GLYCOSIDES OF MARINE INVERTEBRATES.

VII. STRUCTURE OF HOLOTHURIN B FROM *Holothuria atra*

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Holothurin B, which contains a sulfate group in a carbohydrate chain, has been isolated from the holothurian *Holothuria atra*. Its complete structure has been established by chemical and physicochemical methods.

Until recently, the complete structure of the sulfate-containing holothurins A and B from holothurians of the family Holothuriidae had not been established. The present paper gives the structure of holothurin B, which has been isolated from *Holothuria atra*, the holothurian most common in shallow tropical waters.

The acid hydrolysis of holothurin B yielded several holothurinogenins in addition to D-xylose and D-quinovose, and, therefore, like holothurin A from *Actinopyga agassizii* [1], holothurin B consists of a combination of oligosides difficult to separate and differing in details of the structure of the triterpene aglycones. The main aglycone of (I) was identified with a holothurinogenin known previously [2]. It has a UV spectrum with absorption maxima at 237, 244, and 252 nm, which are characteristic for a 7,9(11)-diene, and it was an artefactual product formed from the native precursor under the conditions of hydrolysis. This followed from the UV spectrum of the holothurin itself, which lacked absorption maxima above 210 nm. It is known that compound (I), previously assumed to be a product of the hydrolysis of holothurin A, is actually formed by a dehydration in an acid medium of the native 12α-hydroxyholothurinogenin (II) [3, 4]. We have shown that for holothurin B the aglycone (I) has the same origin. In actual fact, a comparison of the ^{13}C spectra of holothurin B (III), desulfated holothurin B (IV), and the model aglycones (V) and (VI) showed that the natural glycoside (II) has only one double bond, and this occupies the 9(11) position. In addition to the eight CH-OH signals of the atoms of the carbohydrate chain, two other signals of such groups are found in the weak-field region: at 71.5 ppm (C_{12}) and at 80.6 ppm (C_{22}). A singlet signal at 81.4 ppm (C_{25}) shows that the tetrahydrofuran nucleus is already present in the initial glycoside and is not formed under the conditions of hydrolysis. Thus, the characteristics of the ^{13}C NMR spectrum, which are given in Table 1, confirm that the aglycone moiety of the main component of holothurin B is compound (III). The configurations of the C_{20} and C_{22} carbon atoms in compound (III) are given on the basis of the results of an x-ray structural analysis of the related aglycone (VII) [6].

The β configuration of both anomeric carbon atoms of the carbohydrate chain was established by a comparison of the ^{13}C NMR spectra of glycosides (III) and (IV) with spectra of model methyl α- and β-xylopyranosides (VIII, IX) and methyl α-, β-quinovopyranosides (X, XI) [7]. As can be seen from the figures given in Table 1, the signals of the C_2 carbon atoms for the α-isomers of the model glycosides are present in the $\delta \sim 100$-101 ppm region, while for the β-isomers and the anomeric carbon atoms of the carbohydrate chains of compounds (III) and (IV) they are in the weaker field of $\delta \sim 104$-105 ppm. The assignment of the signals in the carbohydrate chains of glycosides (III) and (IV) was made after the determination of its structure by using the characteristic features of the changes in the chemical shifts of the carbon atoms of monosaccharide units on sulfation and on the addition of a new monosaccharide unit [8].

The structure of the carbohydrate chain of holothurin B was established in the following way. On methylation by Hakomori's method, halothurin B (III) gave a compound containing

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TABLE 1. Chemical Shifts of the Signals of Some Carbon Atoms in the ^{13}C NMR Spectra of Holothurin B and Related Compounds

C atom	III	IV	V	VI	VIII	IX	X	XI
3	88,8d	88,8d	78,8d	78,5d	—	—	—	—
9	153,3s	153,8s	151,4s	151,4s	—	—	—	—
11	115,4d	115,5d	110,5d	110,5d	—	—	—	—
12	71,5d	71,5d	—	—	—	—	—	—
17	89,6s	89,6s	52,4d	52,4d	—	—	—	—
18	174,5s	174,4s	175,9s	175,9s	—	—	—	—
20	86,6s	86,6s	83,2s	83,2s	—	—	—	—
22	80,6d	80,6d	—	—	—	—	—	—
25	81,4s	81,4s	77,2s	—	—	—	—	—
C_1^I	105,6d	106,1d	—	—	100,6d	105,1d	—	—
C_2^I	83,1d	83,9d	—	—	72,3d	74,0d	—	—
C_3^I	76,6d	78,0d	—	—	74,3d	76,9d	—	—
C_4^I	75,1d	70,8d	—	—	70,4d	70,4d	—	—
C_5^I	64,0d	66,6t	—	—	62,0t	66,3t	—	—
C_1^{II}	105,1d	105,7d	—	—	—	—	100,3d	104,3d
C_2^{II}	76,0d	76,6d	—	—	—	—	76,2d	75,4d
C_3^{II}	77,3d	77,7d	—	—	—	—	73,9d	76,7d
C_4^{II}	76,6d	77,0d	—	—	—	—	76,2d	76,2d
C_5^{II}	73,3d	73,4d	—	—	—	—	68,7d	78,0d
C_6^{II}	18,5q	18,5q	—	—	—	—	17,6q	17,8q

