STEROL GLYCOSIDES FROM THE FAR EASTERN HOLOTHURIAN *Cucumaria japonica*

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Sterol β-D-xylopyranosides and arabinopyranosides in a ratio of 9:1 have been isolated from a lipid extract of the musculocutaneous sac of the Far Eastern holothurian *C. japonica*. The main components of both glycosidic fractions were identified as derivatives of cholesta-7,22-diene-3β-ol, cholest-22-en-3β-ol, 5α-cholestan-3β-ol, 24-ethylcholesta-7,22-dien-3β-ol, and 24-methylene-, 24-methyl-, and 24-ethylcholest-22-en-3β-ols.

A lipid extract of the musculocutaneous sac of the Far Eastern holothurian *Cucumaria japonica* includes two fractions (A and B) the component of which contain sterol residues [1]. Fraction A has been characterized as a mixture of acid sterol sulfates, mainly of the C_{27}, C_{28}, and C_{29} types. In the present communication we describe the isolation and chemical characterization of fraction B.

For the isolation of fraction B, a chloroform–methanol extract of the musculocutaneous sac of the holothurian was subjected to alkaline methanolysis under mild conditions. The lipophilic substances of the methanolysate were chromatographed on a column of DEAE-cellulose. A mixture of chloroform and methanol (9:1; here and below the volume ratios of the solvents are shown) eluted neutral lipids — fatty acid methyl esters, cerebrosides, and fraction B (see [1, 2]). The chromatography of the neutral lipids on a column of silica gel led to the isolation of fraction B, and its final purification was achieved by recrystallization from chloroform. In TLC on silica gel, the mobility of fraction B after purification did not differ from its mobility when the initial lipid extract was chromatographed, from which it follows that the structures of the components of fraction B had not changed in the process of isolation. Fraction B made up about 1% of the extracted lipids (0.056% of the weight of the dry biomass).

On chromatograms, fraction B gave positive reactions with specific reagents for sterols and glycosides. The IR spectrum (see the Experimental part) contained strong bands of the H–O and C–O bonds of alcohol groups and lacked bands of carbonyl absorption. The acid methanolysis of fraction B formed a mixture of lipophilic products practically identical in their TLC mobilities on silica gel with the sterols from fraction A [1], and a mixture of methyl glycosides. Judging from the mass spectrum of the lipophilic fraction of the methanolysate, it its main components were a saturated C_{27} sterol, three monoenic sterols — C_{27}, C_{28}, and C_{29} — and three dienic sterols — C_{27}, C_{28}, and C_{29}. The total sterols of the methanolysate were acetylated and separated into saturated, monoenic, and dienic compounds by chromatography on silica gel impregnated with silver nitrate. Each fraction of the acetate was analyzed by GLC and by GLC-mass spectrometry. In addition, the fractions were subjected to alkaline methanolysis, and the free sterols were converted into the trimethylsilyl derivatives (TMSDs) and these were analyzed by the same methods.

The saturated sterols was characterized as 5α-cholestan-3β-ol, since its acetate and TMSD had identical retention times on GLC and identical mass spectra to those of authentic samples of the acetate and TMSD of this alcohol. In the mass spectra of all three monoenic acetates the maximum peak was that of the ion with m/z 257 formed as the result of the elimination of the side chains and AcOH from the molecular ions. Consequently, the steroid nucleus in these compounds was saturated, and the double bond must have been located in the side chain. This was also shown by the presence in the spectra of the peaks of ions with m/z 317 [M — side chain (s.c.)], 275 ([M — s.c. — $CHCH_2CH_2$ (from ring D) — H]), and 215 (loss of AcOH by the ion with m/z 275). The strongest peaks in the same mass spectra corresponded to an

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ion with m/z 344, which is characteristic for the fragmentation of the molecular ions of the acetates of Δ^{22}-sterols formed as the result of the cleavage of the $C_{(20)}$—$C_{(22)}$ bond with migration of the hydrogen atom to the neutral fragment [3-5]. The peak of the analogous fragment (m/z 374) is one of the main peaks in the mass spectra of the TMSDs of monoenic sterols. On the basis of the facts given, the monoenic sterols present in fraction B were characterized as cholest-22-en-3β-ol, 24-methylcholest-22-en-3β-ol, and 24-ethylcholest-22-en-3β-ol. The presence of a methyl group at $C_{(24)}$ in the second of the compounds mentioned and of an ethyl group in the same position of the third compound was confirmed by the relatively high intensities of the peaks of the ions [M − 42] and [M − 43], in the mass spectra of their derivatives, and also of the peak of an ion [M − 29] in the spectrum of the acetate of the last-mentioned sterol. The appreciable formation of these ions is due to the allyl position of the isopropyl and ethyl groups of the side chains.

