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TWO NEW POLYHYDROXYLATED STEROLS FROM THE SPONGE *DYSIDEA FRAGILIS*

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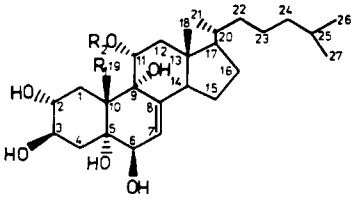
ABSTRACT.—Two new polyhydroxylated sterols **1** and **2** have been isolated from the Black Sea sponge *Dysidea fragilis*. Their structures and stereochemistry have been established by analysis of spectral data. These sterols are biosynthesized from cholesterol.

Dysidea fragilis (family Dysideidae, order Dictyoceratida, identified by Dr. St. Andreev) is the main representative of sponges in the Black Sea. These sponges inhabit shallow waters, mainly between 2 and 20 m. Numerous investigations on the chemical composition of *Dysidea* species have been carried out, and several different groups of natural compounds have been isolated, some of them possessing biological activity. It has been shown that highly functionalized sterols are widespread in this genus (1–5); all the isolated sterols are characterized by oxidation exclusively in rings A and B, as well as at C-11. Sterols isolated from different *Dysidea* sponges show structural differences. *D. fragilis* has not been investigated chemically till now, and for this reason its sterol composition is of interest.

The fresh sponge *D. fragilis* was extracted with MeOH and $CHCl_3$. Polyhydroxylated sterols were isolated by cc followed by hplc separation. Two polyhydroxylated sterols have been isolated, and their structures **1** and **2** determined mainly by spectral methods.



1 R_1=Me, R_2=H
2 R_1=Me, R_2=Ac
3 R_1=CH_2OH, R_2=H

Compound **1** had a molecular composition of $C_{27}H_{46}O_6$, as indicated by hreims of the *m/z* 430 peak $[M-2H_2O]^+$; this is in agreement with ^{13}C-nmr data (Table 1). The eims is typical for polyhydroxylated sterols. Elimination of one, two, three, and four molecules of H_2O from the molecular ion peak, accompanied by elimination of a methyl group and the C_8H_{17} side chain, indicated the presence of at least four hydroxyl groups and a cholesterol-type side chain.

The nmr assignments were made using ^{1}H-nmr double resonance, DEPT, and 2D ^{13}C-^{1}H shift correlation experiments. The ^{13}C-nmr spectrum of compound **1** (Table 1) contained 27 signals. The six signals between 70 and 80 ppm indicated carbon atoms bearing hydroxyl groups. Four of them were assigned to methines and two to quaternary carbons on the basis of DEPT experiments. A quaternary signal at 142.3 ppm and a methine signal at 123.3 ppm showed the presence of a double bond. Five signals for methyl groups (at 13.0, 19.4, 22.2, 23.0, and 23.2) were typical for a sterol having a cholestane structure.

The comparison of ^{13}C-nmr data of sterols **1** and **3** [a polyhydroxylated sterol isolated from *Dysidea etheria* (3)] (Table 1) showed a similarity between these compounds, the only substantial difference being the absence of the CH_2-19 signal and the appearance of an Me

TABLE 1. ^{13}C-nmr Chemical Shift Data Comparison.[a]

