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NATURAL PRODUCTS FROM LAKE BAIKAL ORGANISMS, I. BAIKALOSTEROL, A NOVEL STEROID WITH AN UNUSUAL SIDE CHAIN, AND OTHER METABOLITES FROM THE SPONGE *BAICALOSPONGIA BACILIFERA*

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ABSTRACT.—A free sterol fraction, α-methylglucopyranoside, and fatty acid monoglycerides were isolated from the sponge *Baicalospongia bacilifera*. Structures of the sterols, including a novel minor sterol 24-ethyl-26-norcholesta-5,22*E*,25-trien-3β-ol (baikalosterol) [**1**], were established.

Baikal is the deepest lake on earth. The depth and size of the lake, which contains about 20% of the fresh water in the world, make the life conditions of Baikal similar to those of the sea. The colonization of the lake by some invertebrates began very likely about 50 million years ago and led to unique bentonic biocenoses. For example, sponge fauna include mainly endemic species belonging to the family Lubomirskiidae (1).

It is known that sponges produce the greatest variety of secondary metabolites of any animal group (2,3). However, little work has been reported on natural products of fresh-water sponges (4–6), especially of Baikalian animals (7,8).

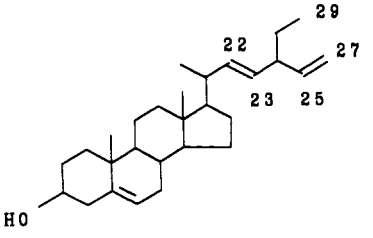
Here we report the isolation and structural elucidation of several secondary metabolites from the sponge *Baicalospongia bacilifera* Dyb. (Demospongiae, family Lubomirskiidae). *B. bacilifera* is a common species of the lake and is present both in shallow water and deep water habitats.

RESULTS AND DISCUSSION

We have collected seven species of Baikalian sponges including *B. bacilifera, Baicalospongia intermedia, Lubomirskia baicalensis, Lubomirskia fusifera, Lubomirskia abietina, Swartchewskia papyracea*, and *Protobaicalospongia primitiva* sp. novae.

Tlc analysis of sponge extracts shows some similarity of chemical composition among all species collected. A series of polar components are predominant while a green pigment, triglycerides, and free sterols are found in the least polar part of these extracts.

We initiated our structural studies on natural products of Baikalian sponges by isolating several secondary metabolites of *B. bacilifera*. The sponge (dry wt 0.66 kg) was



1

extracted with EtOH. The extract was fractioned into hexane and aqueous-EtOH-soluble fractions, and the latter fraction was partitioned between $CHCl_3$ and aqueous EtOH. Free sterols were isolated by chromatography of the hexane fraction. The $CHCl_3$ fraction yielded a mixture of monoglycerides, while the polar aqueous EtOH fraction gave glycosides including α-methylglucopyranoside.

Free sterols (Table 1) were separated by hplc for subfractions SF1–SF6 and purified by repeated chromatography under the same conditions. Comparison of spectral properties and chromatographic behavior with corresponding literature data (9, 10) was used for structural identification of the sterols.

TABLE 1. Sterol Composition of *Baicalospongia bacilifera*.

Sterol	RRt		% in mixture
	hplc	glc	
24-Ethyl-26-norcholesta-5,22*E*,25-trien-3β-ol [**1**]	0.64	1.08	0.5
(24*S*)-Methylcholesta-5,22*E*,25-trien-3β-ol	0.64	1.08	0.2
Cholesta-5,22*E*-dien-3β-ol	0.75	0.91	6.8
24-Methylcholesta-5,24(28)-dien-3β-ol	0.75	1.30	1.4
24ξ-Methylcholesta-5,22*E*-dien-3β-ol	0.88	1.09	10.8
Cholesterol	1.00	1.00	39.2
(24*S*)-Methylcholest-5-en-3β-ol	1.15	1.25	10.1
24ξ-Ethylcholesta-5,22*E*-dien-3β-ol	1.15	1.38	6.4
(24*S*)-Ethylcholest-5-en-3β-ol	1.25	1.60	23.8

Glc-ms analyses demonstrated that SF1 contains a mixture of two minor C_{28}-trien-3β-ols **1** and (24*S*)-methylcholesta-5,22*E*,25-trien-3β-ol. Fraction SF2 gave sterols identified by ^{1}H-nmr and eims spectra as cholesta-5,22*E*-dien-3β-ol and minor amounts of 24-methylcholesta-5,24(28)-dien-3β-ol. The sterol from SF3 was determined to be 24ξ-metyhylcholesta-5,22*E*-dien-3β-ol. Only cholesterol was found in SF4. Two sterols of SF5 proved to be (24*S*)-methylcholest-5-en-3β-ol and minor amounts of 24ξ-ethylcholesta-5,22*E*-dien-3β-ol. The sterol from SF6 was identified as (24*S*)-ethylcholest-5-en-3β-ol (clionasterol).

