Cytotoxic Pheophorbide-Related Compounds from *Clerodendrum calamitosum* and *C. cyrtophyllum*[§]

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Three pheophorbide-related compounds (**1**–**3**) were isolated from the leaves and stems of *Clerodendrum calamitosum*. The methyl ester of **3** (**6**) and the known (10*S*)-hydroxypheophytin a (**7**) also were isolated from leaves of the related plant *Clerodendrum cyrtophyllum*. Compounds **1** and **6** were isolated for the first time as naturally occurring products from a plant source. All structures were elucidated by detailed spectroscopic analysis. Biological evaluation showed that **1** and **2** exhibited strong cytotoxicity against human lung carcinoma (A549), ileocecal carcinoma (HCT-8), kidney carcinoma (CAKI-1), breast adenocarcinoma (MCF-7), malignant melanoma (SK-MEL-2), ovarian carcinoma (1A9), and epidermoid carcinoma of the nasopharynx (KB), and its etoposide- (KB-7d), vincristine- (KB-VCR), and camptothecin-resistant (KB–CPT) subclones. Compound **3** was less cytotoxic than **1** and **2**. Compounds **4**–**6**, the methyl esters of **1**–**3**, showed strongly increased cytotoxicity compared with the parent acids. Interestingly, **6** was the most active derivative among these compounds. Compound **7** was inactive.

Clerodendrum calamitosum L. (Verbenaceae) is a small shrub originally found in Java, but now widely cultivated in Taiwan.[2] The leaves and stems of this plant, known as "Hwa-Shih Tsao", have been used in folk medicine to treat bladder, kidney, and gall stones and as a diuretic.[2] Previous studies by Chen et al.[3] reported that the crude extract of this plant exhibited antihypertensive, sedative, and anti-bacterial effects, but no literature reports describing the cytotoxic effects or the active chemical constituents of this plant have yet appeared. The related *Clerodendrum cyrtophyllum* Turcz (Verbenaceae) is a perennial shrub, indigenous to central and northern Taiwan. Its stems and roots are used in traditional Chinese medicine to treat beriberi, fever, jaundice, leukorrhea, syphilis, and typhoid.[4,5]

In the course of screening medicinal plants for potential antitumor agents, we found that a chloroform-soluble extract of the leaves of *C. calamitosum* showed significant in vitro cytotoxicity against tissue-cultured cells of human epidermoid carcinoma of the nasopharynx (KB). Subsequent bioassay-directed fractionation of this extract led to the isolation of three pheophorbide-type compounds, **1**–**3**. Their structures were established from spectral data, including UV, MS, ¹H and ¹³C NMR, DEPT, COSY, HETCOR, HMBC, HMQC, and NOESY, and by chemical interconversions between these compounds. Among them, **1** has been isolated as a new naturally occurring compound from this plant, although it has been prepared previously semisynthetically.[6]

Lai et al.[7] and Terwel and van der Hoeven[8] have shown that vegetable extracts containing chlorophylls are anti-mutagenic in a bacterial assay system, and chlorophyllin has been shown to be a potent antimutagenic[9–11] and anticarcinogenic agent.[12–15] However, data demonstrating cytotoxic or antitumor activities of chlorophyll-related compounds are very limited. Nakatani et al.[16] and Nakamura et al.[17] reported that pheophorbides showed photocytotoxic effects toward the tumor cells upon direct photoirradiation.

We report herein on the isolation and characterization of pheophorbide-type constituents of *C. calamtosum* and *C. cyrtophyllum* and the cytotoxicity of these compounds against a panel of human tumor cell lines, including A549, HCT-8, CAKI-1, MCF-7, SK-MEL-2, 1A9, and KB and its drug-resistant KB-7d, KB-VCR, and KB-CPT subclones, compared with their structurally related compounds, chlorophyll and chlorophyllin, under ambient light treatment.

