Antimicrobial Activity of New Phorbins from *Jatropha curcas* Linn. (Euphorbiaceae) Leaves

Irene M. Villaseñor* and Flerida A. Cariño

Institute of Chemistry, University of the Philippines, Diliman, Quezon City, Philippines.
E-mail: irene.villasenor@up.edu.ph

* Author for correspondence and reprint requests

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The crude methanol extract of *Jatropha curcas* leaves exhibited activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium phlei*, *Candida albicans*, and *Trichophyton mentagrophytes* but was inactive against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*. In a bioassay-directed fractionation, two new phorbins were isolated and analysed by spectroscopic methods. Isolate **1** was characterized as an analogue of pheophytin b with a phytyl moiety containing three double bonds which are at positions P2/P3, P6/P7, and P10/P11. Compound **2** was characterized as methyl pheophorbide a with 13^2-OH and 17- and 17^1-CH_3 moieties. It is active against *Serratia marcescens*.

Key words: Pheophytin b Analogue, Methyl Pheophorbide a Analogue, *Serratia marcescens*

Introduction

Jatropha curcas Linn. (Euphorbiaceae), although primarily used in the production of biodiesel, has several medicinal applications. Most parts of the plant are used for various human and veterinary ailments. The sap of *J. curcas* showed antimicrobial activity against oral pathogens (Agbelusi *et al*., 2007) and mouth infections in children (Thomas *et al*., 2008). The latex showed a broad spectrum of antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Candida albicans*, and clinical isolates of *Trichophyton* sp. (Oyi *et al*., 2007). The roots were active against reference and against sensitive and resistant hospital strains of *S. aureus* (Atindehou *et al*., 2002). The crude extracts of the stem bark (Igbinosa *et al*., 2009) and leaves (Kalimuthu *et al*., 2010) also showed antimicrobial activities.

Previously isolated from *J. curcas* were curcain (Nath and Dutta, 1991), curcacycline A (Van den Berg *et al*., 1995) and B (Auvin *et al*., 1997), diterpene diesters with a 12-deoxy-16-hydroxyphorbol moiety (Hirota *et al*., 1988; Haas *et al*., 2002), and diterpenoids with lathyrane and podocarpane skeletons (Ravindranath *et al*., 2004a). Deoxypreussomerins from the stems of *J. curcas* showed antibacterial activity (Ravindranath *et al*., 2004b). Six diterpenoids isolated from the roots of *J. podagrica* Hook. were active against *S. aureus* and *B. subtilis* (Aiyelaagbe *et al*., 2007). The present study focused on the isolation and characterization of two phorbins from *J. curcas* leaves that gave positive results against *Serratia marcescens*.

Results and Discussion

The crude methanol extract (JM) of *J. curcas* was inactive against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Saccharomyces cerevisiae*. It exhibited activity against *Staphylococcus aureus*, *Bacillus subtilis*, *Mycobacterium phlei*, *Candida albicans*, and *Trichophyton mentagrophytes* (Table I). The hexane extract (JH) initially showed antifungal activity against *Candida albicans* but, when fractionated into nine fractions labeled JH3 to JH11, only fractions JH3, JH8, and JH9 exhibited incomplete inhibition as indicated by thinning of the fungal growth.

Sequential fractionation of the ethyl acetate extract (JE) gave fractions labeled JE1 to JE15. Subsequent bioautography showed that fractions JE5 to JE8 were active against *Staphylococcus aureus*. Fraction JE6 showed complete inhibition of *Serratia marcescens* and incomplete inhibition of *Staphylococcus aureus*. Purification of JE6 gave TLC-pure spots with Rf values of 0.59 for **1** and 0.48 for **2** in 40% EtOAc/*n*-hexane as developing

Table I. Antimicrobial activity of *J. curcas* leaf extracts.

