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Chemistry and Biochemistry of Chinese Drugs. VII.[1] Cytostatic Pheophytins from Silkworm Excreta, and Derived Photocytotoxic Pheophorbides

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Four chlorophyll derivatives (pheophytins) were isolated from silkworm excreta by column and thin-layer chromatography and their structures were elucidated by spectroscopy.

Among them, two substances, 13^2-hydroxy (13^2-*R,S*) pheophytin a and pheophytin b, have *in vitro* cytostatic activity against hepatoma tissue culture (HTC) cells. The methyl ester derivatives of pheophorbides showed cytotoxic effects towards the tumor cells under illumination. This can be explained by photodynamic destruction of HTC cells by singlet oxygen, as has already been shown in the case of hematoporphyrin. The observation that natural pheophytins have cytostatic effects even in the dark suggests that there is another biological mechanism for the action of these chlorophyll derivatives.

Keywords——chlorophyll derivatives; silkworm excreta; hepatoma tissue culture; photodynamic cytotoxicity; cytostatic activity

Introduction

The excreta of the silkworm (*Bombix mori* L.) have been studied as a source of chlorophyll or carotenoids.[2–4] They have also been used in traditional Chinese medicine, as a drug called *Cán Shā* ("silkworm sand") 蚕沙 against rheumatic and abdominal pains.[5] We have previously demonstrated cytotoxic activity of steroidal derivatives isolated from silkworms infected by a fungal parasite (*Bombyx cum Botryte*),[6] and have now checked the effect of *Cán Shā* extracts on the growth of tumor cells. This has led to the unexpected discovery of the cytostatic and cytotoxic effects of some known and novel chlorophyll derivatives.

Materials and Methods

Cell Cultures and Cytotoxicity Tests——HTC Cells: HTC cells, derived from clone 7288c of a rat Morris hepatoma,[7] are cultured at 37°C in suspension in Swim's 77 medium (Gibco, Flobio SARL-France) supplemented with 10% newborn calf serum (Gibco, Ref. 601). Total and viable cells are counted every 24 h for 3 days, using a "Neubauer" microcytometer, always in parallel with a simultaneous control run, after 15 min incubation with the vital dye Trypan Blue (Gibco, Ref. 525) to differentiate the living and the dead cells. The control cell suspension is adjusted at time 0 to *ca.* 10^5 cells/ml. Multiplication is approximately exponential during the 3 days of the test, and levels off later. The substances to be tested (2.5 mg of 5 mg) are dissolved in N,N-dimethylformamide or in ethanol (100 μl); sterile addition to 75 ml of cell suspension gives a final concentration of 33 or 66 μg/ml of culture medium. We have checked that the solvents, used at this concentration, have no measurable influence on cell multiplication.

3T3 Cells: Mouse 3T3 fibroblasts[8] are a stable, non-tumor cell line. They are grown as a monolayer in 5 ml of Dulbecco's modified Eagle medium (Gibco-Biocult, Ref. H16), supplemented with 0.3% glucose and 10% newborn calf serum, in 25 cm^2 Falcon culture flasks. The tests are performed as above and, for counting, the cells from each flask are recovered and dispersed by trypsinization.

Isolation of Pheophytins 1—4 (Figure 1)——Silkworm excreta were extracted for us with acetone–water (4:1) by Nishin Flour Milling Co. Ltd., Saitama (Japan). The extract (23 g) exhibited weak cytostatic activity at 66 μg/ml on HTC cells. The extract was first fractionated on a Silica gel column (500 g, Merck Kieselgel 60, 70—230 μ) successively with (a) petroleum ether, then 8% ether–petroleum ether, (b) ether and (c) methanol. Fraction (a) 3.6 g (16%), no effect on HTC cells, (b) 16.1 g (70%), inhibitory effects (66 μg/ml) on the growth of the tumor cells, and (c) 2.4 g (10%), no activity.

