* contained a triterpene glycoside fraction precipitated by cholesterol. After repeated gel filtration of the glycoside fraction on Sephadex G-10, we isolated a novel, heretofore undescribed triterpene glycoside called stichoposide B, $[\alpha]_{\text{D}}^{20} = -48^\circ$ (C 0.1006, pyridine). The acid hydrolysis products of stichoposide B contained glucose, xylose, quinovose and 3-OMe-glucose. The main aglycon proved to be identical to stichopogenin IV obtained by us previously at hydrolysing the glycosides of the Pacific holothurian *St. chloronotus* (Elyakov *et al.*, 1973). The said genin was obtained simultaneously from the same holothurian by Rothberg *et al.* (1973), who established its structure as 23-acetoxy-9,11-ene-holostane- β -ol (IV). The i.r. spectra of the acetate of (IV) from *A. multifidus* had absorption bands for the five-membered lactone and acetate at 1760 and 1725 cm^{-1} , respectively. The u.v. spectrum

Fig. 1. Mass spectrum of acetate I. (Holothurin A, *Holothuria surinamensis*).Fig. 2. Mass spectrum of acetate II. (Holothurin A, *Holothuria grisea*).Fig. 3. Mass spectrum of acetate III. (Holothurin A, *Holothuria mexicana*).

did not reveal absorption over 220 nm. The MS showed m/e 556 (M^+), 541 ($M^+ - CH_3$), 496 ($M^+ - CH_3COOH$), 481 ($M^+ - CH_3 - CH_3COOH$), 437 ($M^+ - CH_3 - 2CH_3COOH$), 109, 69, 57, 43. Besides (IV), hydrolysis of stichoposide B produced another aglycon (V), which differed from (IV) in molecular weight. The MS of (V) showed m/e 554 (M^+), 539 ($M^+ - CH_3$), 494 ($M^+ - CH_3COOH$), 479 ($M^+ - CH_3COOH - CH_3$), 435 ($M^+ - CH_3COOH - CH_3 - CO_2$), 419 ($M^+ - 2CH_3COOH - CH_3$).



The data obtained in the study of the acid hydrolysis products of the isolated glycosides are shown in Table 2.

DISCUSSION

The above-cited evidence confirmed our previous suggestion (Elyakov *et al.*, 1973) that Holothuriidae and Stichopodidae contain different glycoside sets. Indeed, in the *Holothuriidae* species studied we mainly detected glycosides of the holothurin A and B type, i.e. substances that with acid hydrolysis produce holothur-

inogenins (I–III) with a chromophorous heteroannular 7,9(II)-diene group and, correspondingly, four or two monosaccharides. The data obtained in the study of Pacific holothurians (Elyakov *et al.*, 1973) indicate to a wide distribution of such glycosides (holothurins) in this family. The results of the present work and the holothurinogenins obtained after hydrolysis of glycoside extracts or crude glycoside fractions from such species as *H. grisea* (Turch *et al.*, 1967), *H. polii*, *H. forskali* and *H. tubulosa* (Habermehl & Volkwein, 1968, 1970) and *Bohadshia koellikeri* (Roller *et al.*, 1969) also confirm the above conclusion. Beyond doubt, holothurins are affined substances, whose structure is based on the same or similar aglycons, or they are substances with very similar monosaccharide content. On the other hand, we have shown that chromatographically homogeneous holothurins isolated from different animal species may differ from one another. Thus, at hydrolysis, holothurin A from *H. surinamensis* produces almost exclusively holothurinogenin I; holothurin A from *A. agassizii* yields a mixture, wherein holothurinogenins I and II prevail; and holothurin A from *H. mexicana* produces a mixture of I and III. Differences in the structure of carbohydrate chains are also likely, though this suggestion needs additional verification.

Structurally, the glycosides of the family Stichopodidae (stichoposides) are based on aglycons with the same skeleton as in holothurinogenins. However, they do not form a chromophorous heteroannular-diene group at acid hydrolysis. The aglycon portion of stichoposide B from *A. multifidus* structurally resembles the glycosides of certain Pacific holothurians from Stichopodidae, e.g. *S. chloronotus*.

It is noteworthy that our attempts to isolate cholesterol-precipitated glycosides from *I. badionotus* proved to be unsuccessful. Thus, *I. badionotus* is the first of the Stichopodidae species studied that apparently does not contain triterpene glycosides. Earlier, the absence of cholesterol-precipitated glycosides was observed solely in species of the order Apoda (Elyakov *et al.*, 1973).

Further studies of Stichopodidae appear to be interesting both for the search of new triterpene saponins and with a view to a final elucidation of the regularities of glycoside distribution among the animals of that family.

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Key Word Index—Glycosides; triterpenoid glycosides; sea cucumber; Holothurioidea; hydrolysis of glycosides; Holothuriidae; Stichopodidae; Cucumariidae; Synaptidae.