In the molecules of the dienic sterols, judging from the mass spectra of their derivatives, one of the double bonds was present in the steroid nucleus and the other in the side chain. This was shown by the following facts. In the spectra of all three dienic acetates the peak of an ion with m/z 315 corresponded to the elimination of the side chain from the molecular ion, and this was accompanied by the stronger peak of the fragment [M − s.c. − 2 H] with m/z 313, which is characteristic for mass spectra of sterols with unsaturated side chains [4]. The splitting out of the side chain and three carbon atoms of ring D corresponded to the appearance of an ion with m/z 273. The peaks of ions with m/z 255, 253, and 213 formed as the result of the loss of AcOH molecules by the fragments mentioned above were among the main peaks for all three spectra.

In the mass spectra of the acetates of the C_{27} and C_{29} dienic sterols one of the strongest peaks in each case was that of an ion with m/z 342, analogous to the ion with m/z 344 considered above in our discussion of the monoenic sterols. An ion of this type with m/z 372 was observed in the mass spectra of TMSDs of these dienic sterols. From this, it may be concluded that in the side chains of the C_{27} and C_{29} sterols the double bond was located between $C_{(22)}$ and $C_{(23)}$. In the mass spectrum of the acetate of the C_{28} dienic sterol, the maximum peak was found at m/z 356 and corresponded to the fragment arising on the cleavage of $C_{(22)}$—$C_{(23)}$ bond with migration of the hydrogen atom to the neutral fraction. This type of decomposition of the molecular ion may be connected with the presence of an exomethylene group in it at $C_{(24)}$ (see [3, 4]). The presence of a $RR^1C{=}CH_2$ grouping in the molecule of the dienic sterol was shown by the IR spectrum of the fraction of dienic acetates: strong ν(C=C) and ν(C—H) bands at 1644 and 888 cm^{-1}, and also a δ(C—H) band at 1412 cm^{-1}. In the mass spectra of the TMSDs of the dienic sterols the peaks of ions with m/z 129 and [M − 129] were of low intensity, which excludes the location of the endocyclic double bond at $C_{(5)}$—$C_{(6)}$ (see [6]). On the other hand, in each of the TMSD spectra a peak at m/z 229 characteristic for the mass spectra of the TMSDs of Δ^7-sterols [7] was extremely strong. As an additional proof of the localization of the double bond at $C_{(7)}$—$C_{(8)}$ we may also consider the presence of a peak at m/z 288 in the spectra of the corresponding acetyl derivatives [8]. Thus, the dienic sterols of fraction B have the structures of cholest-7,22-dien-3β-ol, 24-methylcholest-7-en-3β-ol, and 24-ethylcholest-7,22-dien-3β-ol.

Below, we give the relative amounts of the main sterols present in the structure of the components of fraction B:

Sterol	Relative Amount, %
1. 24-Ethylcholest-7-en-3β-ol	11.6
2. 24-Ethylcholesta-7,22-dien-3β-ol	6.5
3. 24-Methylcholest-7-en-3β-ol	14.7
4. 24-Methylenecholest-7-en-3β-ol	36.0
5. 5α-Cholestan-3β-ol	5.1
6. Cholest-22-en-3β-ol	16.3
7. Cholesta-7,22-dien-3β-ol	9.8

Analysis of the hydrophilic part of the methanolysate of fraction B with the aid of GLC showed that 90% of it consisted of methyl xylosides and about 10% of methyl arabinosides. On this basis, it may be assumed that fraction B consisted of a mixture of sterol xylosides and arabinosides. It has been possible to separate these types of glycosides with the aid of chromatography on a column containing silica gel impregnated with boric acid. A gas-chro-

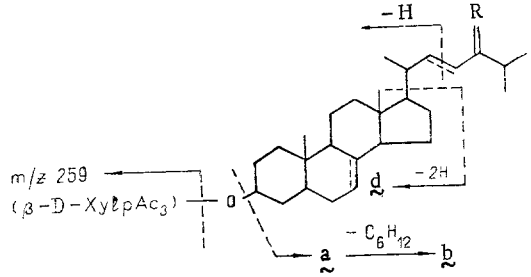
TABLE 1. Main Peaks in the Mass Spectra of the Per-O-Acetyl (Ia) and Per-O-deuteroacetyl (Ib) Derivatives of of Sterol β-D-xylopyranosides

