Unexpected Novel Pheophytin Peroxides from the Leaves of *Biden pilosa*

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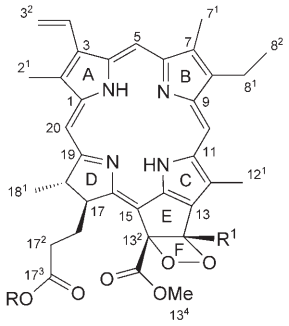
Two new pheophytins, bidenphytins A (**1**) and B (**2**), with peroxide functionalities on ring *E*, were isolated from *Biden pilosa* LINN. var. *radiata* SCH. BIP., a popular Taiwanese folk medicine. Also isolated were the following six known compounds: pheophytin *a* (**3**), (13^2*R*)-13^2-hydroxypheophytin *a* (**4**), (13^2*S*)-13^2-hydroxypheophytin *a* (**5**), (13^2*R*)-13^2-hydroxypheophytin *b* (**6**), (13^2*S*)-13^2-hydroxypheophytin *b* (**7**), and aristophyll-C (**8**). Their structures were elucidated by spectroscopic methods (UV, IR, 1D- and 2D-NMR) and by mass spectrometry (HR-FAB-MS). Possible biosynthetic pathways for **1** and **2** are proposed.

Introduction. – *Biden pilosa* LINN. var. *radiata* SCH. BIP. (Compositae) is a worldwide distributed annual or perennial herb that became rampant in Taiwan only over the past two decades [1]. The whole plant and aerial parts are being used in various folk medicines (or as a popular ingredient in herbal tea) for their anti-inflammatory, antiseptic, liver-protective, blood-pressure-lowering, and hypoglycemic effects [2]. Previous phytochemical examinations on the constituents of *B. pilosa* afforded an acetylacetone [3], polyacetylenes [4], flavonoids [5–7], and a diterpenoid [8]. However, there has been no report on its chlorin-like constituents to date.

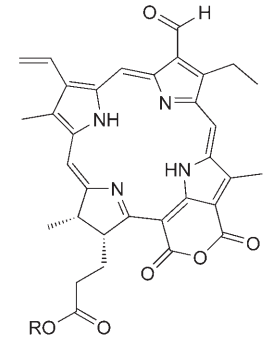
Chlorin (=2,3-dihydroporphyrin) and its derivatives – including chlorophyll, pheophytin, chlorophyllin, pheophobide, and many other closely related analogues, either found in most higher plants, algae, and even bacteria – are interesting compounds for the development of new drugs in photodynamic therapy (PDT), since they strongly absorb light above 670 nm, thereby enabling deeper penetration of radiation through tissue [9]. Among them, some naturally occurring entities have been previously shown to be effective photosensitizing agents, both *in vitro* and *in vivo* [10]. However, these compounds, considering their dihydro and tetrahydro reduction states, can be readily oxidized back to the parent porphyrin by a variety of oxidants, *e.g.*, ferric salts, I_2, or molecular ground-state oxygen ($^3O_2$), which gives rise to loss of their long-wavelength absorption bands [11]. This potential lack of stability promoted us to search other chlorin-like chromophores from natural sources assumed to contain abundant

chlorine derivatives, as assessed from the UV spectra of the corresponding hexane extracts in preliminary screenings.

Herein, we report a phytochemical examination of the hexane-soluble extract of the leaves of *B. pilosa*, which led to the isolation and identification of two novel pheophytins, compounds **1** and **2**, together with six known compounds, **3**–**8**.