Isolation of minor sterols was carried out by acetylation of a part of the free sterol mixture followed by cc on Si gel impregnated with $AgNO_3$. Fractions F3–F1 contained acetates of **1** and 24-methylcholesta-5,24(28)-dien-3β-ol, acetates of (24*S*)-methylcholesta-5,22*E*,25-trien-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol, and a mixture of acetates of other sterols, respectively. F2 and F3 were then separated by hplc to isolate the individual acetates of minor strols.

Acetates of (24*S*)-methylcholesta-5,22*E*,25-trien-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol were structurally identified by comparison of ^{1}H nmr and eims spectra with corresponding literature data (11–13).

Sterol **1** is a novel steroid with an uncommon side chain structure. Examination of its acetate ($C_{30}H_{46}O_2$) clarified that **1** is a C_{28} sterol with three double bonds. The $[M]^+$ peak in the eims of the acetate of **1** is absent, as is typical for acetates of Δ^5-sterols. The intense peak in this spectrum at *m/z* 378 $[M - HOAc]^+$, as well as fragment ions at *m/z* 255 (43%), *m/z* 213 (10%), a multiplet signal at 4.60 ppm (H-3), and singlets at 0.69 ppm and 1.01 ppm (Me-18 and Me-19) in the ^{1}H-nmr spectrum (Table 2), are indeed indicative of the presence of a monounsaturated steroid nucleus of the 3β-hydroxy-Δ^5-type in **1** (10,14).

The side chain structure in the acetate of **1** was established by spin decoupling ex-

periments. Irradiation at 5.72 ppm (H-25) revealed signals of the neighboring olefinic methylene group (H_2-27) at 4.93–5.01 ppm and the allylic proton signal (H-24) as a multiplet at 2.49 ppm. Irradiation of this multiplet in a double resonance experiment collapsed multiplets at 5.21 (H-22, H-23) and 1.37 ppm (H_2-28). The latter signal was also collapsed by the irradiation of the triplet signal at 0.86 ppm (Me-29). Finally the multiplet at 1.95 ppm (H-20) was found by irradiation of signals at 1.02 (Me-21) or 5.21 ppm (H-22). These results could be explained by a 24-ethyl-26-nor-22,25-diene group as the side chain of **1**. The uv spectrum confirmed that these two double bonds are not conjugated.

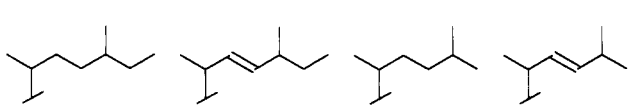
TABLE 2. Chemical Shift in Ppm and Spin-spin Coupling Constants (Hz) of Protons of the Acetate of **1**.

Proton	δ	Proton	δ	H,H	*J*
H-3	4.60 m	H-27	4.93–5.01	22,23	15.0
H-6	5.37 m	H-27′	4.93–5.01	24,25	6.8
H-20	1.95 m	H_2-28	1.37 m	25,27	16.5
H-22	5.21 m	Me-18	0.69 s	25,27′	10.0
H-23	5.21 m	Me-19	1.02 s	27,27′	1.7
H-24	2.49 m	Me-21	1.02 d	28,29	7.0
H-25	5.72 m	Me-29	0.86 t	20,21	6.5

In agreement with the proposed structure, the acetate of **1** has its most intense peak in eims at *m/z* 378 $[M - HOAc]^+$, like other Δ^5 sterols. A prominent peak at *m/z* 349 $[M - C_2H_5 - HOAc]^+$ is connected obviously with the C-24–C-28 allylic cleavage facilitated by both double bonds of the side chain. Peaks at *m/z* 282 [M − C-20–C-22-cleavage − AcOH]$^+$ and at *m/z* 296 [M − C-22–C-23-cleavage − HOAc]$^+$ are indicative of the presence of a 22(23) double bond. The corresponding spin-spin coupling constant (15.0 Hz) was determined by spin decoupling experiment and demonstrated the trans stereochemistry of the double bond. In fact, irradiation at 2.49 ppm (H-24) not only simplified a complicated multiplet of H-22 and H-23 but separated a doublet of H-23 (5.16 ppm, 15.0 Hz) from a doublet of doublets of H-22 (5.25 ppm, 15.0, 7.0 Hz).

