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NEW CHLOROPHYLL-A-RELATED COMPOUNDS ISOLATED AS ANTIOXIDANTS FROM MARINE BIVALVES

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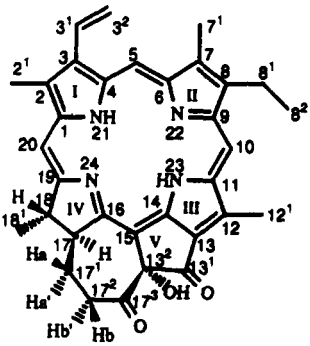
ABSTRACT.—New chlorophyll-a-related compounds chlorophyllone a [**1**], chlorophyllonelactone a [**2**], and chlorophyllonic acid a methyl ester [**3**] as well as pyropheophorbide a [**4**] were isolated as the main antioxidants from the edible parts of the short-necked clam, *Ruditapes philippinarum*. Our continuing studies on new antioxidants have resulted in the isolation of the analogous compounds purpurin 18 [**6**], purpurin 18 methyl ester [**7**], and 13^2-oxopyropheophorbide a [**8**]. Compound **5**, an epimeric isomer of **1**, together with **2**, **3**, and **4**, was isolated from the viscera of the scallop, *Patinopecten yessoensis*. The presence of **1**, **2**, and **3** in the viscera of the oyster *Crassostrea* sp. and **1** in the mixture of attached diatoms (*Fragilaria oceanica, Fragilaria cylindus, Nitzschia closterium, Nitzschia seriata, Cocconeis pseudomarginata*, and *Hyalodiscus stelliger* are predominant) cultured for seedling production of juvenile abalone was also confirmed by spectroscopic evidence.

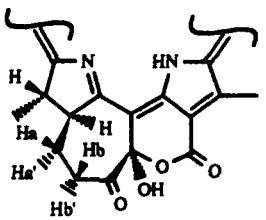
Organisms are well known to have defense mechanisms against oxidation, because peroxides of polyunsaturated fatty acids have shown many kinds of toxicity including mutagenicity (1,2). We focused our research interests on marine organisms containing a large amount of polyunsaturated fatty acids and measured peroxide value (POV) by the conventional antioxidant test (3) and mutagenicity by the *rec* assay (4) of their organic solvent extracts. Positive correlation was observed between POV and mutagenicity of their extracts. Among the extracts of viscera of various kinds of marine fish and bivalves, the extract of the short-necked clam, *Ruditapes philippinarum* Jay (Veneridae), showed a very low POV and mutagenicity. Hplc analysis revealed that this extract contained amounts of tocopherols too small to show the strong antioxidant activity. These observations encouraged us to survey new antioxidants responsible for this low POV of the extract.

We have already communicated the isolation and structure elucidation of pyropheophorbide a [**4**] and new pheophorbide a (PB a) derivatives **1** (5) and **3** (6) from *R. philippinarum* collected in Hamana Lake, Japan (7). Our continuing study revealed the presence of the other similar antioxidants **2**, **6**, **7**, and **8** (Figure 1). As compounds **1**, **2**, **3**, and **8** were found to be new PB-a-related compounds, we were interested in the origin of these compounds. We examined the viscera extracts of the scallop *Patinopecten yessoensis* Jay (Pectnidae) and the oyster *Crassostera* sp. (Limidae), which are also plankton feeders. From the extracts of the scallop collected in Hokkaido, compounds **2**, **3**, and **4** as well as a new member **5** of this type of compound were isolated. The extract of the oyster was found to contain compounds **1**, **2**, and **3**. As **1** was also isolated from the attached diatoms, which were microscopically found to be predominantly composed of *Fragilaria oceanica, Fragilaria cylindus, Nitzschia closterium, Nitzschia seriata, Cocconeis pseudomarginata*, and *Hyalodiscus stelliger*, it is particularly interesting to clarify the origin of these isolated compounds.

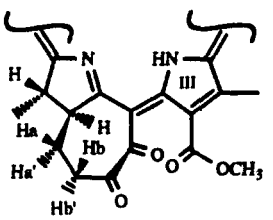
We describe herewith the isolation and structure determination of these PB-a-



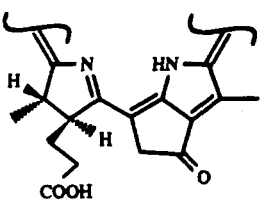
Chlorophyllone a [1]
Short-necked clam
Oyster
Attached diatoms mixture



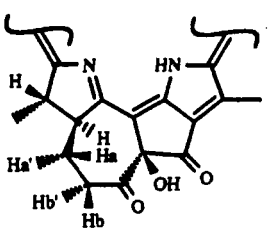
Chlorophyllonelactone a [2]
Short-necked clam
Scallop
Oyster



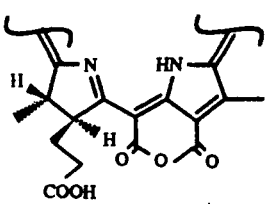
Chlorophyllonic acid a methyl ester [3]
Short-necked clam
Scallop
Oyster



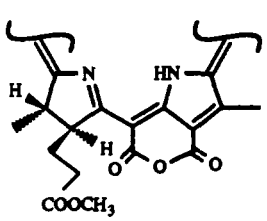
Pyropheophorbide a [4]
Short-necked clam
Scallop



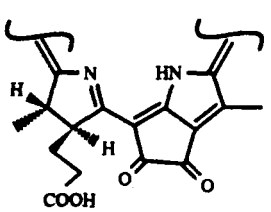
13^2-*epi*-Chlorophyllone a [5]
Scallop



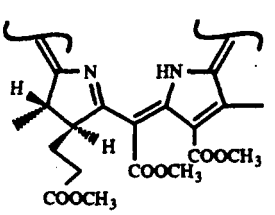
Purpurin 18 [6]
Short-necked clam



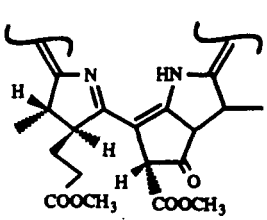
Purpurin 18 methyl ester [7]
Short-necked clam



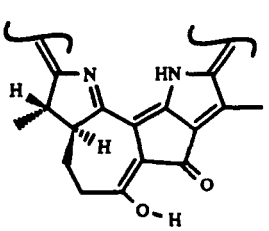
13^2-Oxopyropheophorbide a [8]
Short-necked clam



Chlorin-*p6* trimethyl ester [9]



Pheophorbide a methyl ester [10]



13^2,17^3-Cyclopheophorbide enol [11]
Darwinella oxeata

FIGURE 1. Structures of **1–8** and 13^2,17^3-cyclopheophorbide enol [**11**] and their origin.

related compounds **1–8** in detail, and the biogenetic considerations on these compounds.