Carbon	Compound			
	1		**2**	**3**[b]
	CD_3OD	C_5D_5N	CD_3OD	C_5D_5N
C-1	37.2 t	36.5 t	37.2 t	35.4 t
C-2	73.6 d	73.6 d	73.4 d	73.1 d
C-3	73.3 d	73.3 d	73.0 d	72.4 d
C-4	40.7 t	40.0 t	40.8 t	40.2 t
C-5	78.8 s	78.8 s	78.5 s	77.8 s
C-6	70.7 d	70.2 d	73.0 d	71.3 d
C-7	123.3 d	124.5 d	123.5 d	122.7 d
C-8	142.3 s	141.2 s	141.4 s	143.8 s
C-9	77.5 s	77.3 s	77.4 s	76.5 s
C-10	44.6 s	44.1 s	44.4 s	49.7 s
C-11	73.2 d	73.3 d	74.3 d	73.2 d
C-12	47.4 t	47.3 t	42.8 t	46.5 t
C-13	43.2 s	43.0 s	43.6 s	43.2 s
C-14	51.7 d	51.2 d	51.7 d	51.2 d
C-15	24.2 t	23.6 t	24.1 t	23.4 t
C-16	28.9 t	30.1 t	28.9 t	28.2 t
C-17	57.6 d	56.6 d	57.4 d	56.3 d
C-18	13.0 q	12.9 q	12.7 q	13.0 q
C-19	22.2 q	22.6 q	21.9 q	65.1 t
C-20	37.3 d	36.5 d	37.4 d	36.5 d
C-21	19.4 q	19.1 q	19.4 q	18.9 q
C-22	38.5 t	38.9 t	38.7 t	36.3 t
C-23	25.0 t	24.3 t	25.0 t	24.1 t
C-24	39.3 t	39.9 t	39.3 t	39.7 t
C-25	29.2 d	28.3 d	29.2 d	28.3 d
C-26	23.2 q[c]	23.0 q[c]	23.3 q[c]	22.6 q[c]
C-27	23.0 q[c]	22.8 q[c]	23.1 q[c]	22.9 q[c]
MeCO			172.0	
*Me*CO			21.8	

[a]^{13}C-nmr spectra were recorded at 62.9 MHz. The chemical shift values are given in ppm and referenced to pyridine-d_5 (149.9 ppm) or CD_3OD (49.0 ppm).

[b]Data in this column are from West and Cardellina (3).

[c]Signals could be interchanged.

signal at 22.2 ppm in the ^{13}C-nmr spectrum of **1** which could be assigned to the C-19 methyl group. Similarly, the main differences in the ^{1}H-nmr spectra of these two sterols (Table 2) were in the signals for Me-18 and the lack of the Me-19 signal in the spectrum of **3**. Also the signal for H-11 in **3** is more strongly deshielded than in **1**.

The similarity of the chemical shifts and coupling constants of compounds **1** and **3**, especially those for C-1, C-2, C-3, C-4, C-12 (Table 1), and the protons connected to them (Table 2), and the substantial differences in the ^{13}C- and ^{1}H-nmr chemical shifts and coupling constants of **1** with those reported for the stereoisomeric 5β-cholest-7-ene-2β,3α,5β,6α,9α,11α,19-heptaol (4) proved both structure and stereochemistry of **1** and especially the trans-junction of rings A and B. Further support of the structure and the stereochemistry of the fragment including C-3, C-4, C-6, C-7, C-14 is the very similar pyridine-induced shifts of the protons connected to the above carbons observed for the studied compounds and those published in 1990

TABLE 2. ^{1}H-nmr Chemical Shifts Data Comparisons.[a]

