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Chemical Investigation of the Sponge *Verongia aerophoba*

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The solvent effect on *Verongia aerophoba* metabolites during the extraction and isolation processes were examined in this study. A major compound **(6)**, a stereoisomer of fistularin-3, and a new compound **(2)** were isolated from the acetone extract of dried and ethanol extract of fresh *V. aerophoba* samples respectively. The structures of these metabolites were elucidated using spectroscopic methods.

Introduction

The sponge family *Verongidae* has been studied extensively. Most of the metabolites obtained from this family are 3,5-dibromotyrosine derivatives having biological activities. Within the framework of chemical research carried out on the marine organisms collected from the waters of Turkish seas, samples of the very widespread sponge *Verongia aerophoba* Schmidt (syn. *Aplysina aerophoba*) have been carefully examined. In the first study, sponge samples collected from Gökçeada (Aegean Sea) were preserved in ethanol for approximately four months and extracted with the same solvent. From the extract the known metabolite dienone **(1)** [1] and a new metabolite, dienonediethoxy ketal **(2)**, were isolated as major and minor compounds respectively. Although dienone was found in all *Verongia* species, and its dimethoxy ketal **(3)** [2] as well as its mixed ketals (ethylmethyl ketal) **(4)** [3] were obtained from some *Verongia* species, its diethoxy ketal had not yet been obtained. Following studies with the *Verongia obtusa* samples collected from Gökçeada and Çanakkale, we have also isolated **(3)** and **(4)** from MeOH and EtOH extracts respectively from the fresh sponges. However, a known oxazolidone derivative **(5)** [4,5] was found in all the extracts. All these findings prompted us to examine systematically whether compounds **(2-4)** are natural compounds or artefacts formed with a solvent effect during the extraction or isolation processes. In this paper, we report the results of these examinations and the characteristics of compound **(2)**, which has not been given before. We also report the isolation and structure of the compound **(6)**, a new stereoisomer of fistularin-3, from acetone extract of dried sponges.

Experimental

General producedure

Melting points were determined on a melting point microscope (Reichert) and were uncorrected. Optical rotations were measured with a Warning polarimeter. The IR spectra (KBr) were recorded on a Perkin Elmer

577. Mass spectra were taken on AEI MS 30 and Kratos MS 50 apparatus (reagent gas for CI-MS: NH_3). ^1H and ^{13}C NMR spectra were recorded on 200 and 360 MHz Bruker apparatus in $CDCI_3$, using TMS as the internal standard. The following silica gels were used: Silica gel 60 (Merck) for column chromatography, Silica gel GF_{254} (Merck) for analytical (0.25 mm) and preparative (0.5 mm) TLC. The solvent systems for TLC were: $CHCl_3$/MeOH(10:1) and EtOAc.

Extraction and Isolation

For the first examination, *Verongia* samples were collected from Gökçeada (Aegean Sea) and preserved in EtOH for approximately four months. The ether soluble part of this extract was chromatographed on a silica gel column, eluting with $CHCl_3$, and increasing amounts of CH_3OH was added. From $CHCl_3$ / MeOH (15:1) fractions, a large amount of dienone **(1)** was obtained. The mother liquid of the dienone was allowed to crystallize again and besides dienone crystals, compound **(2)** crystalized in a different form and was separated with pincers. For further studies, sponge material was again collected from Güzelyalı (Çanakkale). The fresh sponge samples were immediately cut into small pieces and put into plastic containers filled with methanol, ethanol and acetone (200, 275, 165 g wet sponge wt. respectively) and macerated four times with these solvents. The sun-dried sample (200 g) was cut into small pieces and extracted four times with acetone at room temperature. Each extract was evaporated *in vacuo* and partitioned between ether and H_2O. The ether layer was concentrated *in vacuo* and chromatographed on a silica gel column (35-70 mesh: 50 × 3.5 cm) eluting with $CHCl_3$ and increasing amounts of CH_3OH. From fractions 5-10 ($CHCl_3$/CH_3OH, 10:1), 16-21 (5:1) and 23 (5:1) of the MeOH extract (5 g), dienonedimethoxy ketal **(3)** (1.430 g), compound **(5)** (320 mg) and dienone **(1)** (74 mg) were obtained. From fractions 21 ($CHCl_3$/CH_3OH, 10:1), 22-24 (5:1) and 26 (3:1) of the EtOH extract (7.4 g), compound **(5)** (70 mg), mixed ketal **(4)** (88 mg) and dienone **(1)** (60 mg) were obtained. The acetone extract of the fresh sponge (10 g) yielded compound **(5)** and dienone **(1)** (fractions 19 and 23 respectively, $CHCl_3$/CH_3OH,5:1). From the dried sponge extracts (3.9 g), compound **(6)** (540 mg from fraction 15, $CHCl_3$/CH_3OH, 10:1) and compound **(5)** (30 mg fraction 22, $CHCl_3$/CH_3OH, 5:1) were isolated. Purification was generally achieved by repeating prep. TLC. Analytically pure samples of **(4)** and **(6)** were obtained by reversed-phase hplc (50 μm, semi-prep. column 15×19 mm, Waters absorbosphere C-18) with 80% MeOH/H_2O.