ISOLATION AND STRUCTURE ELUCIDATION.—Edible parts of the clam *R. philippinarum* collected at Hamana Lake were finely chopped under ice cooling and extracted with

$CHCl_3$-MeOH (2:3). The MeOH layer obtained by partitioning of the concentrated extract between hexane and MeOH was chromatographed on a Sephadex LH-20 column using MeOH as an eluent to give the antioxidative fractions GPX and GPY. The latter fraction was further chromatographed on Si gel to yield four active compounds, **1**, **2**, **3**, and **4**. Purification of fraction GPX in a similar manner gave compounds **6**, **7**, and **8**.

Compound **4** was identified as pyropheophorbide a by direct comparison of its spectroscopic data with those of an authentic specimen. The other compounds also showed characteristic uv and visible absorption spectra [λ max (MeOH) 273–288, 386–410, 498–514, 529–545, 608–642, and 665–698 nm] for the chlorin structure as shown in Figure 2.

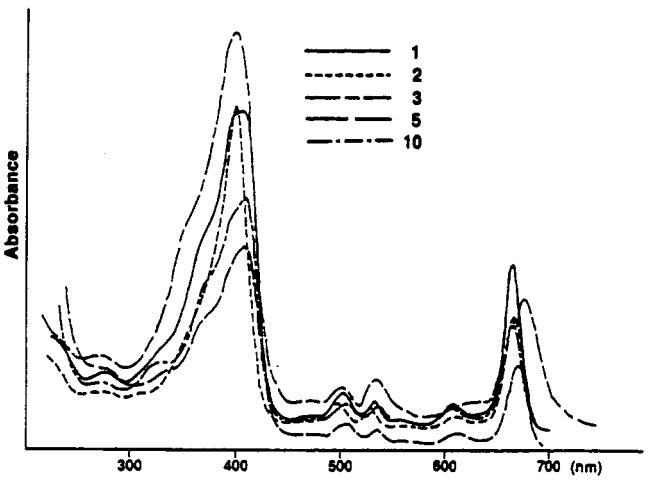


FIGURE 2. Uv and visible absorption spectra of **1**, **2**, **3**, **5**, and **10**.

Compound **1**, dark green solid, hrfabms *m/z* $[MH]^+$ 533.2581 (+2.9 mmu for $C_{33}H_{33}N_4O_3$), was found to be a new compound related to pheophorbide a and designated as chlorophyllone a (5,7).

Compound **2**, isolated as dark green crystals, gave quasimolecular ion at *m/z* $[MH]^+$ 549.2477 (−2.5 mmu for $C_{33}H_{33}N_4O_4$) in the hrfabms, appropriate for a molecular formula of $C_{33}H_{32}N_4O_4$ containing one more oxygen atom than **1**. Comprehensive ^{1}H-nmr analysis (Table 1) revealed that **2** has a structure similar to that of pheophorbide a methyl ester [**10**] with the characteristic protons (5, 10, 20, a vinyl at C-3, vinyl methyls at C-2, -7 and -12, a methyl at C-18, an ethyl at C-8, and two NH protons) for chlorin ring except for around rings IV and V. The signal due to a hydroxyl proton (δ 5.86) was observed in place of H-13^2 of **10** (Table 1). The chemical shifts and coupling patterns of H-17, H_a-17^1, $H_{a'}$-17^1, H_b-17^2, and $H_{b'}$-17^2 of **2** were quite different from those of **1** (Table 2). In the ^{13}C-nmr spectrum (Table 3) of **2** an ester carbonyl carbon signal was observed at higher field (δ 162.26) in place of the ketone carbonyl (δ 195.62) of **1**, and an oxygen-bearing quaternary carbon signal (C-13^2) appeared 9.88 ppm lower field (δ 103.33) than that (δ 93.45) of **1**.

In the HMBC spectrum (Figure 3) of **2**, prominent crosspeaks from $H_{b'}$-17^2 to C-13^2 and C-17^3 and H_b-17^2 to C-17^3 were observed. These observations revealed that C-13^2 and C-17^3 are within three-bond proximity from H_b-17^2 and $H_{b'}$-17^2. Thus **2**, designated as chlorophyllonelactone a, has a structure in which ring V of **1** has been converted to a δ-lactone. The lower shift ($\Delta\delta = 1.53$) of C-13^2 hydroxyl proton (δ 5.86)

TABLE 1. ^{1}H-nmr Spectral Data ($CDCl_3$, 400 MHz).