TABLE I. ^{1}H NMR[a] Chemical Shifts (δ[ppm] from Tetramethylsilane in $CDCl_3$) of Compounds **1**—**9**

Proton				13*S*		13*R*				13*S*		13*R*	
	1	**5**	**9**	**2a**	**6a**	**2b**	**6b**	**3**	**7**	**4a**	**8a**	**4b**	**8b**
7-CHO	—	—	—	—	—	—	—	11.15	11.08	11.21	11.20	11.21	11.20
10-H	9.51	9.46	9.48	9.61	9.54	9.59	9.51	9.66	9.56	9.79	9.78	9.77	9.76
5-H	9.37	9.31	9.37	9.47	9.38	9.45	9.35	10.38	10.29	10.51	10.50	10.48	10.47
20-H	8.55	8.55	8.55	8.63	8.63	8.61	8.60	8.54	8.54	8.61	8.62	8.59	8.59
3-CH	7.99	7.94	7.96	8.04	7.94	8.04	7.94	8.00	7.99	8.04	8.02	8.04	8.02
3′-CH_2	~6.25	~6.22	~6.23	~6.25	~6.20	~6.25	~6.20	~6.31	~6.29	~6.32	~6.31	~6.32	~6.31
13^3-H	6.26	6.25	5.25, 5.11 (2H)	—	—	—	—	6.24	6.23	—	—	—	—
13^3-OH	—	—		5.53	5.51	5.34	5.34	—	—	5.59	5.53	5.34	5.32
P-1-H[c]	4.46	—	—	4.47	—	4.47	—	4.46	—	4.48	—	4.48	—
P-2-H[c]	5.12	—	—	5.18	—	5.18	—	5.15	—	5.13	—	5.13	—
18-H	4.45	4.44	4.48	4.56	4.49	4.56	4.49	4.46	4.46	4.58	4.46	4.58	4.46
17-H	4.16	4.20	4.21	4.15	4.15	4.69	4.68	4.13	4.18	4.14	4.13	4.65	4.63
13^3-OCH_3	3.88	3.88	—	3.73	3.72	3.72	3.72	3.90	3.91	3.75	3.75	3.73	3.75
17^3-OCH_3	—	3.57	3.61	—	[b]	—	[b]	—	3.60	—	[b]	—	[b]
12-CH_3	3.68	3.67	3.66	3.61	3.56	3.63	3.56	3.68	3.66	3.63	3.54	3.65	3.54
2-CH_3	3.40	3.38	3.41	3.43	3.40	3.42	3.39	3.38	3.37	3.40	3.41	3.40	3.41
7-CH_3	3.22	3.17	3.23	3.26	3.18	3.25	3.17	—	—	—	—	—	—

a) CAMECA 250 superconducting NMR spectrometer, operating at 250 MHz.
b) Six peaks observed between the extreme values; no reliable assignment was possible.
c) P-1-H: C-1 ^{1}H of phytyl group, P-2-H: C-2^{1}H of phytyl group.

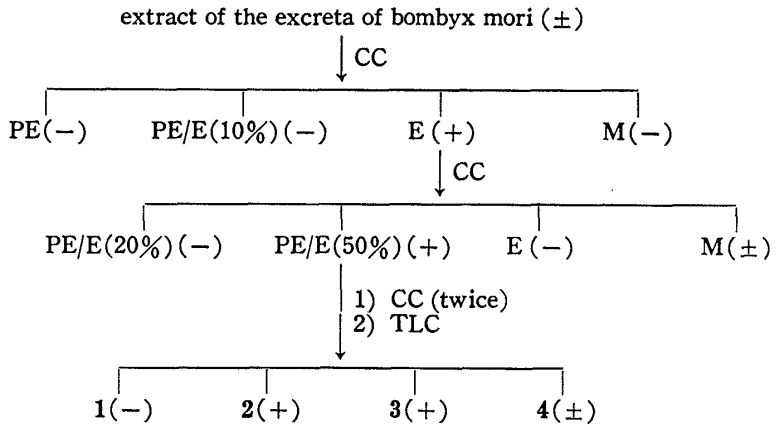


Fig. 1. Isolation of Constituents Responsible for the Cytostatic Effects on HTC Cells, in the 80% Acetone Extract of Silkworm Excreta

E, ether; PE, petroleum ether; M, methanol; (−), inactive; (+), cytostatic; (±), weakly cytostatic.