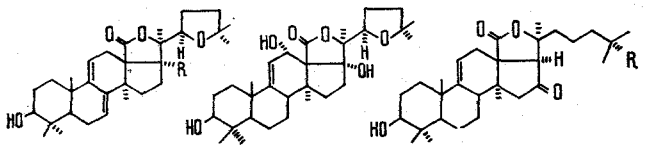
Note: s) singlet; d) doublet; t) triplet; q) quartet.

a sulfate grouping (the absorption band at 830 cm^{-1} remained in the IR spectrum), and the methanolysis of the permethylate obtained followed by acetylation of the products formed showed that in the carbohydrate chain of the glycoside (III) there are a terminal quinovose residue and a xylose residue with a sulfate group in position 2 or 4, since we identified methyl 2,3,4-tri-O-methyl-α- and -β-quinovopyranosides and methyl 2,4-di-O-acetyl-3-O-methyl-α- and -β-xylopyranosides.

The desulfated holothurin B (IV) obtained by solvolytic cleavage of the pyridinium salt of holothurin B (III) gave, on methylation followed by methanolysis, methyl 2-O-acetyl-3,4-di-O-methyl-α- and -β-xylopyranosides. Consequently the sulfate group occupies position 4 in the xylose residue, and the xylose and the quinovose have a 1→2 bond. This conclusion was confirmed independently — by replacing the sulfate group with a formyl group under the action of oxalyl chloride in dimethylformamide on the peracetate of holothurin B (III) [9]. The derivative obtained was then subjected to acetolysis. The only O-formylated products proved to be the peracetates of 4-O-formyl-α- and -β-xylopyranose, which were identified in a chromato-mass-spectrometric study. They had the ion with m/e 245 (M^+ − OAc) that is characteristic for mono-O-formylxylopyranoses, and signals with m/e 185 and 145 showed the presence of the O-formyl group in position 2 or 4, while the presence of a peak with m/e 139 and the absence of one at m/e 125, together with ions having m/e 150 and 114, confirmed that the formyl group was present in position 4. The fragmentation of the formyl derivative was similar to that of the peracetates of xylopyranose [10], but corresponding signals in the mass spectrum were shifted by 14 units in the direction of smaller masses.

The last structural question remaining was the position of attachment of the carbohydrate chain to the aglycone. A comparison of the ^{13}C NMR spectra of glycosides (III) and (IV) and also of the model aglycones (V) and (VI) showed that the signal of the C_3 atom was shifted in the glycosides by almost 10 ppm downfield, which shows that the carbohydrate chain is attached to this particular atom. The complete structure of holothurin B can be represented by formula (III), and that of its desulfated derivative by (IV).

When the work on our paper was already in the state of completion, a report appeared from Japanese authors [11] on the structure of holothurin B from *Holothuria vagabunda*. The structures established in this paper and in the present paper coincide completely, which confirms the correctness of the conclusion made [12] concerning the closeness of the chemical structure of glycosides from different holothurians of the family Holothuriidae.



I. R = OH
VII. R = H

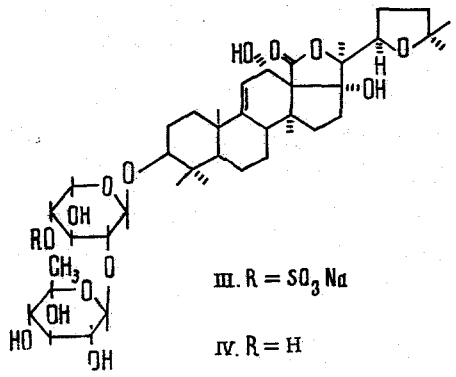
II

V. R = OH
VI. R = H

Scheme 1

EXPERIMENTAL

For chromatography we used type KSK silica gel, Whatman No. 1 paper, and the following solvent systems: 1) chloroform–methanol–water (60:30:1); 2) phenol–water (100:40); 3) benzene–ethyl acetate (3:1); and 4) benzene–ethyl acetate (8.5:1). The glycosides were detected on the chromatograms with sulfuric acid, and the monosaccharides with aniline phthalate at 110°C, 5 min.