Results and Discussion

Ground, air-dried leaves and stems of *C. calamitosum* were extracted with methanol, and the methanol extract was further fractionated successively with *n*-hexane, chloroform, and ethyl acetate. Repeated chromatography of the chloroform fraction led to the isolation of **1**–**3**. The methyl ester derivatives, **4**–**6**, were obtained from **1**–**3**, respectively, by diazomethane methylation. Similarly, the dried leaves of *C. cyrtophyllum* were extracted with methanol, and the methanolic extract was further fractionated successively with *n*-hexane and chloroform. Repeated chromatography of the chloroform fraction led to the isolation of **6** [methyl (10*S*)-hydroxypheophorbide *a*] and **7** [(10*S*)-hydroxypheophytin *a*].

Compound **1**, a dark brown amorphous solid, had a molecular weight of 638 by FABMS. It was identified as purpurin 7 dimethyl ester by comparison of its ¹H and ¹³C NMR data with those in the literature[6,18] and of similar compounds, including **2** and **3**.[19–21] In addition, the structure was supported by NOE correlations of the 9a-methyl protons with both the 5a- and 10b-methyl signals.

Compound **2**, dark green crystals, had a molecular weight of 592 by FABMS. It was identified as pheophorbide *a* by comparison of ¹H and ¹³C NMR spectral data with those reported in the literature.[19–21]

Table 1. ^{1}H NMR Data of **1**–**7** (δ, in CDCl$_3$)$^{a-c}$

position	1	2	3	4	5	6	7^d
1a	3.26 (s)	3.32 (s)	3.42 (s)	3.34 (s)	3.37 (s)	3.43 (s)	3.37 (s)
2a	7.80 (dd, 18, 12)	7.85 (dd, 18, 12)	8.01 (dd, 18, 12)	7.92 (dd, 18, 12)	7.91 (dd, 18, 12)	8.02 (dd, 18, 12)	7.89 (dd, 18, 12)
2b (*E*)	6.21 (d, 18)	6.19 (d, 18)	6.30 (d, 18)	6.30 (d, 18)	6.24 (d, 18)	6.31 (d, 18)	6.20 (dd, 18, 1)
2b (*Z*)	6.06 (d, 12)	6.09 (d, 12)	6.19 (d, 12)	6.15 (d, 12)	6.13 (d, 12)	6.21 (d, 12)	6.11 (dd, 12, 1)
α	9.21 (s)	9.21 (s)	9.48 (s)	9.32 (s)	9.27 (s)	9.47 (s)	9.29 (s)
3a	3.10 (s)	3.09 (s)	3.26 (s)	3.19 (s)	3.14 (s)	3.27 (s)	3.09 (s)
4a	3.58 (q, 8)	3.53 (q, 8)	3.72 (q, 8)	3.59 (q, 8)	3.58 (q, 8)	3.73 (q, 8)	3.53 (q, 8)
4b	1.63 (t, 8)	1.61 (t, 8)	1.70 (t, 8)	1.70 (t, 8)	1.65 (t, 8)	1.71 (t, 8)	1.592 (t, 8)
β	9.54 (s)	9.37 (s)	9.62 (s)	9.60 (s)	9.43 (s)	9.61 (s)	9.47 (s)
5a	3.58 (s)	3.61(s)	3.73(s)	3.53 (s)	3.60 (s)	3.73 (s)	3.70 (s)
7	4.68 (br dd, 9, 2)	4.18 (br dt, 9, 2)	4.18 (dd, 9, 3)	4.68 (br d, 9)	4.21 (br. d, 8)	4.15 (dd, 9, 3)	4.17 (dd, 9, 3)
7a	2.38 (m)	2.61 (m)	2.98 (ddt, 14, 9, 3)	2.37 (m)	2.65 (m)	2.98 (m)	2.95 (m)
7a′	2.10 (m)	2.28 (m)	2.37 (ddd, 14, 9, 6)	2.10 (m)	2.30 (m)	2.35 (m)	2.34 (m)
7b	2.09 (dd, 3, 9)	2.55 (m)	2.64 (dt, 9, 9)	2.08 (dd, 3, 9)	2.52 (m)	2.66 (m)	2.57 (m)
7b′	1.76 (m)	2.21 (m)	2.25 (dt, 14, 9, 4)	1.76 (m)	2.25 (m)	2.27 (m)	2.27 (m)
7d				3.60 (s)	3.67 (s)	3.62 (s)	
8	4.28 (q, 7)	4.42 (dq, 2, 7)	4.49 (q, 7)	4.30 (m)	4.46 (m)	4.48 (q, 7)	4.50 (q, 7)
8a	1.77 (d, 7)	1.80 (d, 7)	1.61 (d, 7)	1.79 (d, 7)	1.80 (d, 7)	1.61 (d, 7)	1.586 (d, 7)
δ	8.45 (s)	8.51 (s)	8.64 (s)	8.49 (s)	8.55 (s)	8.61(s)	8.61(s)
9a	3.84 (s)			3.88 (s)			
10		6.22 (s)			6.27 (s)		
10b	4.12 (s)	3.86 (s)	3.62 (s)	4.14 (s)	3.90 (s)	3.56 (s)	3.62 (s)
NH		−1.69	−1.81		−1.67		
OH-10			5.60 (br. s)			5.31(s)	5.50 (s)