Proton	Compound			
	1		2[b]	3[c]
	C_5D_5N	CD_3OD	CD_3OD	C_5D_5N
H-1α	3.16 t (13.2)	2.10 t (13.6)	2.16 t (13.0)	3.15 m
H-1β	3.34 dd (13.0, 4.4)	2.36 dd (12.6, 5.3)	1.7	3.50 dd (13.4, 4.5)
H-2	4.58 m	3.60 m	3.57 m	4.58 m
H-3	4.79 m	3.77 m	3.75 m	4.84 m
H-4α	2.58 dd (13.8, 4.9)	1.70 dd (14.0, 5.3)	1.7	2.57 dd (13.8, 5, 4)
H-4β	3.09 t (13.2)	2.16 t (13.0)	2.21 t (12.3)	3.15 m
H-6	4.58 m	3.66 m	3.68 m (5.4, 2.2)	4.45 brs
H-7	5.89 brd (4.5)	5.41 dd (5.3, 2.0)	5.44 dd (5.5, 2.1)	5.89 dd (5.7, 1.9)
H-11	4.58 m	4.07 (11.7, 4.2)	5.31 (11.5, 4.9)	5.07 dd (11.5, 4, 7)
H-12α	2.09 t (13.0)	1.6	1.7	2.14 t (11.7)
H-12β	2.37 dd (12.7, 4.6)	2.05 dd (13.0, 4.7)	2.04 dd (12.0, 5.0)	2.40 dd (11.9, 4.9)
H-14	2.81 m	2.49 m	2.58 m	2.99 m
H-15			1.7	1.60
H-16			1.6	1.78
H-17	1.36 dd (9.3, 2.5)	1.19 dd (5.2, 1.5)	1.3	
H-18	0.65 s	0.66 s	0.72 s	0.91 s
H-19	1.80 s	1.20 s	1.13 s	4.72 m
H-20			1.4	
H-21	0.91 d (4.4)	0.98 d (5.9)	0.92 d (5.4)	0.92 s
H-22			1.7	
H-23			1.3	
H-24			1.7	
H-25			1.3	1.47 m
H-26, -27	0.84 d (6.5)	0.88 dd (6.3, 1.0)	0.88 dd (6.6, 0.6)	0.84 d
*Me*CO			2.09 s	

[a]^{1}H-nmr spectra were recorded at 250.1 MHz. The chemical shift values are given in ppm and referenced to TMS. The coupling constants are given in Hz.

[b]The chemical shifts of protons assigned by HETCOR spectra are given with one significant digit after the decimal point, because of the poorer digital resolution of the 2D spectra and the overlapping of the signals in the region 0.5–2 ppm.

[c]Data in this column are from West and Cardellina (3).

by Migliuolo *et al.* (6) for 5α-cholest-7-ene-3β,5,6β,9-tetraol.

On the basis of these results, structure **1** is proposed for the isolated sterol. The stereochemistry of 11-OH was elucidated by nOe experiments. Irradiation of the H-18 signal (0.66 ppm) produced a 5% enhancement of the H-11 signal (4.07 ppm). When the latter signal was irradiated, an enhancement of the Me-18 (8%) and Me-19 (7%) signals was achieved.

Compound **2** has a composition of $C_{29}H_{48}O_7$ according to the highest ion peak in its eims at *m/z* 490 $[M-H_2O]^+$ and its ^{13}C-nmr spectral data (Table 1). The elimination of ketene (*m/z* 448) and HOAc (*m/z* 430) from the above-men-

tioned ion is an indication for the presence of an acetoxy group. These data and comparison of the molecular formula of **2** with that of **1** suggested that the investigated sterol was the monoacetate of **1**. Its eims (elimination of H_2O, HOAc, methyl group, and cholesterol side chain) and cims (elimination of HOAc and H_2O) are in agreement with the proposed structure. The extra signals at 172.0 and 21.8 ppm in the ^{13}C-nmr spectrum of **2** and at 2.1 ppm in its ^{1}H-nmr spectrum compared to the spectra of **1** indicated the presence of an acetate group. The shift of the signal for H-11 at 4.07 ppm (dd) in the spectrum of **1** to 5.31 ppm for **2** indicated that it is a signal of proton geminal to the acetate group. The ^{13}C- and ^{1}H-nmr spectra of the investigated sterol are similar to those reported (3) for the C-11 acetyl derivative of sterol **3**.

Final confirmation of the proposed structure of **2** was obtained by alkaline hydrolysis of **2**, which yielded a product identical to sterol **1** (tlc, ^{1}H-nmr spectrum).