Proton	Compound								
	1[a]	2[a]	3[a]	5[b]	6[a]	7[a]	8[a]	9[a]	10[a]
H-10	9.55 (s)	9.70 (s)	9.67 (s)	9.49 (s)	9.60 (s)	9.63 (s)	9.88 (s)	9.71 (s)	9.53 (s)
H-5	9.50 (s)	9.54 (s)	9.47 (s)	9.39 (s)	9.37 (s)	9.38 (s)	9.85 (s)	9.50 (s)	9.40 (s)
H-20	8.66 (s)	8.66 (s)	8.58 (s)	8.54 (s)	8.57 (s)	8.57 (s)	9.01 (s)	8.66 (s)	8.56 (s)
H-3^1	8.03 (dd)	8.02 (dd)	7.96 (dd)	7.98 (dd)	7.88 (dd)	7.90 (dd)	8.11 (dd)	8.00 (dd)	8.00 (dd)
H-3^2(*E*)	6.31 (dd)	6.34 (dd)	6.31 (dd)	6.28 (dd)	6.29 (dd)	6.31 (dd)	6.36 (dd)	6.32 (d)	6.30 (dd)
H-13^2									6.25 (s)
H-3^2(*Z*)	6.21 (dd)	6.20 (dd)	6.16 (dd)	6.19 (dd)	6.19 (dd)	6.20 (dd)	6.27 (dd)	6.15 (d)	6.19 (dd)
H-8^1	3.68 (q)	3.73 (q)	3.70 (q)	3.66 (q)	3.64 (q)	3.65 (q)	3.79 (q)	3.74 (q)	3.70 (q)
H-12^1	3.71 (s)	3.84 (s)	3.61 (s)	3.66 (s)	3.78 (s)	3.81 (s)	3.86 (s)	3.52 (s)	3.69 (s)
H-2^1	3.44 (s)	3.43 (s)	3.39 (s)	3.38 (s)	3.34 (s)	3.35 (s)	3.51 (s)	3.42 (s)	3.41 (s)
H-7^1	3.26 (s)	3.27 (s)	3.22 (s)	3.23 (s)	3.16 (s)	3.18 (s)	3.36 (s)	3.25 (s)	3.24 (s)
H-18^1	2.20 (d)	1.85 (d)	1.73 (d)	2.21 (d)	1.74 (d)	1.74 (d)	1.88 (d)	1.85 (d)	1.81 (d)
H-8^2	1.70 (t)	1.71 (t)	1.69 (t)	1.69 (t)	1.66 (t)	1.68 (t)	1.75 (t)	1.70 (t)	1.70 (t)
H-18	4.36 (dq)	4.39 (dq)	4.41 (dq)	4.77 (dq)	4.39 (q)	4.39 (q)	4.69 (dq)	4.39 (dq)	4.45 (dq)
H-17	4.91 (dt)	4.42 (ddd)	4.54 (ddd)	3.83 (ddd)	5.19 (dd)	5.20 (dd)	5.18 (ddd)	5.16 (dd)	4.21 (ddd)
H_a-17^1	2.26 (ddt)	2.22 (dddd)	2.39 (ddt)	3.70 (dddd)	1.99 (m)	2.00 (m)	2.83 (m)	1.86 (m)	2.64 (dddd)
$H_{a'}$-17^1	2.90 (dddd)	2.88 (ddt)	2.91 (dddd)	2.67 (dddd)	2.5 (m)	2.5 (m)	2.44 (m)	2.21 (m)	2.32 (dddd)
H_b-17^2	2.81 (ddd)	3.54 (dt)	3.85 (dt)	3.86 (ddd)	2.5 (m)	2.5 (m)	2.75 (m)	2.06 (m)	2.52 (ddd)
$H_{b'}$-17^2	4.36 (ddd)	3.01 (ddd)	3.07 (ddd)	2.97 (ddd)	2.79 (m)	2.73 (m)	2.34 (m)	2.38 (m)	2.23 (ddd)
NH	0.62 (br s)	−0.8 (br s)	−0.67 (br s)	0.98 (br s)	0.23 (br s)	0.26 (br s)	−2.35 (br s)	−0.80 (br s)	0.56 (br s)
NH	−1.90 (br s)	−1.45 (br s)		−1.50 (br s)	−0.07 (br s)	−0.05 (br s)		−1.00 (br s)	−1.61 (br s)
13^2-OH	4.33 (br s)	5.86 (br s)		4.16 (br s)					
-OMe			4.04 (s)			3.60 (s)		4.22 (s)	3.57 (s)
-OMe								4.16 (s)	3.88 (s)
-OMe								3.65 (s)	

[a]Concentration 1 mg/0.6 ml.
[b]Concentration 0.6 mg/0.6 ml.

TABLE 2. ^{1}H-^{1}H Coupling Constants[a] J (Hz, $CDCl_3$).

Protons	Compound								
	1	2	3	5	6	7	8	9	10
$3^1,3^2(E)$	18.0	18.0	17.8	17.8	17.8	17.8	18.3	17.8	18.0
$3^1,3^2(Z)$	11.4	11.7	11.5	11.0	11.6	11.6	12.2	11.5	11.4
$3^2(E),3^2(Z)$	1.5	1.1	1.3	1.5	1.2	1.4	1.6		1.5
$8^1,8^2$	7.7	7.7	7.7	7.7	7.7	7.7	7.5	7.7	7.5
18^1,18	7.3	7.3	7.3	7.0	7.3	7.3	7.6	7.3	7.3
18,17	3.6	1.6	2.2	9.2			1.0		1.6
$17,17^1$a	12.8	11.8	13.4	12.6	8.7	9.0	2.8	8.8	3.2
$17,17^1$a′	3.6	5.6	6.3	1.8	2.2	2.5	8.7	2.6	9.0
17^1a,17^1a′	13.2	12.8	13.4	13.5					14.0
17^1a,17^2b	2.6	9.0	10.0	5.5					6.7
17^1a,17^2b′	13.2	1.6	1.1	12.1					9.9
17^1a′,17^2b	4.3	9.0	10.0	2.3					9.3
17^1a′,17^2b′	3.0	9.0	7.8	5.8					5.2
17^2b,17^2b′	11.2	11.3	12.1	16.0					15.7

[a]Obtained from the spectral data in Table 1.

of **2** than that (δ 4.33) of **1** was reasonably ascribed to the hydrogen bonding between the hydroxyl proton and C-17^3 ketone. The relative stereostructure around rings IV, V, and VI (7-membered ring) of **2** was deduced by a ^{1}H-^{1}H decoupling experiment (Table 2). The large coupling constants of $J_{H\text{-}17,17^1a}$=11.8, $J_{H\text{-}17^1a,17^2b}$=9.0, and $J_{H\text{-}17^1a',17^2b'}$=9.0 Hz indicated trans-diaxial relationship between H-17 and H_a-17^1 and an eclipsed conformation between H_a-17^1 and H_b-17^2 and $H_{a'}$-17^1 and $H_{b'}$-17^2. NOe experiments (Table 4) confirmed the relative stereochemistry at C-17, C-17^1, C-17^2, C-18, C-18^1, and C-13^2 as shown in structure **2**. Baeyer-Villiger type oxidation of **1** with *m*-CPBA did not give **2**.

Compound **3**, chlorophyllonic acid a methyl ester, was isolated as brown crystals, hrfabms *m/z* $[MH]^+$ 563.2686 (+2.8 mmu for $C_{34}H_{35}N_4O_4$). As already communicated (6), **3** has a structure with a sterically congested 1,2-diketone group. Chemical conversion of **2** to **3** was attempted to confirm the structure. Treatment of **2** with 1% HCl/MeOH followed by Si gel chromatography gave compound **7** as a main product, which was identical with the known compound purpurin 18 methyl ester (8), suggesting that **2** was oxidized into chlorin p_6 (9) followed by condensation to give **7**. Thus the

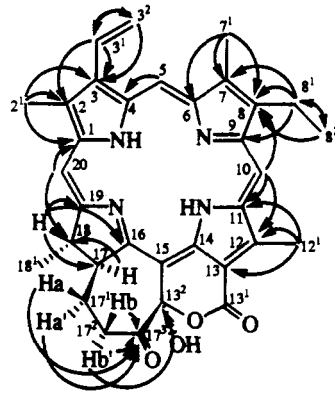


FIGURE 3. HMBC spectral data of **2** ($CDCl_3$, 400 MHz).