Dienone (1)

Yellow powder, m.p. 200 °C.

IR ν_{max} (KBr) 3400, 3130, 1690, 1655, 1420, 1365, 1180, 925, 815 cm^{-1}; the compound was compared on TLC with an authentic sample and they were found identical.

Dienonediethoxy ketal (2)

Colorless crystals, m.p. 183-187 °C.

IR ν_{max} (KBr) 3430, 1660, 1652, 1425, 1170 cm^{-1}.

^1H NMR (see Table 1). EI-MS m/z 380, 382, 384 $[M\text{-}17]^+$, 352, 354, 356 $[M\text{-}45]^+$, 293, 295, 297 $[M\text{-}104]^+$, 265, 267, 269 $[M\text{-}132]^+$ base peak 53.

Dienonediethoxy ketal (3)

Colorless crystals, m.p. 187-189 °C.

IR ν_{max} (KBr) 3395, 3230, 2925, 1665, 1610, 1120, 965, 810 cm^{-1}.

^1H NMR (see Table 1). EI-MS m/z (rel. int.) 338, 340, 342 (31.6;58.8;31.3) $[M\text{-}CH_3O]^+$, 320, 322, 324 (15.1;31.6;18.4) $[M\text{-}CH_3O\text{-}H_2O]^+$311, 313, 315 (6.6;9.2;4.4) $[M\text{-}CH_2CONH_2]^+$, 211 (65.4) $[M\text{-}Br\text{-}HBr]^+$.

Mixed ketal (Dienoneethylmethyl ketal (4)

Colorless crystals, m.p. 183-184 °C.

IR ν_{max} (KBr) 3360, 3290, 2925, 1740, 1700, 1540, 1460, 1235, 1080, 960 cm^{-1}.

^1H NMR (see Table 1). CI-MS m/z (rel. int.) 383, 385, 387 (7.4;13.6;7.4) $[M+1]^+$, 352, 354, 356 (89.3;100;88.2) $[M\text{-}CH_3O]^+$, 338, 340, 342 (84.2;92.6;88.2) $[M\text{-}CH_3CH_2O]^+$, 279, 281, 283 (13.9;21.3;11.0) $[M\text{-}CH_3CH_2OH\text{-}CH_2CONH_2]^+$, 264, 266, 268 (36.7;61.4;38.2) $[M\text{-}CH_3CH_2O\text{-}CH_2CONH_2\text{-}OH]^+$, 246, 248 (79.0;77.2) $[M\text{-}58\text{-}Br]^+$, 215, 217 (54.0;52.9)

Compound (5)

Colorless crystals, m.p. 214-215 °C.

IR ν_{max} (KBr) 3255, 2920, 1765, 1730, 1630, 1550, 1465, 1340, 1230, 1080 cm^{-1}; the compound was compared on TLC with an authentic sample and they were found identical.

Compound (6)

Pale yellow powder, m.p. 128 °C.

IR ν_{max} (KBr) 3380, 2930, 2860, 1660, 1600, 1530, 1455, 1255 cm^{-1}.

^1H NMR (see Table 2). Cl-MS m/z 425, 427, 429, 431 (5.9;15.1;15.8;7.4), 365, 367, 369 (38.2;64.7;33.8), 348, 350, 352 (54.4;100;50.7), 332, 334, 336 (16.9;34.5;16.2), 263, 265, 267 (11.8;15.4;14.7), 225, 227 (33.8;33.2).

$[\alpha]_D 25$=+ 51.60 (c=0.58, MeOH).

Preparation of 6 acetate Compound **(6)** (50 mg) was treated with Ac_2O (10 ml) and pyridine (0.2 ml) and left overnight at room temperature. Water was added, and the resulting mixture was evaporated in vacuo. The product was purified on silica gel using $CHCl_3$/MeOH (10:1) to give **6a** (14 mg) in the form of pale yellow powder, m.p. 181-184 °C.

IR ν_{max} (KBr) 3334, 3066, 2938, 2841, 2366, 1743, 1665, 1540, 1437, 1223, 1089, 988 cm^{-1}.