III. R = SO_3Na

IV. R = H

Scheme 2

The ^{13}C and ^{1}H NMR spectra were taken on a Brüker HX-90E instrument in chloroform and pyridine solutions with tetramethylsilane as internal standard. IR spectra were recorded on a Specord IR 75 in potassium bromide tablets, and UV spectra on a Specord UV-VIS in ethanolic solution. The chromato-mass-spectrometric (GLC-MS) study of the methanolysis products was performed on an LKB 9000S instrument with an ionizing voltage of 70 V, using a 3 m × 3 mm column containing 3% of QF-1 on Chromosorb W. The temperature of analysis was raised from 100 to 200°C at the rate of 2 degrees/min. The carrier gas was helium, 60 ml/min. GLC analysis was carried out on a Pye Unicam 104 chromatograph using 3 mm × 2 m columns containing 5% of QF-1 on Chromaton N, 100-210°C, 2 degrees/min. The carrier gas was helium at the rate of 60 ml/min.

Holothurin B (III). An aqueous ethanolic extract of muscle sacs of the holothurians was concentrated in vacuum. The residue was dissolved in water and the solution was extracted several times with hexane and then with butanol. The butanolic solution was evaporated to dryness. The residue was dissolved in hot ethanol, and the solution was boiled with activated carbon for 0.5 h, filtered, and cooled to room temperature. The microcrystalline precipitate that deposited was separated off. After four crystallizations from ethanol, chromatographically homogeneous (system 1) holothurin B was obtained in the form of colorless needles with mp 216-218°C, $[\alpha]_D^{25}$ −33° (c 1.25; pyridine).

Acid Hydrolysis of Holothurin B. A solution of 50 mg of the glycoside in 10 ml of 2 N HCl was heated at 90-100°C for 2.5 h. The precipitate of aglycones that deposited was separated by extraction with chloroform. After the chloroform had been washed with water and had been concentrated, 16 mg of combined aglycones was obtained (system 3), and these were separated by preparative thin-layer chromatography on KSK silica gel (150-170 mesh) in system 4 (three runs). After crystallization from benzene, aglycone (I) (6.8 mg) had mp 309-312°C; mass spectrum, m/e: 484, 457, 397, 99 (C-20, C-22 cleavage); $\lambda_{max}^{C_2H_5OH}$, nm: 237, 244, 252 (log ε 3.54). According to the literature [1]: mp 315-316°C; mass spectrum: M^+ 484; $\lambda_{max}^{C_2H_5OH}$, nm; 237, 244, 252 (log ε 3.54).

The hydrolysate was neutralized with Dowex anion-exchange resin (HCO_3^-) and after the solution had been evaporated 14 mg of total sugars was obtained with $[\alpha]_D^{25}$ +18.7° (c 1.7; water). Calculated for a mixture of D-xylose and D-quinovose (1:1), $[\alpha]_D$ +25.7° [12]. Paper

chromatography in system 2 led to the identification of xylose and quinovose, and so did the GLC of the peracetates of the aldononitriles obtained from the combined monosaccharides by the method of Easterwood and Heeff [13].

Desulfation of Derivative (IV). An aqueous ethanolic (1:1) solution of 100 mg of holothurin B was stirred with Dowex 50W cation-exchange resin (H^+) for 2 min, and the resin was separated by filtration. The filtrate was neutralized with pyridine and concentrated in vacuum to dryness. The residue (96 mg) was boiled in 50 ml of absolute dioxane containing 1% of pyridine for 1 h. Monitoring by TLC in system 1 showed that under these conditions holothurin B (III) is converted completely into the less polar (IV). The latter was purified by column chromatography on KSK silica gel in the chloroform–methanol (3:1) system. This gave 71 mg of (IV) with mp 280-282°C, $[\alpha]_D^{25}$ −4.5° (methanol).

Methylation and Methanolysis of Glycoside (III). A solution of 25 mg of glycoside (III) in 2 ml of a mixture prepared from 500 mg of sodium hydride and 15 ml of absolute dimethyl sulfoxide at 70°C for 2 hours in an atmosphere of nitrogen was stirred for 2.5 h and was then treated with 1 ml of methyl iodide and left overnight in a nitrogen atmosphere. It was then diluted with 5 ml of water, and the methylation product was extracted with methyl iodide. The extract was washed with saturated aqueous thiosulfate and evaporated to dryness, the residue was dissolved in chloroform, and the solution was filtered through silica gel (10 g). The residue consisted of an amorphous powder (28 mg), $[\alpha]_D^{25}$ +2.8° (c 0.7; chloroform). A solution of 20 mg of the methylation product in 10 ml of anhydrous 10% hydrogen chloride in methanol was boiled for 4 h and evaporated, and then the residue was acetylated in the usual way and the product was analyzed by GLC. Methyl 2,3,4-tri-O-methylquinovopyranosides and methyl 2,4-di-O-acetyl-3-O-methylxylopyranosides were identified.