The fraction b (15.5 g) was again chromatographed on Silica gel (400 g) successively with (d) 20% ether–petroleum ether, (e) 50% ether–petroleum ether, (f) ether and (g) methanol. Fraction (d) 6.6 g (43%), no effect, (e) 5.0 g (32%), inhibitory effects (66 μg/ml), (f) 2.4 g (16%) no effect, and (g) 0.6 g (4%), a slight cytostatic effect (66 μg/ml), but analysis of this fraction has not been done.

The fraction e (5.0 g) was fractionated on a Silica gel (100 g) column successively with (h) 5% ethyl acetate–hexane, (i) and (j) 50% ethyl acetate–hexane, (k) ethyl acetate, then methanol. Fraction (h) 1.2 g (25%), (i) 3.0 g (60%), (j) 0.4 g (8%) and (k) 0.1 g (2%). The first three fractions have inhibitory effects (33 μg/ml), while the last one has no effect. The fraction i (3.0 g) was re-chromatographed on Silica gel with mixtures of hexane–ethyl acetate, increasing the percentage of ethyl acetate: fraction (l) 1.4 g (47%), containing principally compound **1** and fraction (m) 0.5 g (18%), which contains mainly compound **2**. A part of each fraction (l), (m) was purified three times by preparative thin-layer chromatography (TLC) (Merck F 254, 2 mm, hexane–ethyl acetate (4:1) as the developing solvent). Pheophytin a **1** (60 mg) and 13^2-hydroxypheophytin a **2** (135 mg) were obtained. Preparative TLC (three times, hexane–ethyl acetate (4:1) as the developing solvent) of a part of (h) gave **1** (80 mg) and **2** (55 mg). Preparative TLC (3 times, hexane–ethyl acetate (2:1) as the developing solvent) of (j) afforded pheophytin b **3** (9 mg), 13^2-hydroxypheophytin b **4** (9 mg), a mixture of **3** and **4** (50 mg) along with **1** and **2**. Thus the pure products **1** (140 mg), **2** (190 mg), **3** (9 mg), and **4** (9 mg) were obtained.

Identification of Pheophytins 1—4——The following spectroscopic analyses were carried out to determine the structures of the substances **1**—**10**. Nuclear magnetic resonance (NMR) spectra were taken in $CDCl_3$ solution on a Cameca 250 instrument (250 M Hz). Chemical shifts were measured as δ (ppm) from tetramethylsilane, at sample concentrations of 2—5 mg/ml in $CDCl_3$ (Table I). Ultraviolet and visible (UV-VIS) spectra were taken in ether solution on a Jobin Yvon DUOSPAC 203 instrument (Table II). Mass spectra (MS) were obtained by direct introduction of the sample in the ionization chamber on a gold support.[9] Apparatus: double-focusing Thomson-CSF THN-208 B instrument; electron impact source 70 eV; temperature of ionization chamber 220—270°C (Table III). The following chromatographic methods were used for the identification (and to check the purity) of compounds **1**—**10**. Analytical thin-layer chromatography (TLC) was run on Silica gel plates (Merck F 254, 0.25 mm). (Table IV). High performance liquid chromatography (HPLC) was run on a Waters Associates machine, model 450, U6K injector, 6000 A pump; UV detector (410 nm); column dimensions 300 mm × 3.9 i.d.; packing Bondapack C18 (10 μ); mobile phase methanol; flow rate 2.0 ml/min (Table IV).

