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PHAEOPHYTINS FROM A CELL SUSPENSION CULTURE OF THE LIVERWORT *PLAGIOCHILA OVALIFOLIA*

AKIHIKO MATSUO, KANJI ONO,* KIYOSHI HAMASAKI† and HIROSHI NOZAKI†

Division of Environmental Biochemistry, Faculty of Social Information Science, Kure University, Gohara-cho, Kure-city, Hiroshima 724-07, Japan; *Department of Biological Science, Faculty of Science, Kumamoto University, Kumamoto 860, Japan; †Department of Biological Chemistry, Faculty of Science, Okayama University of Science, Ridai-cho, Okayama 700, Japan

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Key Word Index—*Plagiochila ovalifolia*; Bryophytes; Plagiochilaceae; livewort; cell suspension culture; phaeophytins; structural determination.

Abstract—A suspension culture of the liverwort *Plagiochila ovalifolia* was established from callus tissue induced by culturing spores. From the cultured cells, four phaeophytins were isolated as major components and their structures determined by spectroscopic methods. The phaeophytin derivatives showed antibacterial activity. A major sesquiterpenoid, ovalifoliene, found in the mother plant, was detected in the cultures by GC–mass spectrometry.

INTRODUCTION

Liverworts and mosses are phylogenetically placed between the vascular plants and the algae. The bryophytes are believed to belong to a very ancient group that evolved along an evolutionary bypath, and their plant bodies (gametophytes) develop from spores which correspond to the pollens of higher plants.

In spite of the phytochemical and biochemical interests in the chemical constituents of the bryophytes few investigations have been performed on liverworts. It has been shown that they usually elaborate sesquiterpenoids and diterpenoids as well as esters of fatty acids and aromatic acid as their major lipophilic constituents [1–4]. In our previous papers, we isolated from the liverwort *Plagiochila ovalifolia* Mitt. a series of plant-growth-inhibitory active sesquiterpenoids having a 2,3-*seco*-alloaromadendrane framework [5, 6]. Recently, cell culture techniques have been applied to the study of the metabolites of liverworts [e.g. 7, 8]. Here, we describe the investigation of the chemical constituents from a cell suspension culture of the liverwort *P. ovalifolia*.

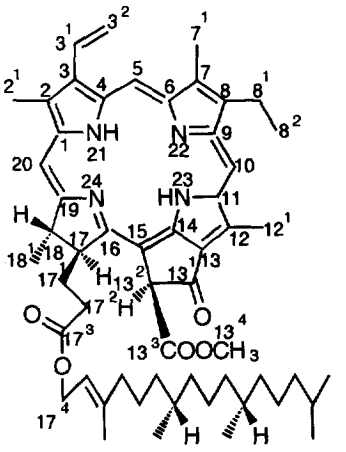
RESULTS AND DISCUSSION

Suspension cells maintained in MSG4 medium at 25° and 3000 lux were harvested at 10–14 day intervals (see Experimental) [9]. The harvested cells were dried, and extracted with methanol, and the extract partitioned against ether. The ether extract, obtained in 4.2% yield, was submitted to a combination of column chromatography and TLC over silica gel to give four phaeophytins (**1**–**4**) and phytol.

The first compound, phaeophytin a (**1**), $C_{55}H_{74}N_4O_5$, mp 109–110°, had a porphyrin system (λ_{max} 670.0, 612.4, 538.4, 507.2 and 413.6 nm) containing an ester group (IR: 1736, 1702, 1620 and 1465 cm^{-1}). Analysis of the ^1H–^{13}C COSY spectrum suggested its relationship to chlorophyll. Furthermore, the ^1H and ^{13}C NMR spectroscopic properties established the presence of 11 methyl groups (Tables 1 and 2). Of these signals four methyls were assigned as three vinyl methyls on the chlorin ring and a methoxycarbonyl methyl. Methanolysis of compound **1** with sodium methoxide in methanol afforded chlorophyllide *a* methyl ester and phytol (Scheme 1), identical with those of the known compounds.

The UV, IR and NMR spectra of the second (**2**) and third (**3**) compounds suggested that they were also phaeophytin derivatives with a hydroxyl group on C-13^2 (hydroxyl proton signal lost on addition of D_2O). These phaeophytin derivatives were finally identified with the known **1**, 13^2-hydroxy-(13^2-*S*)-phaeophytin *a* (**2**) and 13^2-hydroxy-(13^2-*R*)-phaeophytin *a* (**3**), all of which were isolated from silkworm excreta [10].