Type of ion*	Spectrum of the acetates (Ia)		Spectrum of the deutero-acetates (Ib), m/z	Type of ion*	Spectrum of the acetates (Ia)		Spectrum of the deutero-acetates (Ib), m/z
	m/z	I_{rel}, %			m/z	I_{rel}, %	
M_1	672	6	681	b_4	297	18	297
M_2	670	3	679	b_5	287	5	287
M_3	658	6	667	b_6	285	9	285
M_4	656	13	665	b_7	283	8	283
M_5	646	1	655	†	259	66	268
M_6	644	6	653	†	257	17	257
M_7	642	4	651	‡	255	34	255
c	572	29	581	‡	253	15	253
d	529	37	538	‡	229	25	229
d'	531	8	540	†	217	13	224
a_1	397	22	397	‡	215	18	215
a_2	395	9	395	‡	213	18	213
a_3	383	38	383	†	199	37	205
a_4	381	77	381	†	170	25	176
a_5	371	11	371	†	157	85	161
a_6	369	31	369	†	139	64	142
a_7	367	18	367	†	128	28	132
b_1	313	9	313	†	115	25	117
b_2	311	3	311	†	97	100	97,98
b_3	299	24	299				

*The numerical index attached to the letter designation of the ion shows the sterol residue or its fragment forming part of the composition of the ion.
† Fragment of a carbohydrate residue: for their structures see [9].
‡ Fragments of sterol residues (see text).

matographic analysis of the sterol xylosides and arabinosides showed that their compositions were practically identical. The structures of the components of the xyloside subfraction (I) were definitively established on the basis of the results of a mass-spectrometric analysis of the per-O-acetates (Ia), the per-O-trideuteroacetates (Ib), and the TMSDs (Ic), and also from the PMR spectrum of the acetates (Ia).

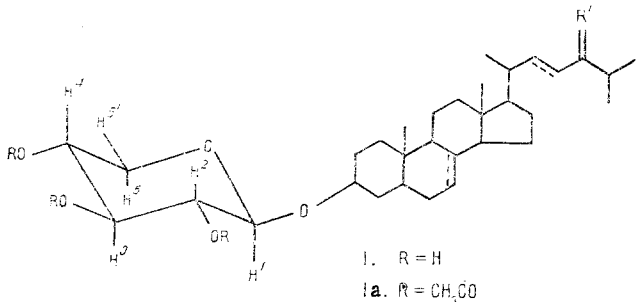
The mass spectrum of the acetyl derivatives (Ia) (Table 1) contained peaks of seven molecular ions, which corresponded to the main components of the fraction, containing residues of different sterols. These peaks were shifted by nine units in the direction of larger mass numbers in the mass spectrum of the deuteroacetate (Ib), from which it follows that the structure of each component included one xylose residue in which all the hydroxy groups, apart from the glycosidic group, were free. Seven fragments of type a (see scheme), formed as the result of the cleavage of the bond between the glycosidic oxygen atom and $C_{(3)}$ of the sterol residue, and also ions of type b, the mass numbers of which showed the loss of 84 mass units (C_6H_{12}) by the ions *a*, corresponded to the molecular ion. The peaks of ions *a* and b retained their positions in the spectrum of the deuteroacetates (Ib).



In the region of high values of m/z, the strongest peaks were those of ions m/z 572 (c) and 529 (d). Both peaks were shifted in the direction of higher mass numbers by nine units

in the spectrum of the deuteroacetate (Ib) and therefore the ions contained a tri-O-acetylxylose residue. The peak of the metastable ion m* 498.8 present in the spectrum of the acetate (Ia) (m* 507.6 in the spectrum of (Ib)) showed that the precursor of fragment c was the molecular ion (M_4^+) with m/z 656 containing a 24-methylenecholest-7-en-3β-ol residue. The conversion of M_4^+ into ion c took place by the elimination of a C_6H_{12} fragment, i.e., as the result of the cleavage of the $C_{(22)}$—$C_{(23)}$ allyl bond of the sterol residue that is characteristic for $\Delta^{24(28)}$-sterols. The formation of ion d took place through the loss by the dienic molecular ions of side chains with two hydrogen atoms. A peak at m/z 531 of lower intensity corresponded to the analogous ion (d') arising from the monoenic molecular ions. The intensities of the peaks corresponding to the cleavage of the $C_{(20)}$—$C_{(22)}$ bond in the Δ^{22}-sterols — at m/z 558 and 560 — were insignificant.