Table II. Ultraviolet and Visible Spectra[a] of Compounds **1**—**4**

Compounds	λ_{max}[b] (α)[c]
1	408(126), 506(14), 535(11), 610(9), 669(65)
2	409(126), 505(14), 533(8), 610(7), 670(64)
3	412(90), 432(203), 525(14), 603(7), 656(43)
4	412(91), 433(204), 526(14), 603(7), 656(41)

a) Jobin Yvon DUOSPAC 203 UV-VIS spectrometer.
b) λ_{max}=nm.
c) α=specific absorption coefficient.

Table III. Mass Spectra[a] of Compounds 1—9

Compounds	Formula	*m/z*
1	$C_{55}H_{74}N_4O_5$	870 (M^+)
2	$C_{55}H_{74}N_4O_6$	886 (M^+)
3	$C_{55}H_{72}N_4O_6$	884 (M^+)
4	$C_{55}H_{72}N_4O_7$	900 (M^+)
5	$C_{36}H_{38}N_4O_5$	606 (M^+)
6	$C_{36}H_{38}N_5O_6$	622 (M^+)
7	$C_{34}H_{36}N_4O_3$	548 (M^+)
8	$C_{36}H_{36}N_4O_6$	620 (M^+)
9	$C_{36}H_{36}N_4O_7$	636 (M^+)

a) Thomson-CSF THN 208 mass-spectrometer: direct introduction, electron impact source, 70 eV.
These spectra were obtained by our new method, on a gold support[9] as described in the text, without field desorption (*cf.* ref. 17, 18). Full details will be published later.

Table IV. t_R (HPLC)[a] and *Rf* (TLC) of Compounds 1—10

Compounds	t_R (min)	*Rf*
1	8.4	0.49[c]
2	6.1, 6.7[b]	0.36[c]
3	6.7	0.33[c]
4	4.8, 5.3[b]	0.27[c]
5	2.8	0.18[c]
6	2.4	0.11[c]
7	2.5	0.08[c] (0.46)[d]
8	2.2	0.06[c] (0.33)[d]
9	3.6	0.13[c]
10	1.7	0.56[e]

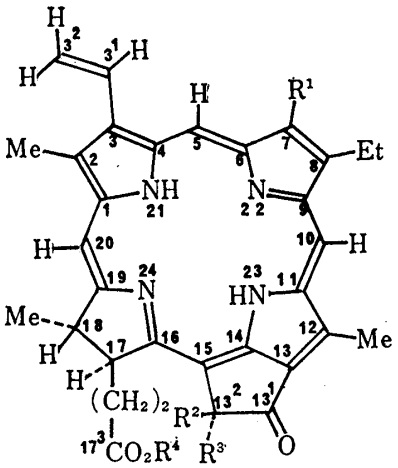
a) Waters Associates model 450 high performance liquid chromatograph; 6000 A pump; U6K injector; UV detector (410 nm). Column dimensions 300 mm × 3.9 I.D.; packing Bondapack C_{18} (10 μ); mobile phase methanol; flow rate 2.0 ml/min.
b) Epimers.
c) Hexane–ethyl acetate (7: 3).
d) Hexane–ethyl acetate (1: 1).
e) Ethyl acetate (Silica gel plates of Merck F 254, 0.25 mm, in all cases).