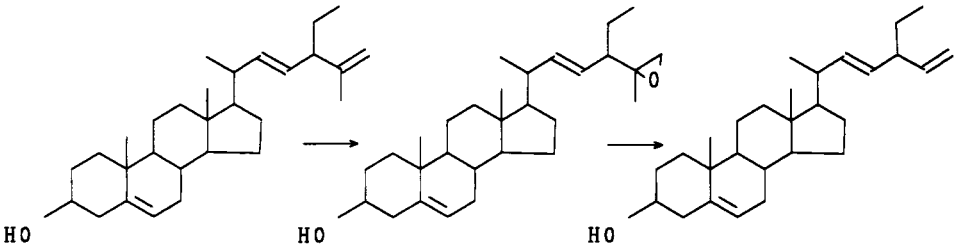
The identification of the unusual side chain structure of **1** raises interesting questions with respect to the biosynthesis of this compound. Alkylated sterols with side chains of **a**, **b**, **c**, and **d** types are widespread in sterol fractions from marine sponges (13) but have not been found from *B. bacilifera*. All these sterols seem to be derived from C_{28} sterols.

Sterol **1** is also a product of dealkylation but derived obviously from a C_{29} sterol. Actually, C_{29} sterols comprise more than 30% of the sterol fraction from *B. bacilifera*. Besides **1**, another sterol component, 24*S*-methylcholesta-5,22*E*,25-trien-3β-ol, from the same sponge contains a 25 double bond. We assume that the precursor of **1** is a minor 25-unsaturated C_{29} sterol **2**, a dehydrogenated derivative of clerosterol, which has been isolated from the green alga *Codium fragile* (15). Dealkylation of **2** could be



a **b** **c** **d**

through the formation of an epoxide **3** (Scheme 1) by analogy to the transformation of fucosterol into desmosterol and cholesterol by insects and some marine invertebrates (16,17).



2 **3** **1**

SCHEME 1

α-Methylglucopyranoside was obtained after repeated cc of the aqueous EtOH extract on XAD-2 resin followed by hplc on Altex C-18 column. It was identified by the direct comparison with an authentic sample of corresponding peracetate after acetylation in the usual manner.

Some glucosides of aliphatic alcohols have been found earlier from marine invertebrates: for example, α-isopropylglucoside from the coral *Sclerophytum capitalis* (18). However, this is the first identification of α-methylglucopyranoside in sponges. Because MeOH was not used in any phase of storage, extraction, or isolation it can be assumed that the α-methylglucopyranoside is not an artifact produced during this investigation.

The $CHCl_3$ extract of the sponge *B. bacilifera* gave a monoglyceride fraction after repeated chromatography on a Si gel column; this fraction was shown to be very complicated by hplc on a C-18 column. This method yielded a monoglyceride mixture of two components. The ^{1}H-nmr spectrum demonstrated that the monoglycerides had fatty acid residues at C-1 of glycerol. In fact, the signal at 4.19 ppm (dt) was due to an acylated hydroxymethyl group of glycerol. The free hydroxymethyl group gave an analogous signal at 3.66 ppm as a multiplet. Protons of the fatty acid gave signals at 5.34 (CH=CH), 2.36 (t, CH_2CO), 2.02 (CH_2-CH=), 1.30 $(CH_2)_n$, and 0.88 ppm (t, Me). Methanolysis of the monoglycerides yielded methyl esters of 16:1 and 18:1w7t fatty acids (1:3) identified by glc on OV-101 capillary column.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The ^{1}H-nmr spectra were recorded on a Bruker WM-250 spectrometer in $CDCl_3$ with TMS as internal standard. Melting points and optical rotation $[\alpha]_{578}^{25}$ were determined on a Boethius and a Perkin-Elmer 141 apparatus, respectively. Mass spectra (eims were measured on LKB 9000S spectrometer (ionizing energy 70 eV). A glass column (150 × 0.5 cm) with 1.5% SE-30 at 280° and flame ionizing detector were used for glc-ms. Glc analyses were carried out: for monosaccharide derivatives on a Zvet-2 (USSR) chromatograph with a glass column 150 × 0.5 cm having 3% QF-1 (110–240°, 4°/min); for methyl esters of fatty acids on a Shimadzu 9A chromatograph equipped with a 25 m × 0.25 mm fused silica column with immobilized OV-101 (215°); for sterol fractions on a Biochrom-1 (USSR) chromatograph with 15 m × 0.25 mm glass column coated with SE-30 (240°). Preparative hplc was conducted utilizing a DuPont 8800 instrument. A column Altex Ultrasphere ODS (25 cm × 4.6 mm) was used. Tlc and cc (lplc) were performed on Si gel L (Chemopol, Czechoslovakia) 5/40 and 40/100μ, respectively.

ANIMAL MATERIAL.—The sponge *B. bacilifera* was collected in August 1989 at depth 20–25 m. Other sponge species were collected in July 1990 from 8–20 m. Locality of the collection is near Bolshie Koty (southwestern part of Lake Baikal). A voucher specimen is on deposit in the marine specimen collection of the Pacific Institute of Bioorganic Chemistry, USSR.