The fourth new compound (**4**), $C_{56}H_{77}N_4O_7$, also gave the characteristic spectra of a porphyrin skeleton (see Experimental). The mass spectrum afforded a $[M]^+$ ion containing to one more CH_2O_2 unit than **1**. The NMR spectra showed the other methoxymethyl signal, but no more carbonyl carbons, compared with



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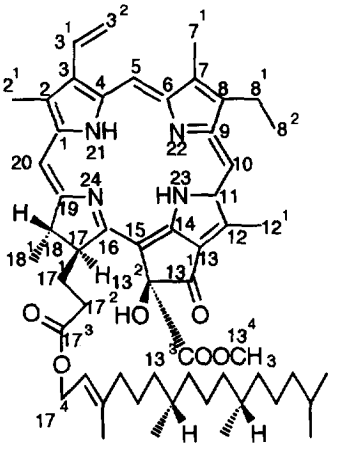

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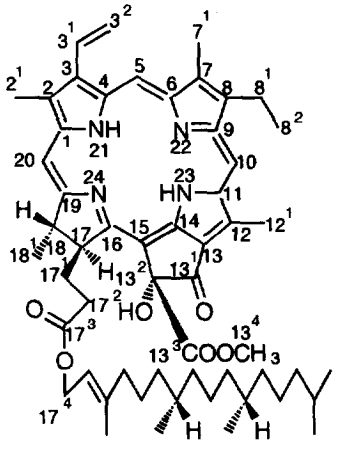

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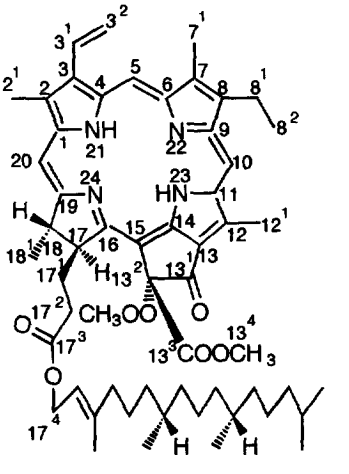

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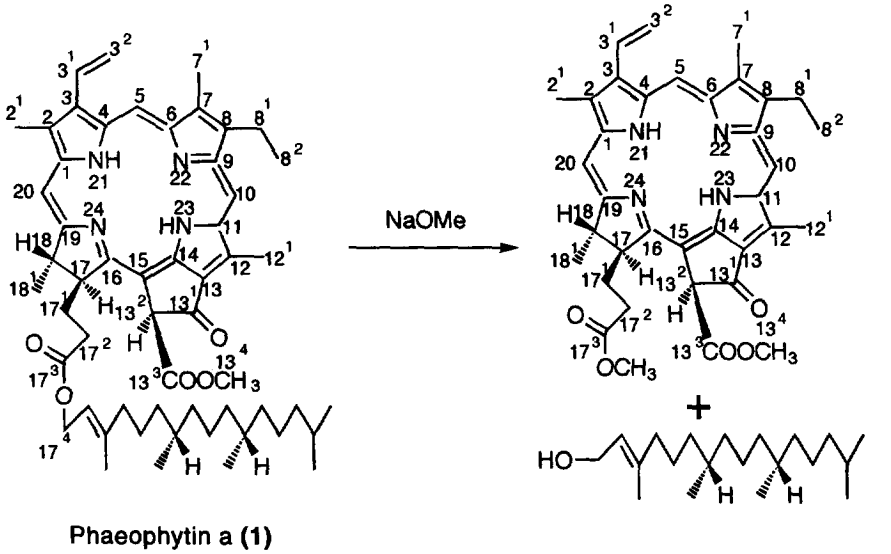

Phaeophytin a (1)

Scheme 1. Methanolysis of compound **1**.