^{a} All assignments were based on COSY, NOESY, and TOCSY experiments. ^{b}s = singlet, d = doublet, t = triplet, m = multiplet, br = broad. ^{c}500 Hz, J values in parentheses in Hz. ^{d}Phytyl proton assignments given in Experimental Section.
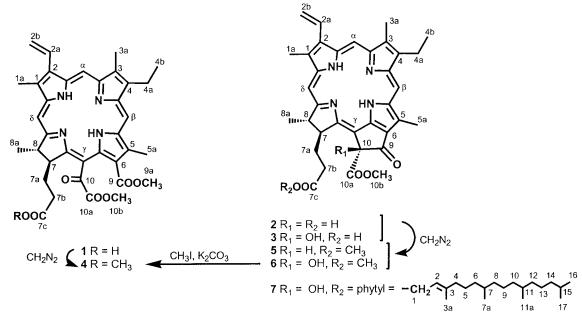

Figure 1. Structures and reactions of pheophorbide *a* related compounds.

Compound **3**, dark green crystals, had a molecular weight of 608 by FABMS and was identified as (10*S*)-hydroxypheophorbide *a* by comparison of ^{1}H and ^{13}C NMR spectral data with literature values.[19]

The detailed proton signal assignments of **1**–**3** are listed in Table 1. All three compounds were converted to the corresponding methyl esters (**4**–**6**) by treatment with diazomethane. Furthermore, **6** underwent ring opening and esterification to give the β-keto ester **4** using methyl iodide in the presence of potassium carbonate.

Compound **6**, a dark green amorphous solid, showed a protonated molecular ion peak at m/z 622 [M$^+$] in the FABMS. Its ^{1}H NMR spectrum and TLC R_f value were identical to those of the methyl ester synthesized from **3** obtained from *C. calamitosum* above. This report is the first description of its isolation as a naturally occurring compound.

Compound **7**, a dark green amorphous solid, showed a protonated molecular ion peak at m/z 886 [M$^+$] in the FABMS. Its ^{1}H and ^{13}C NMR spectra closely resembled those of **6** except for the presence of a phytyl group and the absence of one methyl ester. Compound **7** was identified as (10*S*)-hydroxypheophytin *a* by comparison of its ^{1}H NMR data with those reported in the literature.[16]

All compounds isolated and prepared in this study were assayed for in vitro cytotoxicity against a panel of human tumor cell lines. The results are shown in Tables 2 and 3. Compounds **1**–**3** showed similar trends of tumor specificity, being most cytotoxic against the KB and 1A9 cell lines, less cytotoxic against the CAKI-1 and SK-MEL-2 cell lines, and the least cytotoxic against the HCT-8, A-549, and MCF-7 cell lines. Among these compounds, **2** displayed both strong and broad activity for the KB, 1A9, CAKI-1, SK-MEL-2, HCT-8, A549, and MCF-7 tumor cells, with ED$_{50}$ values of