Authentic Specimens: Pheophytin a,[10,11] pheophytin b,[10,11] and methyl pheophorbide a[10,12] were kindly provided by Prof. N. Nakatani (Osaka, Japan), Dr. E. Zass (Zurich, Switz.), and Dr. H. Fukawa (Saitama, Japan) respectively; 13^2-hydroxypheophytin a was prepared by autoxidation of pheophytin a in methanol in the same manner as in the preparation of 13^2-hydroxychlorophyll a;[13] methyl pheophorbide b was prepared from pheophytin b by ester exchange in methanol in the same manner as described in the literature;[10,12,14] methyl 13^2-decarboxymethylpheophorbide a was prepared by heating methyl pheophorbide a with collidine;[12,14] hematoporphyrin dimethyl ester was prepared from hematoporphyrin by treatment with diazomethane.[14] The structures of the compounds described above were confirmed by their NMR, UV-VIS and mass spectra. The methyl ester derivatives **5**—**8** were obtained from the naturally occurring phytyl esters **1**—**4** by ester exchange in methanol.[12,14]

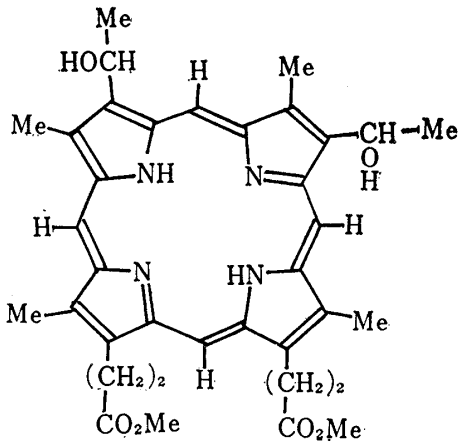
Results

Structures of Pheophytins 1—4 (Figure 2)[15]

1: **Pheophytin a**——The structure of **1** was confirmed by comparison of its NMR spectrum with that of an authentic sample as well as by co-injection of both samples on HPLC. Further confirmation was obtained by ester exchange of **1** in methanol,[12,14] which afforded the compound **5**, identified as methyl pheophorbide a in the same manner as described above.



1—9



10

Compounds	R^1	R^2	R^3	R^4
1	Me	H	CO_2CH_3	Phytyl
2a	Me	OH	CO_2CH_3	Phytyl
2b	Me	CO_2CH_3	OH	Phytyl
3	CHO	H	CO_2CH_3	Phytyl
4a	CHO	OH	CO_2CH_3	Phytyl
4b	CHO	CO_2CH_3	OH	Phytyl
5	Me	H	CO_2CH_3	Me
6a	Me	OH	CO_2CH_3	Me
6b	Me	CO_2CH_3	OH	Me
7	CHO	H	CO_2CH_3	Me
8a	CHO	OH	CO_2CH_3	Me
8b	CHO	CO_2CH_3	OH	Me
9	Me	H	H	Me

Phytyl=(2*E*)−(7*R*, 11*R*)-3,7,11,15-tetramethyl-2-hexadecenyl

Fig. 2. Structures of Compounds **1**—**10**

2: **13^2-Hydroxy (13^2-*R*, *S*) Pheophytin a**——The "compound" **2** gave one spot on Silica gel TLC, but two distinct peaks of 13^2-diastereomers were observed on HPLC (Table IV): **2a** and **2b** (ratio 2: 1, evaluated by HPLC). The structures of the two epimers were established by NMR comparison of the mixture **2** with an authentic specimen obtained by autoxidation of pheophytin a. This identification was supported by co-injection of both samples on HPLC. However, this autoxidation afforded the products **2a** and **2b** in the ratio of 4:5; it is therefore probable that the hydroxylation process is at least partly metabolic. The NMR spectrum of the mixture of **2a** and **2b** (Table I) shows close signals which are attributed to the two epimers and whose relative intensities correspond to the ratio 2: 1, determined by HPLC. Further confirmation was obtained from the NMR spectrum of **6**, a mixture of methyl 13^2-hydroxy (13^2-*R*, *S*) pheophorbides a **6a** and **6b**, prepared by ester exchange of **2** in methanol.[12,14]

3: **Pheophytin b**——The structure of **3** was confirmed by comparison of its NMR spectrum with that of an authentic sample as well as by co-injection on HPLC. Further confirmation was obtained by ester exchange of **3** in methanol,[12,14] which gave the compound **7**, identified as methyl pheophorbide b in the same manner as described for **3**.