TABLE 3. ^{13}C-nmr Spectral Data ($CDCl_3$).[a]

Carbon	Compound						
	1	**2**	**3**	**5**	**6**	**9**	**10**
C-17^3	208.10	203.39	196.9	206.1	177.5[b]	173.5	173.34
C-13^1	195.62	162.26	166.9	193.5	177.2[b]	170.7	189.59
C-19	172.65	173.00	173.1	172.2		170.7	172.15
C-16	163.19	163.55	164.1	162.7		167.3	161.23
C-6	154.43	155.60	155.2	154.6	156.2	155.0	155.58
C-9	150.81	150.07	149.7	150.8	150.1	149.0	150.95
C-14	147.66	133.97[d]	135.2	149.4	139.9	137.7	149.70
C-8	144.79	145.46	145.5	144.9	145.9	145.3	145.11
C-1	142.10	141.65[d]	142.1	142.3	144.1	141.2	142.04
C-11	138.09	131.36[d]	130.2	138.2	139.0[c]	136.0[b]	137.96
C-3	136.28	136.16	136.3	136.3	136.6[c]	135.6[b]	136.47
C-4	136.20	135.88[d]	136.3	136.3	136.6[c]	135.8[b]	136.16
C-7	135.68	136.31	135.7	135.8	137.8[c]	135.9[b]	136.16
C-2	131.53	131.33	130.8	131.5	131.5	130.8	131.79
C-12	129.04	138.41	138.4	129.0	129.2	129.7	129.02
C-3^1	129.04	128.86	128.9	129.0	128.4	129.2	129.02
C-13	127.71	111.49	121.5	129.5	122.6	122.3	129.02
C-3^2	122.78	122.79	122.7	122.7	123.6	122.3	122.64
C-15	105.38	100.23[d]	108.6	105.9	111.5	103.2	105.28
C-10	103.93	104.18	105.9	104.6	107.5	104.7	104.33
C-5	97.99	99.61	101.5	98.2	103.1	100.3	97.47
C-13^2	93.45	103.33[d]	192.0	92.7	176.4[b]	172.8	64.77
C-20	92.84	93.32	93.4	91.6	95.0	93.6	93.09
C-17	51.89	50.06	50.3	53.7	55.0	52.7	51.21
C-18	51.51	51.13	51.2	50.3	49.2	49.5	50.17
C-17^2	40.12	34.81	36.4	43.2	32.2	31.5	29.92[b]
C-17^1	37.99	31.99	29.5	22.8	31.0	31.3	31.12[b]
C-18^1	22.35	23.50	23.6	16.9	23.8	23.5	23.08
C-8^1	19.18	19.27	19.5	19.3	19.3	19.6	19.34
C-8^2	17.27	17.41	17.5	17.3	17.3	17.6	17.32
C-12^1	12.16	12.11	12.5	12.2	12.2	12.5	12.01
C-2^1	12.07	12.11	11.9	11.9	11.9	12.0	12.01
C-7^1	11.11	11.17	11.2	11.1	10.9	11.2	11.08
OMe			52.3				
C-13^3							169.61
OMe						52.1	52.81[c]
OMe						51.4	51.64[c]
OMe						52.6	

[a]**1** 17.8 mg/0.6 ml, 100 MHz; **2** 18.8 mg/0.6 ml, 100 MHz; **3** 3.5 mg/0.1 ml, 22.5 MHz; **5** 1.8 mg/0.1 ml, 22.5 MHz; **6** 2.9 mg/0.1 ml, 22.5 MHz; **9** 2.0 mg/0.6 ml, 22.5 MHz; **10** from Wray *et al.* (16).

[b,c]Interchangeable.

[d]Deuterium shift 0.1–0.19 ppm.

chemical conversion of **2** into **3** has not been successful yet, but the structure of **3** was confirmed by X-ray crystallographic analysis (6). The C-13^2 carbonyl was found to be oriented downward out of the plane of chlorin ring to reduce the steric hindrance around the carbonyl group.

In addition to compounds **1**, **2**, **3**, and **4**, three other pyropheophorbide-a-related compounds **6**, **7**, and **8**, were isolated although in trace amounts.

Compound **6**, isolated as a purplish red material, was found to have a molecular formula of $C_{33}H_{32}N_4O_5$; positive fabms *m/z* $[MH]^+$ 565, $[M]^+$ 564; negative fabms *m/z* $[M]^-$

TABLE 4. NOe Experimental Results on **1**, **2**, and **5**.

Compound	Irradiated proton				
	H-17[a]	H-18[a]	H_a-17[1]	H_b-17[2]	H_b-17[2]
1	H-18[1] 3.5% H-18 1.4 H_a-17[1] 1.9 H_b-17[2] 3.0				
2	H-18[1] 4.5 H_a-17[1] 4.8	H-20 6.7 H-18[1] 4.7 H_a-17[1] 4.6 H_a-17[1] 2.0	H_a-17[1] 23.0 H_b-17[2] 2.8 H-17 9.5 H-18 2.5	H_b-17[2] 11.2 H_a-17[1] 2.0	
5		H-18[1] 6.0 H_a-17[1] 1.4 H-20 2.5 H-17 1.8			H_b-17[2] 20.3 H-17 3.1 H_a-17[1] 2.2

[a]Irradiated.

564, $[M-H]^-$ 563. The ^{1}H-nmr spectrum of **6** closely matched that of pheophorbide a methyl ester [**10**]. The significant differences were as follows: the ^{1}H-nmr spectrum of **6** lacked the signal due to H-13^2 as well as methyl ester signals of **10** at δ 3.57 and δ 3.88 and had a lower (0.98 ppm) shifted H-17 signal (δ 5.19) (Table 1). In the ^{13}C-nmr spectrum (Table 3) three carbonyl carbon signals were observed at δ 177.5, 177.2, and 176.4. These observations as well as ν max at 1749 and 1716 cm^{-1} in the ir spectrum of **6** indicated the presence of α,β-unsaturated acid anhydride structure in which ring V of **10** was converted into a 6-membered acid anhydride in **6**. Thus **6** was unambiguously identified as purpurin 18 (8,9).