Table 1. ^{1}H NMR chemical shifts of the pheophytins **1**–**4** (excluding phytyl moiety)

		1	2	3	4
2^1	*s*	3.39	3.43	3.42	3.32
3^1	*dd*	7.98	8.01	8.01	7.88
3^2 (*E*)	*dd*	6.28	6.30	6.30	6.27
3^2 (*Z*)	*dd*	6.17	6.20	6.20	6.15
5	*s*	9.36	9.47	9.46	9.28
7	*s*	3.21	3.25	3.25	3.15
8^1	*q*	3.66	3.70	3.72	3.64
8^2	*t*	1.68	1.70	1.70	1.66
10	*s*	9.50	9.62	9.61	9.58
12^1	*s*	3.88	3.74	3.72	3.87
13^2-H	*s*	6.26			
13^2-OH	*s*		5.53	5.35	
13^2-OOMe	*s*				4.14
13^4-OMe	*s*	3.68	3.62	3.64	3.60
17	*ddd*	4.21	4.17	4.48	4.31
18	*dq*	4.46	4.50	4.69	4.70
18^1	*d*	1.80	1.61	1.68	1.79
20	*s*	8.55	8.65	8.63	8.48

Table 2. ^{13}C NMR chemical shifts of the phaephytins **1**–**4** (excluding phytyl moiety)

C		1	2	3	4
1	*s*	142.9	142.8	142.9	142.5
2	*s*	131.8	131.8	131.9	131.4
2^1	*q*	12.1	12.1	12.1	12.0
3	*s*	136.5	136.6	136.5	136.6
3^1	*d*	129.0	129.1	129.1	129.0
3^2	*t*	122.8	122.9	122.9	122.6
4	*s*	136.2	136.3	136.4	136.0
5	*d*	97.5	98.0	97.9	97.0
6	*s*	155.5	155.4	155.6	164.4
7	*s*	136.1	136.2	136.3	136.0
7^1	*q*	11.2	11.3	11.3	11.0
8	*s*	145.2	145.8	145.3	143.0
8^1	*t*	19.7	19.5	19.5	19.7
8^2	*q*	16.3	17.4	17.5	17.5
9	*s*	151.0	151.1	151.0	156.4
10	*d*	104.4	104.3	104.2	100.7
11	*s*	137.9	137.8	137.8	138.9
12	*s*	129.1	129.4	129.3	129.6
12^1	*q*	12.2	12.3	12.2	12.9
13	*s*	129.0	127.0	126.3	129.0
13^1	*s*	189.6	192.0	192.0	186.4
13^2	*d*	64.7	89.0	89.1	119.3
13^2 (O_2Me)					53.5
13^3	*s*	173.0	172.8	173.0	173.5
13^4 (OMe)	*q*	53.0	53.4	53.8	53.2
14	*s*	150.0	149.8	150.2	146.0
15	*s*	105.2	107.7	107.6	106.3
16	*s*	161.3	162.5	161.9	166.8
17	*d*	51.1	51.8	50.8	52.6
17^1	*t*	29.8	31.1	31.2	31.1
17^2	*t*	31.2	31.6	31.9	31.8
17^3	*s*	173.0	173.6	173.5	174.0
18	*d*	50.1	50.3	50.2	49.6
18^1	*q*	22.7	22.7	22.7	22.7
19	*s*	172.2	172.4	172.8	168.2
20	*d*	93.1	93.6	93.4	93.7

the former known compounds. Therefore, it was deduced to have structure **4** containing the methyl ether of hydroperoxide on the C-13^2. The α-configuration was deduced by the similarity of the ^{1}H NMR spectra with those of (13^2-*R*)-phaeophytin *a* (**3**). A major sesquiterpenoid, (+)-ovalifoliene, in the mother plant, as well as the diterpenoid, phytol, were detected by GC–mass spectrometry of the extract.

This is the first report of the isolation of phaeophytins **1**–**4** from a liverwort culture. It is known that the phaeophytins **2** and **3** have cytostatic activity [10]. These compounds show also antibacterial activity.

EXPERIMENTAL

General procedures. Mps: uncorr.; IR and $[\alpha]_D$: $CHCl_3$ at room temp.; ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz): $CDCl_3$ with TMS as int. standard; FAB-MS: glycerol as matrix; UV: EtOH; CC: Merk kieselgel 60; TLC and prep. TLC: Merk kieselgel 60 PF_{254}; analyt. plates were visualized under UV radiation, I_2 vapour or spraying with 10% H_2SO_4 in EtOH followed by heating at 120°.

Suspension culture. Mature spores were removed from sporangia that had been surface-sterilized with 1% NaOCl soln for 10 min. Callus tissue was induced by culturing the spores on solid MS medium containing 2% glucose and 0.8% agar [9]. The suspension cells were maintained in MSG4 medium, which consisted of the major and minor salts and the vitamins of MS medium containing 4% glucose and no phytohormones. The suspension culture was routinely subcultured at 10–14 day intervals by pouring *ca* 2 ml cell pellet into 100 ml fresh medium in a 300-ml conical flask. The flasks were placed on a gyratory shaker [120 rpm, 25° and continuous light (300 lux)].