4: **13^2-Hydroxy (13^2-*R*, *S*) Pheophytin b**——Although "compound" **4** was homogeneous as judged by Silica gel TLC, two separable peaks (2: 1) of 13^2-diastereomers were observed on HPLC (Table IV). The structure of **4** was established as follows; UV-VIS spectra: pheophytin b type substance (Table II), MS: $C_{55}H_{72}N_4O_7$ (M^+=900), *i.e.* one oxygen more than

pheophytin b, NMR: no C-13² H, instead, one OH (exchangeable with D_2O) and other protons with very similar chemical shifts to those of pheophytin b. The chemical shifts of the two epimers **4a** and **4b** (ratio 2: 1, evaluated on HPLC) were determined in the same manner as in the case of **2a** and **2b**. These assignments were supported by the NMR spectrum of **8**, a mixture of methyl 13²-hydroxy (13²-*R*, *S*) pheophorbides b **8a** and **8b**, obtained by ester exchange of **4** in methanol.[12,14]

Configuration at C-13² of the Compounds 2, 4, 6, 8

The configuration at C-13² of diastereomeric methyl 13²-methoxypheophorbides has been correlated by Inhoffen's group[16] with the ¹H NMR shifts of 17-H (C-17: R) in the following way. The 17-H is distinctly deshielded by the 13²-methoxy group when C-13² has the *R* configuration, *i.e.* when this methoxy group is located on the same side of the molecular plane as 17-H. The observed shift is +0.35. On the other hand, when C-13² has the opposite configuration *S*, the 17-H signal is little affected ($\Delta\delta$+0.10) by the presence of the 13²-methoxyl. Table I shows that, in our cases, with 13²-hydroxy instead 13²-methoxy groups, a similar dichotomy is observed: between the substances unsubstituted at C-13² (**1**, **3**, **5**, **7**) and the corresponding 13²-hydroxy diastereomers, the shifts observed for 17-H fall into two classes: one class of substances, to which we have assigned the 13²-*S* configuration and which are labelled **2**, **4**, **6**, and **8a**, in which $\Delta\delta$ is small (−0.05—+0.01), and the other one (**2**, **4**, **6**, and **8b**), in which $\Delta\delta$ is large (+0.45—+0.53), as shown below.

	Chemical shifts of C-17¹H (δ ppm)				
		C-13²*S*		C-13²*R*	
1	4.16	**2a**	4.15	**2b**	4.69
3	4.13	**4a**	4.14	**4b**	4.65
5	4.20	**6a**	4.15	**6b**	4.68
[**5** (OCH_3 instead of OH)	4.19		4.29		4.54 ref. 17]
7	4.18	**8a**	4.13	**8b**	4.63

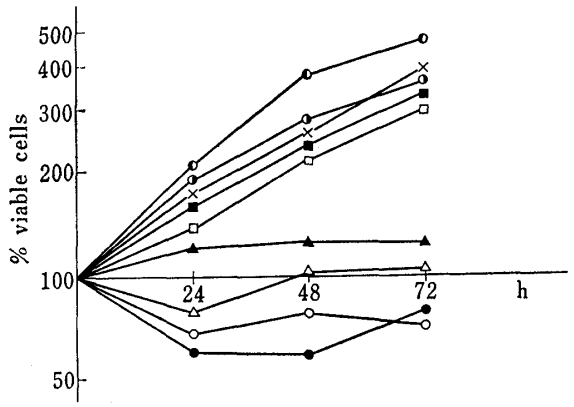


Fig. 3. Cytotoxicity of Natural Pheophytins **1**—**4** towards HTC Cells, at a Dose of 33 μg/ml in the Culture, under Illumination and in the Dark

All cultures, immediately after addition of the compounds to be tested, are normally transferred to an incubator in a dark room. For the irradiated cultures, the cells are incubated in an incubator with a glass-door and the light source, a 150-watt "Floodlight," is placed at a distance of 1 m from the culture flasks. ◐: control. ×: **1**, illumination. ◑: **1**, dark. ○: **2**, illumination. ●: **2**, dark. △: **3**, illumination. ▲: **3**, dark. □: **4**, illumination. ■: **4**, dark.