1 R^1 = H
2 R^1 = OH



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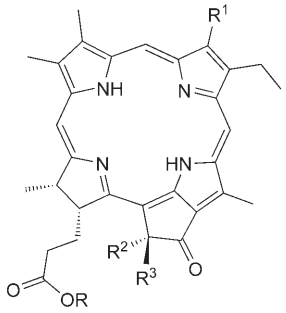
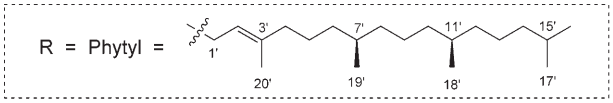


3 – 7

	R^1	R^2	R^3
3	Me	CO_2Me	H
4	Me	OH	CO_2Me
5	Me	CO_2Me	OH
6	CHO	OH	CO_2Me
7	CHO	CO_2Me	OH



Results and Discussion. – Compound **1** was optically active ($[\alpha]_D^{25} = -44.6$), and exhibited UV absorption bands at 404, 505, 534, 612, and 669 nm, indicating a chlorophyll derivative [12]. HR-FAB-MS Analysis showed the $[M+H]^+$ ion at *m/z* 887.5684 (calc. 887.5687), in accord with the molecular formula $C_{55}H_{74}N_4O_6$. The prominent differences in the ^{1}H-NMR spectrum of **1** (*Table*), as compared with that of pheophytin *a* (**3**) [13], were the presence of one extra *singlet* at δ(H) 7.23 (H–C(13^1)) as well as the absence of a *singlet* at δ(H) 6.26 ((H–C(13^2)). These differences were also reflected in the ^{13}C-NMR spectrum (*Table*), in which the C=O resonance at δ(C) 189.6 (C(13^1)) and a tertiary C-atom at δ(C) 64.7 (C(13^2)) in **3** were replaced by a methine at δ(C) 79.0 and a quaternary C-atom at δ(C) 98.9, respectively, accompanied

with significant shifts of C(13) (δ(C) 129.0 *vs.* 113.3), C(14) (150.0 *vs.* 163.4), and C(15) (105.2 *vs.* 134.6), probably caused by some substituent or conformational changes nearby. To account for these differences, both C(13^1) and C(13^2) of **1** must be oxygenated, and a C–O–O–C functionality between them should be formed to fit these changes and match the molecular formula.

Table. *^{1}H- and ^{13}C-NMR Data of* **1** *and* **2** (without the signals of the phytyl moiety). Recorded at 500/125 MHz, resp., in $CDCl_3$; δ in ppm, *J* in Hz.

Position	**1**		**2**	
	δ(C)	δ(H)	δ(C)	δ(H)
1	143.0		141.2	
2	131.6		131.5	
2^1	12.0	3.36 (*s*)	12.0	3.41 (*s*)
3	136.4		136.2	
3^1	128.9	7.95 (*dd*, *J* = 17.8, 11.5)	128.9	7.93 (*dd*, *J* = 17.8, 11.6)
3^2	122.7	6.15 (*d*, *J* = 11.5), 6.28 (*d*, *J* = 17.8)	122.6	6.13 (*d*, *J* = 11.6), 6.28 (*d*, *J* = 17.8)
4	136.2		136.0	
5	99.1	9.37 (*s*)	99.5	9.42 (*s*)
6	155.9		155.6	
7	136.3		136.4	
7^1	11.2	3.20 (*s*)	11.1	3.16 (*s*)
8	145.5		145.4	
8^1	19.5	3.64–3.70 (*m*)	19.4	3.60–3.66 (*m*)
8^2	17.5	1.66 (*t*, *J* = 7.7)	17.5	1.64 (*t*, *J* = 6.5)
9	150.0		149.9	
10	104.8	9.57 (*s*)	104.0	9.66 (*s*)
11	137.3		138.7	
12	131.5		131.4	
12^1	12.2	3.76 (*s*)	12.4	3.87 (*s*)
13	113.3		111.3	
13^1	79.0	7.23 (*s*)	102.0[a])	
13^2	98.9		100.5[a])	
13^3	170.3		170.9	
13^4	53.0	3.51 (*s*)	54.1	3.76 (*s*)
14	163.4		161.0	
15	134.6		149.9	
16	162.5		166.4	
17	52.6	4.50–4.56 (*m*)	53.7	4.07–4.13 (*m*)
17^1	30.6	2.25–2.31 (*m*), 2.59–2.65 (*m*)	31.3	1.85–1.91 (*m*), 2.57–2.63 (*m*)
17^2	30.8	1.97–2.03 (*m*), 2.43–2.49 (*m*)	32.2	2.18–2.24 (*m*), 2.46–2.52 (*m*)
17^3	173.1		173.3	
18	50.5	4.45 (*q*, *J* = 7.3)	50.2	4.45 (*q*, *J* = 7.1)
18^1	22.6	1.56 (*d*, *J* = 7.3)	22.6	1.61 (*d*, *J* = 7.1)
19	172.7		171.1	
20	93.2	8.48 (*s*)	93.9	8.70 (*s*)