Table 2. Cytotoxicity (ED_{50})[a] of Pheophorbide *a* Compounds and Pheophytins against Human Tumor Cell Lines[b]

compound	KB	A-549	HCT-8	CAKI-1	1A9 (OVCAR)	MCF-7	SK-MEL
1	0.70	1.8	1.6	1.3	0.64	1.6	1.1
2	0.46	1.5	1.5	0.88	0.48	1.6	0.91
3	1.3	>4	>4	3.0	1.2	>4	4.0
4	0.20	0.49	0.79	0.55	0.20	0.73	1.1
5	0.23	0.83	1.0	0.62	0.26	0.95	1.2
6	0.30	0.47	0.40	1.9	0.27	0.45	0.84
7	12	15	14	19	22	8.8	14
chlorophyll	63	NT[c,d]	NT[c,d]	NT[c,d]	64	NT[c,d]	NT[c,d]
copper sodium chlorophyllin	38	39	>40	NT[d]	31	>40	NT[d]

[a] Cytotoxicity as ED_{50} in μg/mL for each cell line is the concentration of compound that causes a 50% reduction in absorbance at 562 nm relative to untreated cells using SRB assay. [b]Cell lines cultured in RPMI-1640, 10% (v/v) FBS, 100 μg/mL kanamycin. [c]ED_{50} values of chlorophyll against KB and 1A9 cell growth were >6 μg/mL—not considered to be significant and not further evaluated. [d]NT = not tested.

Table 3. Cytotoxicity of Pheophorbide *a* Compounds against Drug-Resistant Cell Lines

compound	KB-7d[a]	KB-VCR[a]	KB-CPT[a]
1	0.7	0.5	1.0
2	1.0	0.6	1.1
6	0.8	>57	1.1
VP-16	119	153	1.0
Taxol	1.0	15	1.0

[a] Values are fold-resistance relative to activity in KB cells.

0.46, 0.48, 0.88, 0.91, 1.5, 1.5, and 1.6 μg/mL, respectively. Compound **1** showed similar but slightly lower cytotoxicity, while **3** was less potent. This latter compound exerted significant activity against the KB and 1A9 cell lines with ED_{50} values of 1.3 and 1.2 μg/mL, respectively, moderate activity against the CAKI-1 cell line with an ED_{50} value of 3.0 μg/mL, borderline activity against the SK-MEL-2 cell line, and insignificant activity (ED_{50} > 4 μg/mL) against the HCT-8, A549, and MCF-7 cell lines. The cytotoxicities of chlorophyll (commercial mixture of a and b) and chlorophyllin were also evaluated for comparison purposes. The ED_{50} values of chlorophyllin against the KB, 1A9, and A549 cell lines were not significant. Chlorophyll was also inactive against the KB and 1A9 cell lines, with ED_{50} values significantly greater than 4 μg/mL. Therefore, chlorophyllin and chlorophyll were not evaluated further. In addition, (10*S*)-hydroxypheophytin a (**7**) was not active, as shown in Table 2.

The methyl esters **4**−**6** showed increased cytotoxicity against the cell lines tested except for the SK-MEL-2 cell line, when compared with the corresponding acid. For example, **4**−**6** were in turn 4-fold, 2-fold, and 4-fold more cytotoxic against KB cells in comparison with **1**−**3**, respectively. In addition, all three methylated derivatives showed parallel trends of cell line specificity to those of the parent compounds and had similar cytotoxicity against KB and 1A9 cells, with ED_{50} values ranging from 0.2 to 0.30 μg/mL; however, cytotoxicity in other cell lines varied widely. For example, **4**−**6** showed varying cytotoxicity against HCT-8 cells, with ED_{50} values of 0.79, 1.0, and 0.40 μg/mL, respectively. Methylation did not affect the cytotoxicity of **1** and **2** against SK-MEL-2 cells, whereas methylation markedly changed the cytotoxicity of **3** against SK-MEL-2 cells (ED_5 for **3**, 4 μg/mL; for **6**, 0.84 μg/mL). In general, **6** showed potent and broad spectrum activity against all seven cell lines tested, with ED_{50} values ranging from 0.27 to 1.9 μg/mL, while the parent acid **3** was not significantly cytotoxic against the A549, HCT-8, and MCF-7 cell lines.