Finally, the molecular formulae of the compounds **1**—**9** were confirmed by our new method of MS spectrometry,[9] which permits observation of the molecular ions of the compounds **1**—**4**, otherwise very difficult to observe[14,17,18] (Table III).

Thus the structures **1**—**4** were established. The compounds **2** and **4** are newly identified natural products. Obara and Nozaki[3] had described 26 years ago the isolation of about 2% of "chlorophylls" from silkworm excreta, but their structures and biological activities have not previously been studied.

Biological Activity. Effect of Light

The pheophytins **2** and **3** are cytostatic (33 μg/ml) (Fig. 3). The methyl esters of pheophorbides, **5**—**9**, display much stronger activity, being cytotoxic towards HTC cells at 33 μg/ml. However, for compound **5**, this activity is only significant under illumination (Fig. 4). The same is true of the further

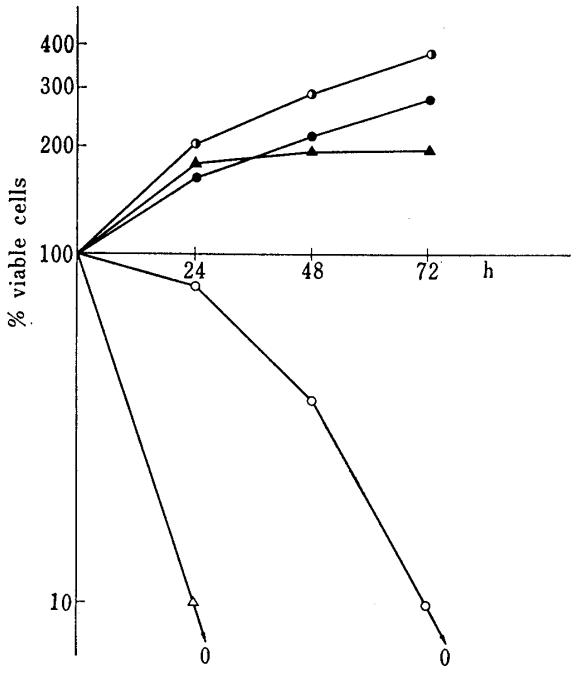


Fig. 4. Cytotoxicity of Methyl Ester of Pheophorbide a **5** (33 μg/ml and 66μg/lml) towards HTC Cells under Illumination and in the Dark

Irradiation conditions were the same as in Fig. 3. ◑: control. ○: **5**, illumination (33 μg/ml). ●: **5**, dark (33 μg/ml). △: **5**, illumination (66 μg/ml). ▲: **5**, dark (66 μg/ml).

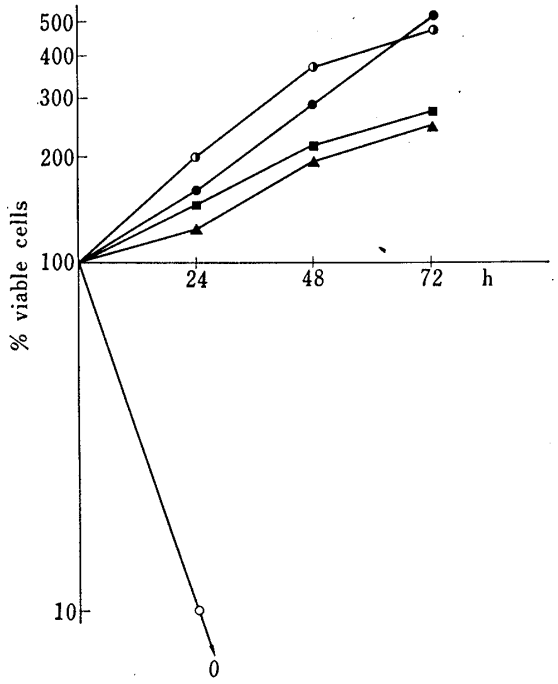


Fig. 5. Cytotoxicity of the Dimethyl Esters of Pheophorbides **6**—**9** and of Hematoporphyrin **10** (33 μg/ml) towards HTC Cells, under Illumination and in the Dark