[a]) Assignment may be interchanged.

The partial structure of rings *E* and *F* of **1** was further confirmed by the following key HMBC long-range correlations: H–C(13^1) to C(13^2), C(13^3), C(14), and C(15) (*Figure*). Considering the configuration of pheophytin derivatives, it is known that HO–C(13^2) exhibits a prominent deshielding effect on H–C(17) (δ(H) 4.50–4.56) when located on the same side of the porphyrin ring [14][15]. In the NMR spectrum of **1**, the signal for H–C(17), indeed, appeared at δ(H) 4.50–4.56, which indicated that H–C(17) and the C–O–O–C moiety were *cis*-oriented. The absolute configurations at both C(13^1) and C(13^2) were, thus, analogously assigned as (*R*) by spectroscopic correlation with pheophytin *a* (**3**) [13]. From these data, the structure of **1** was identified as 13^1,13^2-peroxypheophytin a[1]), and named *bidenphytin A*.

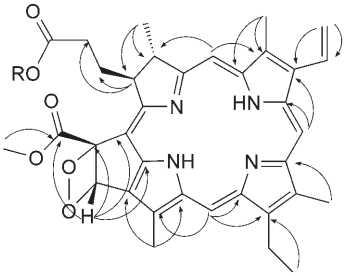


Figure. *Selected HMBC* (H → C) *correlations of* **1**

Compound **2** was optically active ($[\alpha]_D^{25} = -5.4$) and had the molecular formula $C_{55}H_{74}N_4O_7$, as deduced by HR-FAB-MS and ^{13}C-NMR spectroscopy (*Table*). The ^{1}H- and ^{13}C-NMR spectra of **2** were very similar to those of **1**, except that C(13^1) of **2** was shifted from δ(C) 79.0 to lower field (δ(C) 102.0; interchangeable with δ(C) 100.5), indicating that the methine group at C(13^1) of **1** was replaced by an OH group in **2**, in accord with the change in the molecular formulae. Further analysis of the 2D-NMR data, including COSY, NOESY, HSQC, and HMBC, allowed the complete assignment of the ^{1}H- and ^{13}C-NMR spectra of **2**, as illustrated in the *Table*. Accordingly, compound **2** was identified as 13^1-hydroxy-13^1,13^2-peroxypheophytin *a*, and named *bidenphytin B*.

The six known compounds, pheophytin *a* (**3**) [13], (13^2R)-13^2-hydroxypheophytin *a* (**4**) [16], (13^2S)-13^2-hydroxypheophytin *a* (**5**) [16], (13^2R)-13^2-hydroxypheophytin *b* (**6**) [17], (13^2S)-13^2-hydroxypheophytin *b* (**7**) [17], and aristophyll-C (**8**) [18], were identified by comparison of their ^{1}H- and ^{13}C-NMR as well as EI-MS data with those reported in the literature.

Bidenphytins A (**1**) and B (**2**), two rare four-membered peroxide-containing pheophytins, might be readily produced *via* a series of conventional chemical reactions in biosynthetic systems from pheophytin *a* (**3**) as starting material. In the proposed pathway (*Scheme*), **3** is reduced, dehydrated, and then oxidized by atmospheric oxygen (*i.e.*, by triplet oxygen; $^3O_2$) to afford **1** [19]. An alternative route can also be proposed, in which **2** is generated by a series of electron-transfer reactions in the presence of $^3O_2$ from **3**.