Compounds **1**, **2**, and **6** were also evaluated against drug-resistant cell lines (Table 3). Cross-resistance usually is seen against natural products in at least one of two drug-resistant cell lines, as shown for taxol and etoposide (VP-16) (Table 3). However, the cytotoxicity of **1** and **2** was not affected adversely by MRP (KB-7d) or P-glycoprotein (MDR) overexpression (KB-VCR). The cytotoxicity of **6** was not adversely affected in the KB-7d and KB-CPT cell lines; however, cytotoxicity decreased significantly in the KB-VCR cell line. Interestingly, compared with the parental cell line, the growth of the two multidrug-resistant cell lines was more susceptible to **1** (about 2-fold for the MDR derivative).

Compounds **1**−**3** are structurally closely related. Compounds **2** and **3** both contain the same cyclopentanone ring and differ only in the substitution at the C-10 position (H and OH, respectively), while **1** is a ring-opened analogue with a ketone group at C-10. Both **1** and **2** showed comparable cytotoxicity, while **3** was less cytotoxic than **1** and **2**. Methylation of the COOH-7c groups in **1** and **2** led to derivatives (**4**, **5**) with equal or slightly higher potency than the parent compounds. However, methylation of the COOH group in **3** gave **6**, which showed strongly increased cytotoxicity compared with **3**. Except in the CAKI-1 cell line, **6** was as potent or more potent than **4** and **5**. These findings may indicate that, in the parent acid **3**, intramolecular hydrogen bonding occurs between the OH-10 and COOH-7c groups, and this interaction is subsequently prevented by methylation of **3** at the 7c-position, allowing the C-10 hydroxy group to interact more freely with a target receptor. On the other hand, we found that chlorophyll, chlorophyllin, and the pheophytins showed no significant cytotoxicity. This lack of activity might be due to the long-chain phytyl group at the 7c-COOH, which could limit the penetration through the tumor cell membrane and thus decrease drug action. Detailed structure−cytotoxicity relationships remain to be explored.

Although some reports regarding antimutagenic and anticarcinogenic effects of chlorophyll and chlorophyllin have been published, data demonstrating antitumor activity of chlorophyll-related compounds have rarely been reported. In 1981, Nakatani et al. reported that 13²-hydroxy-(13²-*R,S*)-pheophytin a and -pheophytin b, isolated from silkworm excreta,[16] have in vitro cytostatic activity against hepatoma tissue culture (HTC) cells at a dose of 33 μg/mL in culture medium, and six methyl ester derivatives of pheophorbides showed cytotoxic effects toward HTC cells at 33 or 66 μg/mL under direct photoirradiation. In the dark, all these substances are only weak inhibitors of cell multiplication. The authors explained the observed phenomena as photodynamic destruction of HTC cells by singlet oxygen[22,23] and superoxide radical.[24] Also, Nakamura et al.[17] reported that pheophorbides (**2** and **3**) from leaves of *Neptunia oleracea* exhibited photocytotoxicity against Raji cells with photoirradiation.

In our study, **1** and **2**, extracted from the herb *C. calamitosum*, were identified as potent cytotoxic agents

against seven solid tumor cell lines without direct illumination. We demonstrated that methyl ester derivatives **5** and **6**, which were also studied by Nakatani et al.,[16] displayed much stronger cytotoxicity against tumor cell lines tested, without direct photoirradiation. Previous papers[25,26] postulating the mechanism of action for cytotoxic pheophorbides focus on photodynamic cytotoxicity involving several steps: penetration and probable fixation of the compounds in cellular membranes, photosensitized formation of highly reactive singlet oxygen and superoxide radical, and damage to essential cellular components including nucleic acids, proteins, and lipids. Our recent findings suggest that the cytotoxic effect of chlorophyll-related compounds can occur through mechanisms other than phytodynamic action. Mechanism of action studies are currently in progress.

Experimental Section

General Experimental Procedures. Melting points were determined on a Fisher-Johns melting apparatus and are uncorrected. The optical rotation was measured on a JASCO DIP-1000 digital polarimeter. The UV–vis spectrum was recorded on a Shimadzu UV-2101PC UV–vis scanning spectrophotometer. NMR spectra were measured on a Varian Inova 500 MHz spectrometer with TMS as internal standard. Chemical shifts are reported in δ (ppm). FABMS were measured on a VG analytical VG-70E mass spectrometer. Precoated Si gel plates (Kieselgel 60 F_{254} 0.25 mm, Merck) were used in TLC analysis for monitoring isolation and separation. Chlorophyll was obtained from Carl Roth GmbH, Germany, and chlorophyllin from Spectrum, Inc., Gardena, CA.