In the region of low mass numbers there were the peaks of fragments of the carbohydrate moieties of the molecular ions (with m/z 259, 217, 199, 170, 157, 139, 128, 115, 97) which are always present in the mass spectrum of tri-O-acetylpentosides [9]. The assignment of the peaks mentioned was confirmed by their corresponding shifts in the spectrum of the deuteroacetates (Ib). The same region contained the peaks of ions consisting of fragments of the steroid moieties of the molecules with m/z 257, 255, 253, 215, and 213. These peaks retained their positions in the spectrum of the deuteroacetate (Ib).



I. R = H
Ia. R = CH_3CO
Ib. R = CD_3CO
Ic. R = Me_3Si
R' = H_2; CH_2; Me, H or Et, H

In the mass spectrum of the TMSD (Ic), the peak at m/z 204 was the maximum peak, which indicated the pyranoid form of the ring of the carbohydrate residue [10]. An analysis of the PMR spectrum of the acetyl derivatives (Ia) led to the same conclusions. The protons at $C_{(5)}$ of the xylose residue corresponded to two quadruplets in the spectrum: the axial H^5 signal at δ 3.30 ppm (J_{H^5,H^4} = 7.5 Hz, $J_{H^5,H^{5'}}$ = 12 Hz) and the equatorial $H^{5'}$ signal at δ 4.06 ppm ($J_{H^{5'},H^4}$ = 4 Hz, $J_{H^{5'},H^5}$ = 12 Hz). The appearance of the signal from the anomeric proton at H^1 at δ 4.54 ppm and its splitting with J_{H^1,H^2} = 7 Hz shows the anomeric β configuration of the xylopyranose residue. An estimate of the molecular optical rotation of the subfraction of xylosides (I) on the basis of Klyne's rule permitted this residue to be assigned to the D series. Thus, the main glycosidic subfraction consisted of a mixture of β-D-xylopyranosides of the sterols (I). It must be added that the chemical shift of the proton at $C_{(3)}$ of the sterol aglycones — δ 3.50 ppm (multiplet) — corresponded to the equatorial β orientation of the oxygen substituent. The mass spectra of the per-O-acetyl derivatives of the arabinosides were practically identical with the spectrum of the acetates (Ia) considered above. Consequently it may be assumed that the minor subfraction of the glycosides consisted of sterol arabinopyranosides.

Sterol glycosides have so far been detected in a limited number of species of marine animals. Of the compounds of this class, only the β-D-xylopyranosides isolated from holothurians of the genera *Stichopus* and *Isostichopus* have been characterized by an adequate degree [11-13]. The xylosides investigated in the present work differ from those described in the cited communications by the composition of the sterols. A feature of the former may be considered to be a high content of 24-methylenecholest-7-en-3β-ol β-D-xylopyranoside.

EXPERIMENTAL

For column chromatography we used silica gel L (Lachema, Czechoslovakia) with particle dimensions of 100-160 μm, which was first treated by a method described previously [2]. The

treated silica gel was impregnated with 10% boric acid [14]. TLC was performed in the same way as in [1]. To detect the substances on the chromatograms, we also used reagents for glycosides: the anthrone reagent [15] and periodate–Schiff reagent [16]. We used the instruments described in [1] for GLC, GLC-mass spectrometry, mass spectrometry, and IR spectroscopy. The mass spectra were obtained at an energy of the ionizing electrons of 70 eV and an accelerating voltage of 3.5 kV; the evaporation temperature of the samples was 70-80°C. The PMR spectrum was recorded on a XL-100 instrument (Varian) in $CDCl_3$ at a working frequency of 100 MHz.

Isolation of Fraction B. The lipids of the musculocutaneous sac of *C. japonica* (3.2 g) were subjected to alkaline methanolysis, and the lipid fraction of the methanolysate was chromatographed on DEAE-cellulose as described previously [1, 2]. The neutral lipids (2.4 g) were eluted from the ion-exchanger with $CHCl_3$–MeOH (9:1) and were dissolved in 15 ml of $CHCl_3$, and the solution was deposited on a column (35 × 3 cm) filled with silica gel in $CHCl_3$. The column was washed with 1 liter of $CHCl_3$, after which elution was continued with $CHCl_3$–MeOH mixtures in ratios of 40:1, 30:1, 20:1 (500 ml each), and 10:1 (700 ml). The eluate was collected in 10-ml fractions, which were analyzed with the aid of GLC in the $CHCl_3$–MeOH–water (80:20:2) system. The initial fractions eluted by the last mixture of solvents contained sterol glycosides contaminated with cerebrosides. These fractions were combined and evaporated, and the residue was dissolved in boiling $CHCl_3$. The solution was left for a day at 2-4°C, after which 29 mg of fraction B was filtered off with R_f 0.7 (in the system given above), 0.45 ($CHCl_3$–MeOH–conc. NH_4OH (85:15:1.5)). ν_{max}^{KBr} (cm^{-1}): 3400 (HO), 3050 (CH of a vinyl bond), 1643 (C=C in RR'C=CH_2), 1410 (CH in RR'C=CH_2), 1378 and 1385 (CH in CMe_2), 1050 (C–O), 888 (CH in RR'C=CH_2).