EXTRACTION AND ISOLATION.—Crude EtOH extract of the sponge *B. bacilifera* (0.66 kg dry wt) was evaporated in vacuo at low temperature to give a dark green oil (10.9 g). This oil was dissolved in 300 ml of EtOH-H_2O (9:1). The hexane solubles were extracted by partitioning four times with equal volumes of hexane. The aqueous phase was then partitioned twice against $CHCl_3$. The hexane, $CHCl_3$, and H_2O solubles were concentrated at reduced pressure to give 5.70, 2.12, and 3.06 g of residues, respectively. The hexane extract was chromatographed over Si gel [$CHCl_3$-EtOH (20:1)] and Sephadex LH-20 [$CHCl_3$-EtOH (1:1)] to obtain a free sterol fraction (0.521 g, 0.08%) after crystallization from MeOH. The $CHCl_3$ extract was subjected to chromatography on Si gel [$CHCl_3$-EtOH (10:1)] and yielded 0.04 g (0.006%) of oily monoglyceride mixture. Separation of this fraction by hplc (MeOH) gave 4 mg of monoglycerides which formed, after methanolysis, methyl esters of 16:0 (25%) and 18:1w7t (75%) fatty acids. The most polar extract was chromatographed over XAD-2 resin ($H_2O \rightarrow$10% EtOH) to provide a mixture of monosaccharides and glycosides (1.6 g). This mixture gave, after hplc in H_2O, 0.2 g (0.03%) of α-methylglucopyranoside, identified after acetylation by direct comparison with an authentic sample of peracetate of α-methylglucopyranoside.

SEPARATION OF THE STEROL FRACTION.—The sterol mixture was subjected to hplc (elution with MeOH), and subfractions SF1–SF6 were isolated. Repeated hplc of SF2–SF6 was conducted by elution with Me_2CO-MeOH-H_2O (45:45:6). Obtained sterols were identified after crystallization from MeOH by ^{1}H-nmr and eims studies as well as glc analyses. Results of this identification are given in Table 1.

ISOLATIONS OF ACETATES OF MINOR STEROLS **1** AND (24*S*)-METHYLCHOLESTA-5,22*E*,25-TRIEN-3β-OL.—Acetylation of the free sterol fraction (400 mg) with Ac_2O and pyridine afforded 410 mg of acetates, which were further separated on a column with Si gel impregnated with $AgNO_3$ (20%) using hexane-C_6H_6 (7:3). Separation control was by tlc on Si gel impregnated with $AgNO_3$ (20%) in the system hexane-C_6H_6 (16:13). Fraction 1 (F1) (355 mg) contained the mixture of mono- and diunsaturated acetates of sterols cholesta-5,22*E*-dien-3β-ol, 24ξ-methylcholesta-5,22*E*-dien-3β-ol, cholesterol,(24*S*)-methylcholest-5-en-3β-ol, 24ξ-ethylcholesta-5,22*E*-dien-3β-ol, and (24*S*)-ethylcholest-5-en-3β-ol. F2 (5.5 mg) contained acetates of (24*S*)-methylcholesta-5,22*E*,25-trien-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol. F3 (7.5 mg) contained acetates of **1** and 24-methylcholesta-5,24(28)-dien-3β-ol. F2 and F3 were further subjected to hplc (95% Me_2CO) to isolate the acetates.

24-Ethyl-26-norcholesta-5,22E,25-trien-3β-ol acetate (baikalosterol acetate).—White plates (2.1 mg): mp 143–145° (MeOH), $[\alpha]^{25}_{578}$ −70 (*c* = 0.05, $CHCl_3$); eims *m/z* (% rel. int.) [M − HOAc]$^+$ 378 (100), [M − HOAc − Me]$^+$ 363 (6.0), [M − HOAc − C_2H_5]$^+$ 349 (2.3), 336 (1.0), [M − side chain − 2H]$^+$ 313 (0.7), [M − HOAc − C-23–C-24-cleavage]$^+$ 309 (2.3), [M − HOAc − C-20–C-22-cleavage]$^+$ 282 (10.0), 267 (4.0), 255 (43.0), 229 (4.2), 213 (10.0); ^{1}H nmr see Table 2.

(24S)-Methylcholesta-5,22E,25-trien-3β-ol acetate.—White plates (1.3 mg): mp 151–153° (MeOH), $[\alpha]^{25}_{578}$ −50 (*c* = 0.01, $CHCl_3$) [lit. (9) mp 154°, $[\alpha]^{25}_D$ −53.7 (*c* = 1.08, $CHCl_3$)]; ^{1}H nmr and eims identical with those reported by Knights (9) and Rubinstein *et al.* (10).

24-Methylcholesta-5,24(28)-dien-3β-ol acetate.—This acetate (6 mg) was identified with an authentic sample by direct comparison of ^{1}H-nmr, eims, and glc behavior.

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