Compound **7**, a purplish red solid, $C_{34}H_{34}N_4O_5$, positive fabms *m/z* $[MH]^+$ 579, negative fabms *m/z* $[M]^-$ 578, $[M-H]^-$ 577; ir 1750, 1718 cm^{-1}, showed a ^{1}H-nmr spectrum very similar to that of **6** with a slight difference in extra methyl protons (δ 3.60) assignable to a methyl ester group, but with no change of uv absorption of **6**. Compound **7** was thus identified as a methyl ester of **6** and seemed to be an artifact produced during MeOH extraction. This is, to our knowledge, the first report of isolation of **6** and **7**, although they have been already synthesized (8,9).

Compound **8**, named as 13^2-oxopyropheophorbide a, yellowish brown solid, positive fabms *m/z* $[MH]^+$ 549, negative fabms *m/z* $[M]^-$ 548; ir 1706 cm^{-1}, showed the presence of the characteristic protons (5, 10, 20, a vinyl, methyls attached to C-2, -7, -12, and -18, an ethyl at C-8, and two NH protons) for chlorin ring. These features indicated that **8** has a structure similar to that of pheophorbide a except around ring V. In the ^{1}H nmr of **8** the H-17 proton was found at δ 5.18, shifted 1 ppm down-field from its position in **10**. The lower field shift of this proton also occurs in the spectra of **6** and **7**, suggesting an anisotropic effect due to a carbonyl at C-13^2. Hence we propose the 1,2-diketone structure for **8**.

Compounds **1**, **2**, **3**, and **8** were new chlorophyll-a-related compounds, and we were interested in their orgin. As bivalves are well known to feed on wafting diatoms and detritus, these compounds seem to originate from chlorophylls in diatoms and to be metabolized by (1) themselves, (2) microorganisms symbiotic with the diatoms, (3) digestive fluids in the alimentary tract of the clam, or (4) microorganisms symbiotic with the clam. Chlorophyll-a-related compounds in the viscera extracts of the other bivalves, the scallop *P. yessoensis* and the oyster *Crassostera* sp., as well as in the extract of attached diatoms mixture cultured for abalone seedling production were analyzed as follows.

Viscera (2.3 kg) of the scallop *P. yessoensis* was finely chopped under ice cooling and extracted with $CHCl_3$-MeOH (1:1). The concentrated extract was partitioned between hexane and MeOH, and the concentrated MeOH layer was successively subjected to Sephadex LH-20 and Si gel cc to give **2** (1.3 mg), **3** (0.4 mg), **4** (1.0 mg), and a new PB-a-related compound **5**, 13^2-*epi*-chlorophyllone a (1.2 mg). Compound **1**, however, was not detected in the extract.

Compound **5**, dark green solid, hrfabms *m/z* $[MH]^+$ 533.2585 (+3.2 mmu for $C_{33}H_{33}N_4O_3$), ir ($CHCl_3$) 1719 cm^{-1}, has the same molecular formula as **1**. Comparative studies of their ^{1}H-nmr spectra revealed that **5** was a chlorin derivative. The signals due to H-17 and H_b-17^2 were shifted to 1.08 and 1.39 ppm higher field, respectively, than those of **1**. H_a-17^1 and $H_{b'}$-17^2 signals were shifted to lower field than those of **1**. The chemical shift and the coupling constants of successive spin systems, H-18, H-17, H_a-17^1, $H_{a'}$-17^1, H_b-17^2 and $H_{b'}$-17^2, were quite different from those of **1**. These observations suggested that **5** was an epimer of **1** at C-13^2, which was confirmed as follows.

Large coupling constants, $J_{H\text{-}17,17^1{}_a}$=12.6 and $J_{H\text{-}17^1{}_a,17^2{}_{b'}}$=12.1 Hz, and nOe's of H-17 (3.1%) and $H_{a'}$-17^1 (2.2%) by irradiation at $H_{b'}$-17^2 (Table 3) suggested their 1,2-trans diaxial relationships. The lower field shift of H_a-17^1 in **5** compared with that of **1** is ascribable to the anisotropic effect of 13^2-OH, which has the 1,4-diaxial conformation with H_a-17^1. The upper field shifts of H-17 and $H_{b'}$-17^2 in **5** are due to decreased anisotropic effect by 13^2-OH caused by the stereostructural change of **1** into **5**. Larger coupling constant ($J_{H\text{-}18,17}$=9.2 Hz) in **5** than that ($J_{H\text{-}18,17}$=1.6 Hz) in **10** (Table 3) indicated that methyl protons (H-18^1) of **5** were in the same plane of the chlorin ring, whereas H-18^1 of **10** are directed downward out of the chlorin ring. This conformational change of the ring VI increased the anisotropic effect from chlorin ring to H-18^1 to result in its lower field shift ($\Delta\delta$=0.40 ppm) in **5** than that of **10** (Table 1). The upper field shift ($\Delta\delta$=−15.2 ppm) of C-17^1 of **5** from that of **1** is due to the steric compression effect caused by the conversion of 13^2-OH configuration. Thus the structure of **5** was unambiguously established to be the epimer of **1** at C-13^2.

Viscera (1.9 kg) of the oyster *Crassostera* sp. were extracted with $CHCl_3$-MeOH (1:1) under ice cooling. The MeOH layer obtained by partitioning between MeOH and hexane was purified with Sephadex LH-20 and Si gel cc to give 3.9 mg of **1**. Hplc showed the peaks of **2** and **3** in the fraction eluted faster than **1** on the Si gel cc. The oyster viscera contained **1** as well as **2** and **3**.

Dried attached diatoms mixture (18 g), mainly consisting of *F. oceanica, F. cylindus, N. closterium, N. seriata, Coc. pseudomarginata,* and *H. stelliger,* was extracted with $CHCl_3$-MeOH (1:2). The EtOAc layer from the extract was subjected to Sephadex LH-20 cc [$CHCl_3$-MeOH (1:3)] followed by hplc to give **1** (0.6 mg).

Chlorophyll-related compounds are well known to promote lipid oxidation under light (10), but to show antioxidative activity in the dark (11). Compounds **1–4** and **6–8** showed stronger antioxidative activity at a dose of ca. 3 μg than that of 20 μg of α-tocopherol in the dark, suggesting that these compounds contribute to the antioxidative activity of the short-necked-clam extract. Compound **5** from the scallop also showed antioxidative activity. The antioxidative mechanism of chlorophylls is thought to be due to radical scavenger effect (11).