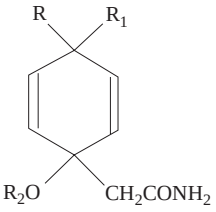
^1H NMR (see Table 2). Cl-MS m/z 536, 538, 540 (14.7;25.7;13.2), 475, 477, 479 (5.2;11.4;5.9), 395, 397, 399 (6.6;13.9;8.5), 379, 381, 383 (13.2;22.0;11.8), 303, 305, 307 (53.3;100;52.2), 277, 279, 281 (11.0;17.6;11.0).

$[\alpha]_D 25$=+ 100 ° (c=0.3, $CHCl_3$).

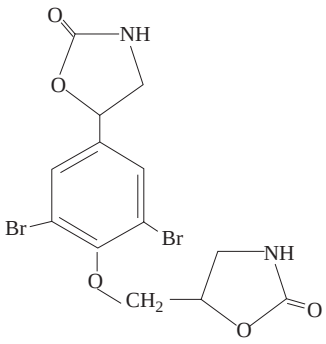
Results and Discussion

To examine the above-mentioned subject, *V. aerophoba* was collected several times in the same month of the year from the same site. A part of the fresh sponge was cut into small pieces and divided into four parts. Three of these were preserved immediately in methanol, ethanol and acetone and extracted with these solvents (f-Me, f-Et, f-Ac respectively). The fourth part of the sponge was dried in hot sunshine in a short time and three solvent extracts of the dried sponge with the same solvents were prepared (d-Me, d-Et, d-Ac). All these extracts were examined by TLC in order to observe solvent effects. TLC examination of the ether soluble parts of all the extracts showed that **(1)** and **(5)** were present in all the extracts as major compounds, whereas the dried sponge extracts contained **(1)** in very small amounts. Ketals of dienone were present only in the methanol and ethanol extracts of the fresh sponge, namely **(3)** in f-Me and **(4)** in f-Et. Later, compound **(2)** could not be detected in f-Et. The fresh sponge extracts contained a major spot at the same Rf value on TLC plates. During attempts to isolate this compound by prep. TLC we observed that it decomposed on the chromatography plate. This was demonstrated clearly by a two-dimensional TLC experiment; during the second development of the plate, some other spots were produced in addition to this major spot, which was the dienone. After these results two fresh extracts and one dried extract (f-Me, f-Et, d-Ac) were worked up by a preparative scale using column chromatography. The compound **(5)** from all extracts, dienone and its dimethoxy ketal from f-Me, and dienone and its mixed ketal from f-Et were

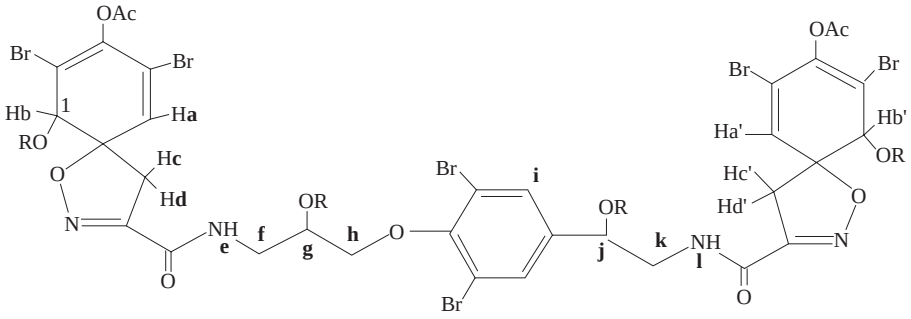
isolated in pure state as major compounds. The d-Ac extract gave **(5)** as minor and compound **(6)** as major compounds. In view of these findings, we suggest that compounds **(1-4)** are artefacts and formed during the extraction processes. The reason why we did not isolate diethoxy ketal again from f-Et is that we probably did not keep the sponge in ethanol for long enough. The known compounds **(1,3-5)** were identified from their spectroscopic data and by comparison with data in the literature.



1 R= R_1= O, R_2= OH
2 R= R_1= OCH_2CH_3, R_2= OH
3 R= R_1= OCH_3, R_2= OH
3a R= R_1= OCH_3, R_2= OAc
4 R=R_1=OCH_3,=R1=OCH_2CH_3, R_2= OH
4a R=OCH_3, R1=OCH_2CH_3, R_2= OAc



5



6 R=H
6a R=$COCH_3$

Scheme 1.