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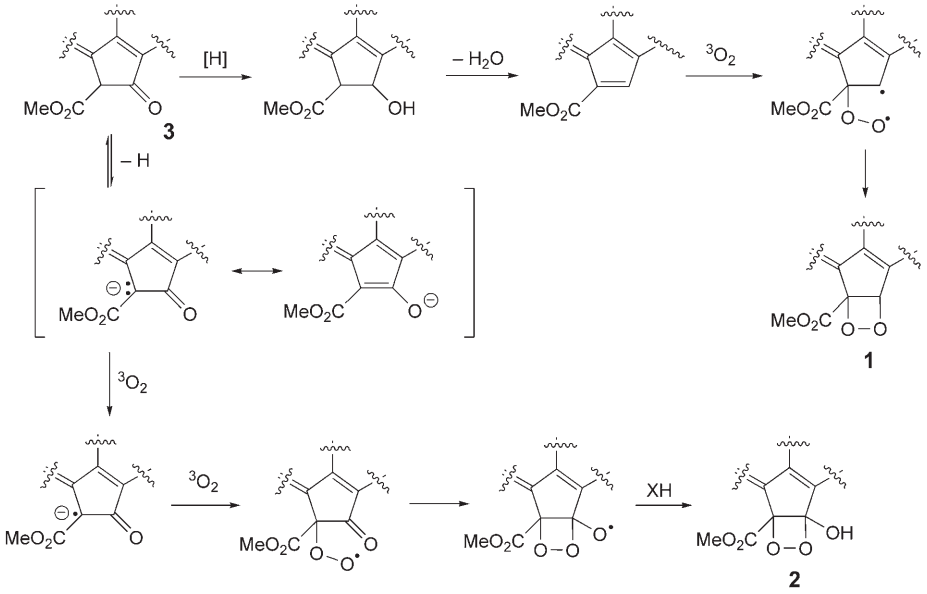
[1]) For systematic names, see *Exper. Part.*

Scheme. *Proposed Biosynthesis of* **1** *and* **2** *from pheophytin* a (**3**)



Although novel pheophytin derivatives have been obtained and cumulated from natural sources [12][20], the biosynthetic pathway of pheophytins is still poorly understood. To disclose the biosynthesis of these class of compounds, more pheophytin-like intermediates and labeling experiments will be required.

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Experimental Part

General. HPLC: *Hitachi L-7000* system with *Merck LiChrospher Si* (7 μm, 10 × 250 mm) semiprep. column. Column chromatography (CC): silica gel *60* (70–230 mesh; *Merck*). Optical rotations: *Jasco DIP-1000* digital polarimeter. IR Spectra: *Nicolet Magna-550* spectrophotometer; KBr pellets; in cm^{-1}. UV Spectra: *Shimadzu UV-1601PC* spectrometer; λ_{max} (log ε) in nm. ^{1}H- and ^{13}C-NMR Spectra: *Bruker AM-300* (at 300/75 MHz, resp.) or *DMX-500SB* spectrometers (at 500/125 MHz, resp.); in $CDCl_3$ soln.; δ in ppm rel. to Me_4Si, *J* in Hz. MS: *Finnigan MAT-95S* and *Jeol SX-102* mass spectrometers; in *m*/*z* (rel. %).

Plant Material. The leaves of *Biden pilosa* L. var. *radiata* SCH. BIP. were collected in Tainan, Northern Taiwan, in June 2003, and identified by Mr. *Nien-yung Chiu*, Institute of Chinese Pharmaceutical Science, China Medical College. A voucher specimen (200106BP2) has been deposited at the Taipei Medical University, Taipei, Taiwan.