Plant Material. In September, 1997, leaves and stems of *Clerodendrum calamitosum* were collected in Taipei, Taiwan, and leaves of *Clerodendrum cyrtophyllum* were collected in Cha-Yee, Taiwan. Dr. Zuei-Ching Chen, Department and Graduate Institute of Botany, National Taiwan University, Taipei, Taiwan, identified both species. Voucher specimens are deposited at the Department of Biochemistry, Taipei Medical College.

Extraction and Isolation of 1–3. The freshly collected leaves and stems (4 kg) of *C. calamitosum* were air-dried, ground, and extracted repeatedly with MeOH. The combined MeOH extracts were evaporated in vacuo to yield a dark greenish syrup. The concentrated syrup was mixed with Celite, dried in a vacuum oven, and packed on a Si gel column, then eluted successively with *n*-hexane, CHCl₃, EtOAc, and MeOH. Isolation of cytotoxic principles from the MeOH extract was guided by bioassay-directed fractionation. Because the CHCl₃ fraction showed more potent cytotoxicity than the others in the KB cell assay, this fraction was subjected to Si gel column chromatography using CHCl₃–MeOH (100:1→1:1) and MeOH as eluents. The eluted fractions were monitored by TLC and combined into 13 pooled fractions. The active fractions were chromatographed repeatedly over Si gel, eluting with EtOAc, EtOAc–MeOH (10:1→1:1), CHCl₃, CHCl₃–MeOH (10:1→1:1), then MeOH, and over microcrystalline cellulose eluting with CHCl₃ to give active principles **1** (45 mg) and **2** (140 mg). Compound **3** (265 mg) was obtained from the EtOAc fraction by repeated cellulose microcrystalline chromatography eluting with CHCl₃.

Purpurin 7 dimethyl ester (1): dark brown amorphous solid; mp 196–198 °C (softened); $[\alpha]^{24}_D$ +76.7° (*c* 0.07, CHCl₃); UV–vis (CHCl₃) λ_{max} (log ϵ) 681 (4.25), 548 (3.91), 506 (3.81), 430–330 (flat top, 4.35), 279 (4.04) nm; ¹H NMR data, see Table 1; ¹³C NMR δ 131.4 (s, C-1), 11.9 (q, C-1a), 136.3 (s, C-2), 128.7 (d, C-2a), 122.6 (t, C-2b), 135.6 (s, C-3ᵃ), 11.0 (q, C-3a), 145.7 (s, C-4), 19.4 (t, C-4a), 17.5 (q, C-4b), 129.6 (s, C-5), 12.9 (q, C-5a), 119.4 (s, C-6), 52.5 (d, C-7), 31.3 (t, C-7a), 30.8 (t, C-7b), 178.0 (s, C-7cᵇ), 49.6 (d, C-8), 23.0 (q, C-8a), 167.7 (s, C-9), 53.2 (s, C-9a), 186.4 (s, C-10), 166.5 (s, C-10a), 51.9 (q, C-10b), 142.7 (s, C-11), 135.8 (s, C-12ᵃ), 156.1 (s, C-13), 149.0 (s, C-14), 138.6 (s, C-15), 136.6 (s, C-16), 164.0 (s, C-17), 174.3

(s, C-18ᶜ), 100.7 (d, C-α), 106.3 (d, C-β), 106.1 (s, C-γ), 93.7 (d, C-δ) (*a,b* assignments[29] may be interchangeable); FABMS *m/z* 639 [M + H]⁺.

Pheophorbide *a* (2): dark green amorphous solid; ¹H NMR data, see Table 1; FABMS *m/z* 593 [M + 1]⁺.

(10*S*)-Hydroxypheophorbide *a* (3): dark green amorphous solid; ¹H NMR data, see Table 1; FABMS *m/z* 608 [M]⁺.