BIOGENETIC CONSIDERATIONS.—Many degradation pathways of chlorophyll a have been reported (12). Enzymatic degradation of chlorophyll a to pyropheophorbide a has been confirmed in a mutant strain of the micro alga, *Chlorella fusca* (13). The fact that **1–3** and **5–8** are isolated here as naturally occurring chlorophyll-a-related compounds and that **5** is an epimer of **1** at C-13^2 strongly suggests that both **1** and **5** are enzymatically produced in each bivalve or diatoms and that new degradation pathways

of chlorophyll a to **1–3** and **5–8** are present. Compound **1** was also isolated from the mixture of attached diatoms, indicating that **1** was produced by the attached diatoms themselves.

Compound **4**, produced by decarboxylation of PB a, seems to be converted into 13^2,17^3-cyclopheophorbide enol [**11**] by Claisen type condensation followed by oxidation to give **1** and **5** (Scheme 1). Compound **11**, which was first synthesized by Falk *et al.* (14), was recently isolated from the sponge *Darwinella oxeata* (15) and is also considered to originate from chlorophylls contained in the diatoms symbiotic with the sponge.

Compound **2** was assumed to be directly produced by Baeyer-Villiger type oxidation of **1** or to be derived from **1** via **3** by the loss of two electrons together with the ring V opening under the acidic conditions, followed by intra-molecular cyclization (Scheme 1). Compound **3** may be derived from **2** by ring V opening or directly produced by oxidation of **1** or **5** under acidic conditions. A hypothetical degradation pathway of chlorophyll a to **1**, **2**, and **3** is summarized in Scheme 1.

Treatment of **2** with anhydrous 1% HCl/MeOH gave **7**, suggesting that compounds **6** and **7** may be derived from **2** by oxidative degradation under acidic conditions. When **1** was hydrolyzed by aqueous KOH followed by treatment with CH_2N_2, chlorin *p6* trimethyl ester [**9**] was obtained. This result indicates that **1** can be oxidized to chlorin *p6* under basic conditions; the chlorin *p6* is then methylated to yield **9** (Scheme 2).

Additional ring formation in **1** and **5** was suggested to occur enzymatically, because each epimer **1** or **5** was individually present to each bivalve. Attempts to convert **1** into **2** by *m*-CPBA treatment, or by air oxidation under neutral and basic conditions, were not successful. These observations indicate that the enzymatic process from **1** to **2** may be significant.

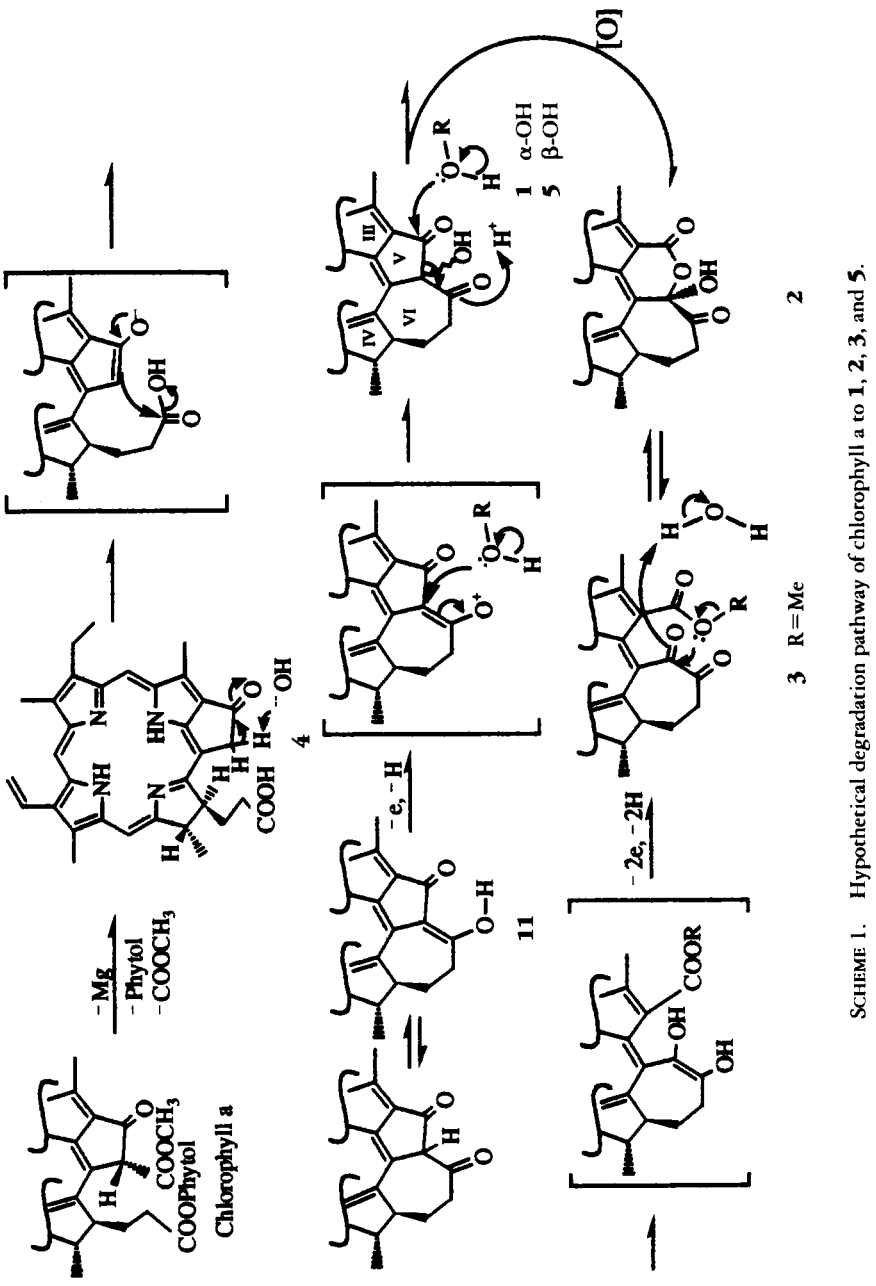
EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Flash chromatography columns were made using Wakogel C-300. YMC-Packed A-012 SIL 5 μm (6 mm×150 mm) and Develosil ODS-10 10 μm (20 mm×250 mm) columns were used in the analytical and preparative hplc, respectively. C.I.G. Prepacked Si gel column (22×100 mm) was used in the medium pressure liquid chromatography (mplc). All solvents used in the extraction and separations were distilled prior to use. Ir spectra were obtained on a JASCO A-102 diffraction grating infrared spectrophotometer in $CHCl_3$. Uv spectra were recorded on Hitachi model U-3210 spectrophotometer in MeOH. The ^{1}H-nmr and ^{13}C-nmr spectra were run in $CDCl_3$ on JEOL JNM GSX-400 or JEOL JNM-FX90Q spectrometers. HMBC and HMQC spectra were recorded on JEOL JNM GSX-500 or JEOL JNM GX-400 spectrometers. Mass spectra were obtained on a JEOL JMS-DX303 HF mass spectrometer with a JMA-DA 5000 mass data system. 3-Nitrobenzyl alcohol (NOBA) was used as a matrix for fabms and hrfabms.