Extraction and Isolation. The air-dried leaves of *B. pilosa* (4.5 kg) were successively extracted with hexane, AcOEt, and MeOH (3 × 60 l) at r.t. Evaporation of the org. solvent from each soln. under reduced pressure gave the corresponding hexane-soluble (62 g), AcOEt-soluble (72 g), and MeOH-soluble (49 g) extracts. The hexane-soluble part was absorbed on 70 g of SiO_2, and then subjected to CC (800 g SiO_2; hexane/AcOEt gradient) to afford five fractions: *Fr. 1* (hexane/AcOEt 10:1.5; 3000 ml), *Fr. 2* (hexane/AcOEt 10:2; 3200 ml), *Fr. 3* (hexane/AcOEt 10:3; 3500 ml); *Fr. 4* (hexane/AcOEt 10:3.5; 3700 ml), and *Fr. 5* (hexane/AcOEt 10:4; 2400 ml). Repeated purification of *Fr. 1* (4.6 g) by CC

(SiO_2; hexane/AcOEt 10:1) followed by HPLC purification afforded **3** (26 mg). *Fr. 2* (6.7 g) was subjected to CC (SiO_2; hexane/AcOEt 10:1.6) followed by HPLC to afford **4** (10 mg) and **5** (20 mg). *Fr. 3* (2.6 g) was purified by CC (SiO_2; hexane/AcOEt 10:2.1) followed by HPLC to afford **1** (3 mg). *Fr. 4* (4.9 g) was subjected to CC (SiO_2; hexane/AcOEt 10:2.9) followed by HPLC, which afforded **6** (4 mg) and **7** (7 mg). *Fr. 5* (3.5 g) was purified by CC (SiO_2; hexane/AcOEt 10:3.2) followed by HPLC to provide **8** (2 mg) and **2** (11 mg).

Bidenphytin A (= *Methyl (1*E,*3*R,*6*S,*11*Z,*16*Z,*20*Z,*23*S,*24*S*)-18-Ethenyl-13-ethyl-14,19,23,28-tetramethyl-24-(3-oxo-3-{[(2*E,*7*R,*11*R*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]oxy}propyl)-4,5-dioxa-9,25,26,27-tetraazaheptacyclo[20.2.1.1^{7,10}.1^{12,15}.1^{17,20}.0^{2,8}.0^{3,6}]octacosa-1,7,10(28),11,13,15(27),16,18,20,22(25)-decaene-3-carboxylate*; **1**). UV (MeOH): 404, 505, 534, 612, 669. $[\alpha]_D^{25} = -44.6$ ($c = 0.76$, $CHCl_3$). IR (neat): 3331, 2960, 2920, 2853, 1732, 1698, 1469. ^{1}H- and ^{13}C-NMR: see *Table* . HR-FAB-MS (pos.): 887.5684 ($[M+H]^+$, $C_{55}H_{75}N_4O_6^+$; calc. 887.5687).

Bidenphytin B (= *Methyl (1*E,*3*R,*6*S,*11*Z,*16*Z,*20*Z,*23*S,*24*S*)-18-Ethenyl-13-ethyl-6-hydroxy-14,19,23,28-tetramethyl-24-(3-oxo-3-{[(2*E,*7*R,*11*R*)-3,7,11,15-tetramethylhexadec-2-en-1-yl]oxy}propyl)-4,5-dioxa-9,25,26,27-tetraazaheptacyclo[20.2.1.1^{7,10}.1^{12,15}.1^{17,20}.0^{2,8}.0^{3,6}]octacosa-1,7,10(28),11,13,15(27),16,18,20,22(25)-decaene-3-carboxylate*; **2**). UV (MeOH): 414, 521, 557, 601, 655. $[\alpha]_D^{25} = -5.4$ ($c = 0.98$, $CHCl_3$). IR (neat): 3320, 2953, 2924, 2869, 1725, 1699, 1466. ^{1}H- and ^{13}C-NMR: see *Table*. HR-FAB-MS (pos.): 903.5657 ($[M+H]^+$, $C_{55}H_{75}N_4O_7^+$; calc. 903.5636).

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