Isolation of Sterol Xylosides and Arabinosides. A solution of 25 mg of fraction B in 0.5 ml of $CHCl_3$ was deposited on a column (18 × 1.2 cm) filled in $CHCl_3$ with silica gel impregnated with boric acid. Elution was performed successively with mixtures of $CHCl_3$ and isopropanol in ratios of 15:1, 10:1, 9:1, 8:1, 7:1, and 6:1 (50 ml each), these mixtures being saturated with boric acid. Fractions with a volume of 5 ml were collected and were analyzed with the aid of TLC on silica gel impregnated with sodium tetraborate [17] in the $CHCl_3$–MeOH–water–15 M aqueous ammonia (280:70:6:1) system [17]. The fractions containing chromatographically homogeneous xylosides (R_f 0.75) and arabinosides (R_f 0.65) were grouped and evaporated. The boric acid was distilled off with methanol from the residues. This gave 19 mg of sterol β-D-xylopyranoside (I), mp 206-210°C, $[\alpha]_D^{22}$ −12° (c 0.2; $CHCl_3$–MeOH, 2:1), and 1.8 mg of arabinosides.

The acetates (Ia) were obtained by treating the xylosides (I) with a mixture of acetic anhydride and pyridine (1:1) at 20°C for 16 h; R_f 0.6 (benzene–ethyl acetate (3:2)). IR spectrum (in a film of the substance, cm^{-1}): 1738, shoulder at 1745 (C=O), 1244 (C–O in an acetate), 1082 and 1050 (C–O in a glycoside). The per-O-acetates of the sterol arabinosides were obtained similarly. The per-O-trideuteroacetates (Ib) were obtained under the same conditions with $(CD_3CO)_2O$ in pyridine.

The trimethylsilylation of the xylosides (I) was carried out by treating them with trimethylsilylimidazole in $CHCl_3$ at 25°C for 2 h.

Acid Methanolysis of the Sterol Glycosides. A mixture of 1-15 mg of glycosides (xylosides, arabinosides, or fraction B) and 1.5 ml of a 0.3 M solution of dry HCl in MeOH was heated at 80°C in a hermetically sealed tube for 2 h. After cooling, the mixture was neutralized with Dowex 2 × 8 (carbonate form), evaporated to dryness, and distributed in a $CHCl_3$–MeOH–Water (10:5:3) two-phase system. Free sterols were obtained from the lower phase. After evaporation of the upper phase, methyl glycosides were obtained which were subjected to trimethylsilylation. The TMSDs were analyzed with the aid of GLC using a hollow column (45 × 0.3 mm) with silicone-30 under the temperature conditions: 140-240°C (2 deg/min), rate of flow of helium 3 ml/min. Authentic samples of methyl pentoside TMSDs were used as standards.

Analysis of the Sterols. The free sterols obtained as the result of the methanolysis of the glycosides were acetylated. The acetates were separated into fractions of saturated, monoenic, and dienic compounds by chromatography on silica gel impregnated with 20% of silver nitrate [1]. Aliquots of the fractions were converted into the TMSDs [1]. The acetates and TMSDs were analyzed by GLC and GLC-mass spectrometry under the conditions described in [1].

CONCLUSION

From a lipid extract of the musculocutaneous sac of the Far Eastern holothurian *Cucumaria japonica* a fraction of sterol pentosides has been isolated 90% of which consisted of β-D-xylo-

pyranosides and about 10% of arabinopyranosides. The main components of the two subfractions were identified as glycosides of 5α-cholestan-3β-ol, cholest-22-en-3β-ol, cholesta-7,22-dien-3β-ol, 24-ethylcholesta-7,22-dien-3β-ol, and 24-methyl-, 24-methylene-, and 24-ethylcholest-22-en-3β-ols.

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