Recent investigations on *D. fragilis* sterols (Ts. Milkova *et al.*, unpublished results) have shown that the main sterols in this sponge are cholesterol and 7-dehydrocholesterol. The isolated polyhydroxy sterols **1** and **2** contain C_{27}-skeletons and could be biosynthesized by biological oxidation of cholesterol. In order to determine whether this assumption is correct we incubated *D. fragilis* with 0.1 mCi [4-^{14}C]cholesterol for 48 h. The organisms were kept alive in an aquarium during the incubation period. A mixture was isolated by preparative tlc. Mass spectral data as well as later hplc experiments showed that it contained only **1** and **2**. The activity of the sterol mixture was 317 dpm/mg. Other biosynthetic experiments with sponges showed higher activities, but for longer incubations; it was not possible to keep sponges alive for longer periods.

These data showed that in *D. fragilis* dietary cholesterol suffered biological oxidation, which produced polyhydroxylated sterol **1**. Recently we found that in the same sponge $\Delta^{5,7}$-sterols were produced from the dietary Δ^5-sterols (Ts. Milkova *et al.*, unpublished results). Further biological oxidation of the C-5 double bond can produce the hydroxyl groups at C-5 and C-6, characteristic for the isolated polyhydroxylated sterols, while the C-7 double bond remains untouched.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were recorded on a Bruker WM 250 spectrometer. NOe and HETCOR 2D experiments were performed using standard Bruker microprograms. The ^{13}C- and ^{1}H-nmr chemical shifts and coupling constants of the investigated compounds are given in Tables 1 and 2.

Mass spectra were obtained with a JEOL D-300 double focusing mass spectrometer with a resolution of 2000.

EXTRACTION AND ISOLATION OF POLYHYDROXYLATED STEROLS.—*D. fragilis* was collected in August 1988 near Varna (depth 5–15 m). A voucher specimen is deposited in the Museum of Natural History, Sofia. The animals were immediately dipped in MeOH and transported to the laboratory. The fresh sponges (307 g dry wt after extraction) were extracted twice with MeOH and then with $CHCl_3$. The combined extracts were concentrated, diluted with H_2O, and extracted three times with $CHCl_3$. After evaporation of the extracts, an oily residue (19.6 g) was obtained. It was subjected to cc on Si gel (500 g) and by elution with increasing concentrations of Me_2CO in $CHCl_3$ a mixture of polyhydroxylated sterols **1** and **2** was isolated (79 mg). This mixture was subjected to reversed-phase hplc on an ODS-2 column (250 × 10 mm). The mobile phase was MeOH-H_2O (80:20). Pure compounds **1** (11 mg) and **2** (15 mg) were isolated as glasses.

Sterol **1**.—Eims (70 eV) *m/z* (rel. int.) 448 (4), 430 (9), 412 (15), 397 (10), 394 (10), 299 (2), 281 (8), 55 (100); hrms *m/z* 430.3071 ($C_{27}H_{42}O_4$ requires 430.3083); $[\alpha]^{25}D$ −20.4° (c = 0.3, MeOH).

Sterol **2**.—Eims (70 eV) *m/z* (rel. int.) 490 (2), 448 (10), 430 (22), 412 (10), 394 (4), 317 (11), 299 (16), 281 (6), 43 (100); cims (400 eV) *m/z* (rel. int.) 491 (55), 431 (100), 413 (90), 395 (92), 377 (12).

HYDROLYSIS OF **2**.—A solution of **2** (7 mg) in 10% KOH/MeOH was refluxed and extracted with CH_2Cl_2. After preparative tlc on Si gel with

hexane-Me_2CO (1:4), pure **1** (4 mg) was isolated, identical with authentic material (tlc, ^1H nmr).

BIOSYNTHETIC EXPERIMENTS.—A sample of *D. fragilis* (9.15 g dry wt after extraction) was dried partially with filter paper, and an H_2O suspension of 0.1 mCi [4-^{14}C]cholesterol with Tween 40 was dropped on the sponge. After 48 h incubation in an aquarium, a mixture of sterols **1** and **2** was isolated by preparative tlc [hexane-Me_2CO (1:4)], and activity of 317 dpm/mg was found for this mixture.

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