Methylation of 1–3. To ethyl ether solutions of **1** (20 mg), **2** (10 mg), or **3** (10 mg) was added excessive fresh diazomethane ethyl solution. Each solution was stood overnight, and the solvent was removed in vacuo. The residue was purified by a cellulose microcrystalline column eluting with CHCl₃ to give **4** (16 mg), **5** (8 mg), or **6** (8 mg), respectively. The structures of all methyl esters were confirmed from the presence of one extra methoxy group signal in the proton NMR spectra (Table 1).

Methylation of 6. To a dichloromethane solution of **6** (10 mg) were added 0.5 mL of methyl iodide and 10 mg of K₂CO₃, and the mixture was stirred at room temperature overnight. After filtration and evaporation, the residue was purified by a microcrystalline cellulose column eluting with CHCl₃ to give **4** (8 mg).

Extraction and Isolation of 6 and 7. The dried leaves (15 kg) of *C. cyrtophyllum* were extracted with MeOH. The MeOH extract was treated as described above to give an *n*-hexane extract (IM1) and three CHCl₃ extracts (IM2-1, -2, and -3). Bioassay-directed fractionation was used to isolate the cytotoxic principles. The first CHCl₃ fraction (IM2-1) was subjected to Si gel column chromatography using CHCl₃–MeOH (100:1 → 1:1) and finally MeOH as eluent. The eluted fractions were monitored by TLC and combined into 10 fractions. The active fractions were chromatographed repeatedly over Si gel eluting with CHCl₃–MeOH (100:1 → 1:1), MeOH, benzene–EtOAc (10:1 → 3:1), and acetone to give **6** (107 mg) and **7** (57 mg).

Methyl (10*S*)-hydroxypheophorbide *a* (6): dark green amorphous solid; ¹H NMR data, see Table 1; ¹³C NMR δ 131.7 (s, C-1), 12.0 (q, C-1a), 136.3 (s, C-2), 128.9 (d, C-2a), 122.7 (t, C-2b), 136.08 (s, C-3ᵃ), 11.0 (q, C-3a), 145.0 (s, C-4), 19.3 (t, C-4a), 17.3 (q, C-4b), 129.3 (s, C-5ᵇ), 12.2 (q, C-5a), 126.9 (s, C-6ᵇ), 51.8 (d, C-7), 31.4 (t, C-7a), 31.1 (t, C-7b), 174.0 (s, C-7c), 51.7 (q, C-7d), 50.3 (d, C-8), 22.6 (q, C-8a), 191.9 (s, C-9), 89.0 (s, C-10), 172.3 (s, C-10aᶜ), 53.4 (q, C-10b), 142.0 (s, C-11), 136.11 (s, C-12ᵃ), 155.2 (s, C-13), 150.9 (s, C-14), 137.7 (s, C-15), 149.8 (s, C-16), 162.4 (s, C-17), 172.8 (s, C-18ᶜ), 97.8 (d, C-α), 104.0 (d, C-β), 107.6 (s, C-γ), 93.6 (d, C-δ) (*a–c* assignments may be interchangeable); FABMS *m/z* 622 [M⁺].