Methylation and Methanolysis of the Glycoside (IV). In a similar manner to that described above, 25 mg of (IV) yielded 23 mg of methylated derivatives in the form of an amorphous powder the methanolysis of which led to methyl 2,3,4-tri-O-methyl-α,β-quinovopyranosides and methyl 2-O-acetyl-3,4-di-O-methyl-α,β-xylopyranosides (GLC and GLC-MS analysis).

Replacement of the Sulfate Group by the Formyl Group in Glycoside (III). Holothurin B (9 mg) was acetylated with a mixture of 0.2 g of acetic anhydride and 0.13 ml of absolute pyridine at room temperature for 24 h. After dilution with ice water, the reaction mixture was treated with chloroform. The organic layer was separated off and dried, and elimination of the chloroform yielded 8.5 mg of a product which was dissolved in 0.3 ml of absolute DMFA, and the resulting solution was treated at 0°C with 0.1 ml of oxalyl chloride. After the mixture had been heated in the water bath at 60°C for 2 h, 0.2 ml of pyridine was added and after further heating at 60°C for 2 h 2 ml of ice water was added to the reaction mixture and it was treated with chloroform.

The organic layer was separated off and dried over sodium sulfate and, after elimination of the chloroform, 6.5 mg of formylation product was obtained. A solution of 3 mg of this substance in 0.1 ml of a mixture prepared from 0.3 ml of concentrated acetic acid and 10 ml of acetic anhydride was kept at room temperature for 4 days, and then 0.8 ml of ice water was added and the mixture was treated with chloroform. The organic layer was separated off, washed with water, dried over sodium sulfate, and evaporated under reduced pressure in the presence of barium carbonate. Yield 2.8 mg. The products of acetolysis were studied by gas–liquid chromatography and chromato-mass-spectrometry and were identified as the α and β isomers of the peracetate of 4-O-formylxylopyranose.

SUMMARY

Holothurin B, containing a sulfate group in the carbohydrate chain, has been isolated from the holothurian *Holothuria atra*. The structure of this compound has been established.

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ALKALOIDS OF THE EPIGEAL PART OF *Convolvulus krauseanus*

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The epigeal part of *Convolvulus krauseanus* collected in two vegetation periods — flowering and fruit-bearing — has been studied for the first time. Five bases have been isolated. Convolvine, convolamine, and convolidine proved to be known, phyllalbine was isolated from this species for the first time, and colvolicine proved to be a new base for which the structure of (±)-N-acetoxy-3α-veratroyl-nortropine has been established.

Three alkaloids (convolvine, convolamine, and convolidine) have been isolated previously from the roots of *Convolvulus krauseanus* Rgl. et Schmalh. collected at the end of of the vegetation periods in the environs of the village of Bakhmal (Turkestan range) [1]. We have studied the total bases of the epigeal part of this plant collected in the same place in two vegetation periods. The total alkaloids were isolated by chloroform extraction of the plant moistened with 8% ammonia solution. It was found that the epigeal part collected in the vegetation period (June 3, 1978) contained 0.82% of total alkaloids, and that collected in the fruit-bearing period (August 13, 1978) contained 0.16%. The combined materials obtained were separated into phenolic and nonphenolic fractions, and the latter were separated with citrate-phosphate buffer solutions having pH 6.8 and 5.6. Convolvine with a small amount of convolamine was extracted mainly into the buffer with pH 6.8, and convolamine with traces of other bases into the buffer with pH 5.6. The mother solution contained practically no convolvine or convolamine.

The combined phenolic bases were treated successively with acetone and methanol. Crystals deposited with mp 209-210°C (chloroform—methanol), M^+ 291. A comparison of the IR, mass, and NMR spectra of the base obtained with the spectra of the known alkaloid phyllalbine, which was first isolated from *Phyllanthus discoides* [2], and also a mixed melting point showed their identity. Thus, this is the first time that phyllalbine has been isolated from this species of *Convolvulus*.

After the removal of the phyllalbine, the mother liquor was treated with methanol. The insoluble part was filtered off, and it proved to be a mixture of two bases. They were separated by fractional crystallization. One of the bases was phyllalbine, and the other, with mp 214-215°C, was convolidine, which was first isolated from the roots of this plant.

The mother liquor from the combined alkaloids (from the plant collected in the fruit-bearing period) after the maximum elimination of colvolvine and convolamine was deposited on a column of silica gel and eluted successively with petroleum ether (70-100°C), hexane, ether, chloroform, and a mixture of chloroform and methanol. From the ethereal fractions, a base crystallized with mp 184-185°C, R_f 0.85 (system 1: chloroform—methanol—NH_4OH (8:2:0.1)). The subsequent ethereal fractions yielded a white crystalline substance with mp 144-145°C

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