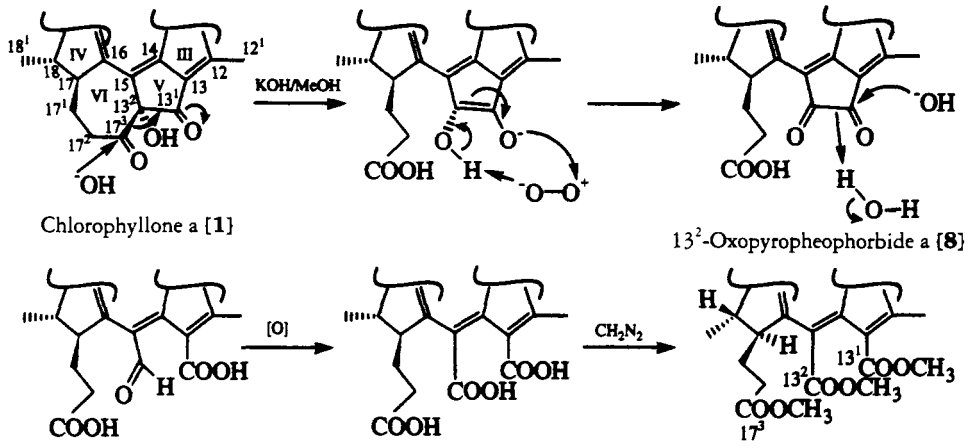
EXTRACTION AND ISOLATION OF **1**, **2**, **3**, **4**, **6**, **7**, AND **8** FROM THE CLAM.—Edible parts (3.5 kg) of the clam *R. philippinarum* collected at Hamana Lake, Shizuoka prefecture, Japan in 1989 (voucher specimen deposited at Department of Applied Biological Chemistry, Shizuoka University) were finely chopped with ice cooling and extracted with $CHCl_3$-MeOH (2:3). The combined extract was concentrated in vacuo and partitioned between hexane and MeOH. Isolation was guided by the antioxidative activity judged by the ferric thiocyanate method slightly modified as shown below. The MeOH layer was concentrated and subjected to Sephadex LH-20 column chromatography (MeOH) to give two antioxidative fractions GPX and GPY. Fraction GPY was purified by repeated Si gel cc (C_6H_6/EtOAc, $CHCl_3$/EtOAc, EtOAc/MeOH, $CHCl_3$/Me_2CO, Me_2CO/MeOH) to yield the new PB-a-related compounds chlorophyllone a [**1**] (22.4 mg), chlorophyllonelactone a [**2**] (19.8 mg), and chlorophyllonic acid a methyl ester [**3**] (3.5 mg) together with PB a [**4**].

Si gel cc ($CHCl_3$/MeOH) of fraction GPX gave purpurin 18 [**6**] (2.9 mg), purpurin 18 methyl ester [**7**] (0.9 mg), and a new related compound, 13^2-oxopyropheophorbide a [**8**] (1.2 mg).

EXTRACTION AND ISOLATION OF **2**, **3**, **4**, AND **5** FROM THE VISCERA OF THE SCALLOP.—The frozen viscera (2.3 kg) of the scallop *P. yessoensis* collected at Rausu, Hokkaido, Japan, in 1989 (voucher specimen deposited at Department of Applied Biological Chemistry, Shizuoka University) were finely chopped with



SCHEME 1. Hypothetical degradation pathway of chlorophyll a to **1**, **2**, **3**, and **5**.



SCHEME 2. Speculative conversion mechanism of **1** to **9**.

ice cooling and immediately extracted with $CHCl_3$-MeOH (1:1). After removal of the organic solvents, the resultant aqueous solution was successively extracted with hexane and EtOAc. The combined organic layer was concentrated and partitioned between hexane and MeOH. The concentrated MeOH layer was subjected to Sephadex LH-20 cc (MeOH) to give orange- and dark green-colored fractions, the latter being futher purified on a Sephadex LH-20 (MeOH) column. The fractions supposed to contain chlorophyll a derivatives were combined based on their tlc profiles. The combined fraction was successively chromatographed on Si gel columns ($CHCl_3$/EtOAc/MeOH, $CHCl_3$/EtOAc), Sephadex LH-20 column (MeOH), and mplc [$CHCl_3$-MeOH (9:2)] to yield **2** (1.3 mg), **3** (0.4 mg), **4**, and 13^2-*epi*-chlorophyllone a [**5**] (1.2 mg).

EXTRACTION AND IDENTIFICATION OF **1**, **2** AND **3** FROM THE VISCERA OF THE OYSTER.—The viscera (1.9 kg) of the oyster *Crassostrea* sp. collected in Hamana Lake in 1990 (voucher specimen deposited at Department of Applied Biological Chemistry, Shizuoka University) gave, after an isolation procedure similar to that outlined above, **1** (3.9 mg) and fractions, in which the presence of **2** and **3** was confirmed by hplc [YMC Packed A-012 ($CHCl_3$), monitored at 400 nm, at a flow rate of 1.0 ml/min] at Rt 5.7 and Rt 4.0 min, respectively.

ISOLATION OF **1** FROM ATTACHED DIATOMS.—Cultured diatoms mixture, mainly consisting of *F. oceanica, F. cylindus, N. closterium, N. seriata, Coc. pseudomarginata,* and *H. stelliger* (dry wt 18 g) at a climax growth stage, collected at Shizuoka Prefecture Fish Farming Center in 1990 (voucher specimen deposited at Department of Applied Biological Chemistry, Shizuoka University), was immersed in 2 liters of MeOH for 8 days. The extract was concentrated and extracted with EtOAc. The EtOAc extract was purified by Sephadex LH-20 (MeOH) and Si gel cc ($CHCl_3$/MeOH) to give **1** (0.6 mg).