(10*S*)-Hydroxypheophytin a (7): dark green amorphous solid; ¹H NMR data, see Table 1 for pheophorbide skeleton; for phytyl protons, δ 4.57 (2H, dd, *J* = 2, 7 Hz, H-P1), 5.23 (1H, dt, *J* = 2, 7 Hz, H-P2), 1.59 (3H, s, H-P3a), 0.86 (3H, s, H-P7aᵃ), 0.84 (3H, s, H-P11aᵃ), 1.50 (1H, sept, *J* = 7 Hz, H-P15), 0.79 (1H, d, *J* = 7 Hz, H-P15aᵇ), 0.81 (1H, dd, *J* = 7 Hz, H-P16ᵇ); ¹³C NMR δ 131.7 (s, C-1), 12.0 (q, C-1a), 136.3 (s, C-2), 129.0 (d, C-2a), 122.7 (t, C-2b), 136.2 (s, C-3ᶜ), 11.1 (q, C-3a), 145.0 (s, C-4), 19.3 (t, C-4a), 17.3 (q, C-4b), 127.0 (s, C-5ᵈ), 12.2 (q, C-5a), 129.3 (s, C-6ᵈ), 51.8 (d, C-7), 31.7 (t, C-7a), 31.2 (t, C-7b), 173.5 (s, C-7c), 50.4 (d, C-8), 22.7 (q, C-8aᵉ), 191.9 (s, C-9), 89.0 (s, C-10), 172.3 (s, C-10a), 53.4 (q, C-10b), 142.0 (s, C-11ᶠ), 136.1 (s, C-12 ᶜ), 155.2 (s, C-13), 150.9 (s, C-14), 137.8 (s, C-15), 149.9 (s, C-16), 162.5 (s, C-17), 172.8 (s, C-18), 97.8 (d, C-α), 104.1 (d, C-β), 107.7 (s, C-γ), 93.6 (d, C-δ), for phytyl carbons, 61.5 (t, C-P1), 118.0 (d, C—P2), 142.7 (s, C-P3ᶠ), 16.3 (q, C-P3a), 39.8 (t, C-P4), 25.0 (t, C-P5), 36.7 (t, C-P6), 32.6 (d, C-P7), 19.71 (q, C-P7aᵍ), 37.4 (t, C-P8ʰ), 24.4 (t, C-P9), 37.33 (t, C-P10ʰ), 32.8 (d, C-P11), 19.65 (q, C-P11aᵍ), 37.27 (t, C-P12ʰ), 24.8 (t, C-P13), 39.4 (t, C-P14), 27.9 (d, C-P15), 22.60 (q, C-P15aᵉ), 22.66 (q, C-P16ᵉ) (*a–h* assignments may be interchangeable); FABMS *m/z* 886 [M⁺].

Growth Inhibition Assay. The SRB assay previously described by Skehan et al.[27,28] was adapted to the measurement of cellular viability, but with some modification. This assay is based on the spectrophotometric determination of sulforhodamine B (SRB), a pink aminoxanthine dye, bound to cellular protein.[29] Drug stock solutions were prepared in

DMSO and stored at −70 °C. Upon dilution into culture medium, the final DMSO concentration was ≤2% DMSO (v/v), a concentration without effect on cell replication. The human tumor cell line panel consisted of epidermoid carcinoma of the nasopharynx (KB), three subclones resistant to etoposide (KB-7d), vincristine (KB-VCR), and camptothecin (KB-CPT), respectively, lung carcinoma (A549), ileocecal carcinoma (HCT-8), renal cancer (CAKI-1), breast adenocarcinoma (MCF-7), malignant melanoma (SK-MEL-2-2), and ovarian carcinoma (1A9). All stock cultures were grown in T-25 flasks containing 4 mL of RPMI-1640 medium supplemented with 25 mM HEPES, 0.2% (w/v) sodium bicarbonate, 10% (v/v) fetal bovine serum, and 100 μg/mL kanamycin at 37 °C in a humidified atmosphere containing 5% CO_2. Freshly trypsinized cell suspensions were seeded in 96-well mictrotiter plates at densitites of 2500−10 000 cells per well. Initial seeding densities varied among the cell lines to ensure a final absorbance reading in control (untreated) cultures in the range 1−2.5 A_{560} units. Tumor cells were incubated at 37 °C for 72 h in the presence of various concentrations of drugs from DMSO-diluted stock. Cultures were monitored briefly at daily intervals by microscopic examination. Cytotoxic action generally developed after 1 day exposure and resulted in crenulation and shrinkage of cells. After 3 days, attached cells were fixed with ice-cold 50% trichloroacetic acid and then stained with 0.4% (w/v) sulforhodamine B (SRB) (Sigma Chemical Co., St. Louis, MO). The absorbance at 562 nm was measured using an automated microculture plate reader (Molecular Devices, Menlo Park, CA) after solubilizing the bound dye. The ED_{50} values, the drug concentration resulting in 50% growth inhibition, were interpolated from dose−response data. Each test was performed in triplicate, and absorbance readings varied no more than 5%. ED_{50} values determined in independent tests varied no more than 30%.

References and Notes

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