Irradiation conditions were the same as in Fig. 3. ◑: control. ○: **6**, **7**, **8**, **9**, **10**, illumination. ▲: **6**, dark. ■: **7**, dark. ●: **8**, dark.

chlorophyll derivatives **6**—**9**, as well as of the dimethyl ester of hematophorphyrin **10** (Fig. 5). In the dark, all these substances are only weakly inhibitory of cell multiplication (substance **9** was not tested in the dark).

We have also checked, and eliminated, the possibility that the observed cytotoxicity might be due to a competitive capture, by the added porphyrins, of iron ions: the results are unchanged after addition of ferric ions, in the case of the dimethyl ester of hematoporphyrin **10**, *i.e.*, when the culture medium was supplemented with 1 and 3 eq. Fe^{3+}, no difference was observed with respect to the control cultures either in the dark or under illumination.

3T3 Tests——The products **2** and **3** are neither cytotoxic nor cytostatic at the concentration of 66 μg/ml. Substance **5** slightly inhibited the growth of non-tumor cells at 33 μg/ml. The other compounds were not tested.

Discussion

Photodynamic cytotoxicity has already been observed with certain porphyrins,[19] including hematoporphyrin, which is even used in the diagnosis and treatment of malignant tumors.[20] Similar photodynamic cytotoxicity has been observed with pheophorbide a and pyropheophorbide a *in vivo*[21] and recently *in vitro*.[22] We have now shown that it is a common property of pheophorbides.

The observed phenomena can be explained on the basis of previously postulated biochemical steps:[19,23] penetration and probably fixation of the compounds **5**—**10** in the membrane, followed by photosensitized formation of singlet oxygen according to equations 1 and 2 (P: porphyrin). The high reactivity of singlet oxygen might be at least in part responsible for the cytotoxic effects.[23]

$$P \xrightarrow{h\nu} P^{*1} \longrightarrow P^{*3} \quad (1)$$

$$P^{*3} + O_2^3 \longrightarrow P + O_2^1 \quad (2)$$

Another deactivation reaction of excited state photosensitizer was recently confirmed:[24] a one-electron transfer reaction with a molecule of oxygen produces the superoxide radical, which also has potentially damaging effects on living cells[25] (equation 3). Therefore, the participation, however limited, of the superoxide radical in the cytotoxic effect is also possible.

$$P^{*3} + O_2^3 \longrightarrow P^{+} + O_2^{-} \quad (3)$$

In contrast to the results described above, natural pheophytins **2** and **3**, which have a phytyl side-chain, also display cytostatic activity in the dark. Cell division was inhibited, but no dead cells were observed (Fig. 3). This suggests another mode of action: because of the long chain, it is possible that the molecule of the chlorophyll derivative cannot completely enter the membrane and its action may be limited to the level of the membrane-bound extrinsic enzymes.

Biological activities apparently independent of oxygen transport were described in 1962 for "chlorophyllides" of unknown structures in algae (irradiation of chlorophyll derivatives gave antibacterial substances),[26] while similar activities were recently described for the derivatives of hematoporphyrin[19a,c,20,23] and for the pheophorbides,[21,22] as we mentioned above, and also for a chlorin, bonellin, isolated from a marine echurian worm.[27]

Our findings here suggest that at least one more mechanism can be added to the biological actions of porphyrins.

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