Chlorophyllone a [**1**].—Dark green solid: hrfabms (glycerol matrix) *m/z* $[MH]^+$ 533.2581 (+2.9 mmu for $C_{33}H_{33}N_4O_3$); ir ($CHCl_3$) 1722 cm^{-1}; uv λ max (MeOH) 277, 408, 503, 534, 608,665 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

Chlorophyllonelactone a [**2**].—Dark green crystalline solid: hrfabms *m/z* $[MH]^+$ 549.2477 (−2.5 mmu for $C_{33}H_{33}N_4O_4$); ir ($CHCl_3$) 1722 cm^{-1}; uv λ max (MeOH) 282, 400, 498, 529, 610, 667 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

Chlorophyllonic acid a methyl ester [**3**].—Brown crystals: hrfabms *m/z* $[MH]^+$ 563.2686 (+2.8 mmu for $C_{34}H_{35}N_4O_4$); ir ($CHCl_3$) 1731, 1693 cm^{-1}; uv λ max (MeOH) 278, 400, 504, 540, 610, 677 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

Pyropheophorbide a [**4**].—Uv λ max (MeOH) 273, 324, 410,508, 539, 609, 666 nm.

13^2-epi-*Chlorophyllone a* [**5**].—Dark green solid: hrfabms *m/z* $[MH]^+$ 533.2585 (+3.2 mmu for $C_{35}H_{33}N_4O_3$); ir ($CHCl_3$) 1719 cm^{-1}; uv λ max (MeOH) 276, 408, 505, 535, 612, 670 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

Purpurin 18 [**6**].—Purplish red solid: positive fabms *m/z* $[MH]^+$ 565, $[M]^+$ 564; negative fabms *m/z* $[M]^-$ 564, $[M-H]^-$ 563; ir ($CHCl_3$) 1749, 1716 cm^{-1}; uv λ max (MeOH) 275, 361, 408, 478, 508, 545, 639, 698 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

Purpurin 18 methyl ester [**7**].—Purplish red solid: positive fabms *m/z* $[MH]^+$ 579, $[M]^+$ 578; negative fabms *m/z* $[M]^-$ 578; ir ($CHCl_3$) 1750, 1718 cm^{-1}; uv λ max (MeOH) 276, 359, 407, 478, 508, 545, 642, 697 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

13²-Oxopyropheophorbide a [**8**].—Yellowish brown solid: positive fabms *m/z* $[MH]^+$ 549, $[M]^+$ 548; negative fabms *m/z* $[M]^-$ 548; ir ($CHCl_3$) 1706 cm^{-1}; uv λ max (MeOH) 288, 386, 514, 618, 676 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

ANTIOXIDANT ASSAY.—Thiocyanate method by Fukuda *et al.* (3) was modified as follows. Each sample dissolved in appropriate solvent (ca. 30 μl) was absorbed on a paper disk (8×1.5 mm) in a 10 ml sample tube. After evaporation of the solvent, 200 μl of EtOH, 200 μl of 2.5% linoleic acid in EtOH, 400 μl of 5×10^{-2} M phosphate buffer (pH 7.0) and 200 μl of distilled H_2O were added. The mixture in a stoppered sample tube was kept in a sonicator for a few minutes to dissolve the sample out of the paper disk and incubated at 40°. At intervals during the incubation, 100 μl of the mixture was taken into a test tube and mixed with 3 ml of 75% EtOH, 100 μl of 30% ammonium thiocyanate, and 100 μl of 2×10^{-2} M $FeCl_3$. After 3 min the absorbance at 500 nm was measured.

ATTEMPTED *m*-CPBA OXIDATION OF CHLOROPHYLLONE A [**1**].—To an anhydrous solution of **1** (0.5 mg) in C_6H_6-EtOAc (1:1) (0.3 ml) was added a trace amount of *m*-CPBA in anhydrous C_6H_6 with ice cooling. After 2 h of the reaction, tlc [C_6H_6-EtOAc (6:1)] showed neither **1** nor **2**, although oxidation reaction certainly occurred.

ATTEMPTED AIR OXIDATION OF **1**.—A solution of **1** (0.1 mg) in the mixture of $CHCl_3$-MeOH (1:5) (2 ml) was kept for 4 months at ambient temperature. The resultant solution was directly analyzed by hplc, which did not give a peak due to **2**. Compound **1** was recovered.

OXIDATIVE REACTION OF CHLOROPHYLLONELACTONE A [**2**] UNDER ACIDIC CONDITIONS.—To an anhydrous solution of **2** (2.6 mg) in 2 ml of C_6H_6-EtOAc (1:1) was added a trace amount of anhydrous 1% HCl/MeOH in the presence of several particles of molecular sieves 3Å with ice cooling. After 68 h stirring, 5 ml of $CHCl_3$ was added. The $CHCl_3$ solution was washed with brine (5 ml×2), concentrated and subjected to Si gel cc (hexane/$CHCl_3$) to afford a dark brown fraction (0.8 mg). Purpurin 18 methyl ester [**7**] was found to be a main product in this fraction based on its ^1H-nmr and fabms data.

HYDROLYSIS OF CHLOROPHYLLONE A [**1**] WITH AQUEOUS KOH.—To a solution of **1** in MeOH (5 ml) containing a small amount of Et_2O was added 1 M KOH/MeOH (1 ml). After 14 h stirring, the reaction mixture was acidified to pH 3 by 1 M HCl diluted with 5 ml of H_2O and extracted with Et_2O (5 ml×3). The Et_2O layer was washed with brine and treated with an excess of etheral CH_2N_2. After removal of excess CH_2N_2 under N_2, the residue was applied to a Si gel column equilibrated with $CHCl_3$, and developed with a mixture of $CHCl_3$-MeOH (20:1, 5:1) to afford chlorin *p*6 trimethyl ester [**9**] as a major product.

Chlorin p6 *trimethyl ester* [**9**].—Dark green substance: fdms *m/z* $[M]^+$ 624; uv λ max (MeOH) 275, 397, 498, 528, 613, 667 nm; ^1H nmr see Table 1; ^{13}C nmr see Table 3.

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