Extraction of cultured cells. The cells were collected by filtering the suspension culture maintained in the above MSG4 medium at 10–14 day intervals. The whole cells (60 g), after being dried in the shade for several days, were extracted twice with MeOH for 1 week at room temp. The solvent was removed under red. pres. and the extract (6.7 g) was then partitioned with Et_2O. Removal of the solvent under red. pres. gave a viscous oil (2.5 g).

Isolation of the constituents **1**–**4**. The Et_2O extract (4.2% yield) was first submitted to CC over silica gel using hexane containing increasing amounts of EtOAc as eluent to give 10 frs. Frs 7 and 8 were then applied to prep. TLC on silica gel to give the following compounds in order of elution: **1** (6.4 mg), **4** (3.4 mg), **2** (8.5 mg) and **3** (7.7 mg). The physical constants and spectroscopic properties of these compounds are listed below.

Phaeophytin a (**1**). $C_{55}H_{74}N_4O_5$; FAB-MS *m/z* (rel. int.): 871 $[M+1]^+$ (100), 593 (39), 533 (40), 459 (50); mp 109–110°; UV λ_{max} nm (log ε): 670.0 (0.46), 612.4 (0.08), 538.4 (0.10), 507.2 (0.11), 413.6 (1.03); IR ν_{max} cm^{-1}: 1736, 1702, 1620, 1465.

13^2 - *Hydroxy* - (13^2 - S) - *phaeophytin a* (**2**).

$C_{55}H_{74}N_4O_6$; FAB-MS *m/z* (rel. int): 887 $[M+1]^+$ (100), 607 (80), 549 (50), 503 (49), 461 (60); mp 110–112°; $[\alpha]_D$ −57.14° (*c*, 0.01); UV λ_{max} nm (log ε): 670.0 (1.58), 612.4 (0.83), 536.0 (0.87), 506.4 (0.99), 404.8 (1.64); IR ν_{max} cm^{-1}: 3028, 1741, 1713, 1620, 1466.

13^2 - *Hydroxy* - (13^2 - R) - *phaeophytin a* (**3**). $C_{55}H_{74}N_4O_6$; FAB-MS *m/z* (rel. int.): 887 $[M+1]^+$ (85), 607 (100), 549 (58), 503 (62), 461 (43); mp 108–110°; $[\alpha]_D$ −27.3° (*c*, 0.02); UV λ_{max} nm (log ε): 668.0 (1.66), 612.4 (1.06), 536.8 (1.08), 506.8 (1.17), 410.0 (1.68); IR ν_{max} cm^{-1}: 3031, 1740, 1710, 1617, 1455.

Phaeophytin a hydroperoxide (**4**). $C_{56}H_{77}N_4O_7$; HR-FAB-MS *m/z* (rel. int.) 917.5678 $[M]^+$ (95), 870 (35), 829 (45), 637 (50), 593 (28), 551 (100), 491 (73), 459 (45); UV λ_{max} nm (log ε): 684.0 (1.58), 547.2 (0.83), 505.6 (0.87), 409.2 (0.99); IR ν_{max} cm^{-1}: 2857, 1800, 1724, 1601, 1212.

Methanolysis of compound **1** *with MeONa*. An excess of MeONa, prepd freshly in MeOH (10 ml), was added to **1** (2 mg) in dry MeOH (1 ml), and the mixt. was stirred at room temp. for 4.5 hr. The reaction mixt. was poured into a satd soln of NH_4Cl and extracted with Et_2O. The products were analysed by TLC.

GC–MS analyses of Frs 1 *and* 5. GC–MS analyses were carried out with a single focusing mass spectrometer under the following conditions: CBP-1 (methylsilicone chemical bond-type) 12.5 m × 0.33 mm; 100–300° (16° min^{-1}) column temp.; EI ionization mode; 70 eV ionization energy; 3.0 kV ionization current; 200° ion source temp.

Test of antimicrobial activity. The antimicrobial activity of the phaeophytin derivatives, **1**, **2**, **3** and **4**, on the bacteria *Escherichia coli* and *Bacillus subtilis* was tested in the following manner. Discs (8 mm in diam.) of each of the bacteria were placed on a tryptone–yeast–glucose–agar medium with or without each of the test compounds, and cultured for 24 hr at 30°. The inhibitory activity was obtained by measuring the diameter of colonies.

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