The IR spectrum of compound **(6)** showed the presence of hydroxyl and amide (3380, 2930, 1660, 1530 cm^{-1}) groups. Acetylation of **(6)** yielded a tetraacetate **(6a)**, confirming the presence of four hydroxyl groups. Although **(6)** failed to exhibit a molecular ion either in EI-MS or CI-MS, in CI-MS the ions at m/z 365, 367, 369 ($C_{10}H_9Br_2NO_4$) and at 348, 350, 352 ($C_{11}H_{10}Br_2O_3$) corresponded to spirocyclohexadienyisoxazole and substituted aromatic moities respectively. The ^{1}H-NMR spectrum of **(6a)** (Table 2) revealed signals [δ 3.07, 3.08 and 3.42, 3.47 (each 1H, d, J=18.4 Hz, H_c, H_d and Hc′, Hd′); 5.86 and 5.82 (each 1H, s, H_b, $H_{b'}$); 6.31 (2H, s, H_a, $H_{a'}$)] corresponding to two dibromospirocyclohexadienyl-isoxazole ring systems [6, 8]. In addition, the ^{1}H-NMR spectrum of **(6)** contained signals arising from two methoxyl groups, [δ 3.77 and 3.78 (each 3H, s)], and a signal at δ 7.50 (2H, s) of an isolated aryl signal that indicated a symmetrically tetrasubstituted aromatic ring. Therefore, compound **(6)** has the molecular formula $C_{31}H_{30}Br_6N_4O_{11}$. A review of the literature revealed that the ^{1}H-NMR spectrum of **(6a)** was very similar to those of fistularin-3 tetraacetate and isofistularin-3 tetraacetate [6, 7]. Comparison of ^{1}H-NMR spectra of **(6a)** and the mentioned tetraacetates revealed small but significant differences; protons H_b

and $H_{b'}$ showed two distinct signals in the spectra of **(6a)** and isofistularin-3 tetraacetate at δ 5.82 and 5.86, whereas the value for fistularin-3 tetraacetate was at δ 5.86 as a unique broad singlet (2H). The signals due to the methylene protons of the two isoxazole rings were shown at δ 3.07, 3.08, 3.42 and 3.47 in **(6a)** whereas the values for isofistularin-3 tetraacetate were at δ 3.09 (2H), 3.41 and 3.46 and for fistularin-3 tetraacetate at δ 3.06, 3.08, 3.45 and 3.47. Compound **(6)** showed an optical rotation of + 51.6° compared to + 102° [9] and +104.2° [6] for fistularin-3 and +108° for isofistularin-3 [7]. All these spectral data and a direct comparison of the ^{1}H-NMR spectra of **(6a)** and fistularin-3 tetraacetate suggest that **(6a)** is a stereoisomer of fistularin-3 tetraacetate, and therefore **6** is 1-*epi*-fistularin-3.

Table 1. ^{1}H-(200 MHz, in $CDCl_3$) NMR Spectral Data of **2, 3** and **4**

position	δH(j,Hz)		
	2	**3**	**4**
H_3,H_5	6.98 bs,	7.26 s	7.02 s
	7.35 bs,		
CO$\underline{NH_2}$	6.72 s	6.77 s	6.03 bs
OH	5.95 s	5.23 s	5.02 bs
OCH_3		3.22 s	3.16 s
OCH_3		3.17 s	
$\underline{CH_2}CH_3$	2.98-3.31 m		3.40 q, 6.9
$\underline{CH2}CONH_2$	2.38 s	2.52 s	2.79 s
$CH_2\underline{CH_3}$	1.12 t, 7		1.28 t, 7

Table 2. ^{1}H-(360 MHz, in $CDCl_3$) NMR Spectral Data of **6** and **6a**

position	δH(j,Hz)	
	6	**6a**
$H_{a'}$	6.27 s	
H_a	6.28 s	6.31 s
H_b	4.05 s	5.86 s
$H_{b'}$	4.07 s	5.82 s
H_c,H_d	2.98, 3.01 d, 18.1	3.08, 3.07 d, 18.4
	3.82 - 3.85 m	3.42, 3.47 d, 18.4
H_e	7.58 m	7.08 t, 8.1
H_f	3.28-3.35 m	3.96 m
	3.47-3.52 m	3.80 m
H_g	4.25 t, 7.2	5.27 quint., 4
H_h	3.94 dd, 8.6;17.3	4.14 dd, 8.3; 4.2
		4.21 dd, 8.3; 4.2
H_i	7.55 s	7.50 s
H_j	4.76 bd, 10.1	5.73 dd, 8.4;3.7
H_k	3.28-3.35 m	3.53 m
	3.47-3.52 m	3.75 m
H_I	7.43 t, 5.8	6.82 t, 8.1
OCH_3	3.74, 3.76 s	3.77, 3.78 s
OAc		2.16, 2.17 s

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