Microorganism	Test sample[a]	Average diameter of zone of inhibition ± SD [mm (AI[b])]			
		50 mg/ml (10 mm i.d.)	10 mg/ml (10 mm i.d.)	1 mg/ml (10 mm i.d.)	30 μg (6 mm i.d.)
S. aureus	JM	19.3 ± 1.2 (0.9)	–(0)	13.7 ± 1.0[b] (0)	
	JH	13.0 ± 0[c] (0)	16.0 ± 1.0[b] (0)	12.0 ± 0 (.1)	
	JE	21.3 ± 0.3 (1.1)	12.7 ± 0.6[b] (0)	14.3 ± 1.2[b] (0)	
	JE6		19.3 ± 1.2[b] (0)		
	Chloramphenicol				28 (3.7)
S. marcescens	JE6		17.3 ± 0.6 (0.7)		
	1 + **2**		14.3 ± 1.2 (0.4)		
	2		19.0 ± 1.0 (0.6)		
	Chloramphenicol				25 (3.2)
B. subtilis	JM	14.0 ± 0 (0.4)	–(0)	–(0)	
	JH	15.0 ± 0 (0.5)	12.7 ± 0.6 (0.3)	–(0)	
	JE	21.7 ± 0.6 (1.3)	17.7 ± 0.6 (0.8)	14.3 ± 0.6 (0.4)	
	Chloramphenicol				27 (3.5)
M. phlei	JM	12.0 ± 0 (0.2)	17.7 ± 0.6 (0.8)	–(0)	
	JH	14.3 ± 0.6 (0.4)	17.3 ± 0.6 (0.7)	14.7 ± 0.6 (0.5)	
	JE	24.3 ± 1.2 (1.4)	21.3 ± 1.2 (1.1)	19.3 ± 1.2 (0.9)	
	1 + **2**		20.0 ± 0[b] (0)		
	2		14.7 ± 2.3[b] (0)		
	Chloramphenicol				33 (4.5)
C. albicans	JM	16.0 ± 3.5[b] (0)	20.0 ± 0 (1.0)	16.3 ± 2.9 (0.6)	
	JH	18.7 ± 3.2 (0.9)	–(0)	17.0 ± 0 (0.7)	
	JE	20.0 ± 0 (1.0)	–(0)	17.3 ± 2.0 (0.7)	
T. mentagrophytes	JM	15.7 ± 0.6[b] (0)	–(0)	12.7 ± 0.6 (0.3)	
	JH	16.3 ± 1.2[b] (0)	14.3 ± 0.6[b] (0)	15.7 ± 1.2[b] (0)	
	JE	18.7 ± 0.6 (0.9)	13.7 ± 1.2 (0.4)	15.3 ± 1.2[b] (0)	

[a] JM, methanol extract; JH, hexane extract; JE, ethyl acetate extract.
[b] AI = (diameter of clearing zone – diameter of agar well)/diameter of agar well.
[c] Thinning of growth; incomplete inhibition.

solvent. Pure **2** was more active against *Serratia marcescens* than a combination of **1** and **2**.

The ^{13}C NMR spectrum of **2** showed 37 carbon signals that are characteristic of a phorbin skeleton (Sobolev *et al*., 2005). Analysis of the 1D and 2D NMR data (Table II) showed that **2** is similar to methyl pheophorbide a (Katz and Brown, 1983) with HSQC cross-peaks showing three methine singlets of C-5, C-10, and C-20; five methyl singlets of sp^2 C-2^1, C-7^1, C-12^1 and of sp^3 C-8^2 and C-18^1; and two methoxy singlets of C-13^4 and C-17^4. The literature values of the proton

Table II. NMR data (δ in ppm; J in Hz) of **1** and **2**; literature values in parentheses.

C	**2** ^{13}C NMR[a]	HSQC	HMBC	COSY	**1** ^{1}H NMR[b]
1	142.0 (141.3)				
2	131.8 (131.1)				
3	136.2 (135.7)				
4	136.6 (135.3)				
5	98.0 (96.4)	9.49, 1H, s (9.15)	136.6, 136.2 (^2J)		9.53, 1H, s (9.43)
6	155.4 (155.0)				
7	136.3 (135.3)				
8	145.2 (144.2)				

Table II continued.

C	**2** ^{13}C NMR[a]	HSQC	HMBC	COSY	**1** ^{1}H NMR[b]
9	151.0 (150.7)				
10	104.2 (103.6)	9.63, 1H, s (9.32)			9.78, 1H, s (10.2)
11	137.8 (137.2)				
12	129.1 (128.3)				
13	126.9 (128.3)				
14	149.8 (149.0)				
15	107.6 (104.8)				
16	162.4 (160.5)				
17	51.8 (51.0)	(4.13)			4.22, 1H, m (4.20)
17-CH_3	29.7	1.26, 3H, s	0.85 (J^4)		
18	50.3 (49.9)				4.47, 1H, m, J = 7.0 (4.25–4.60, m)
19	172.8 (171.4)				
20	93.6 (92.6)	8.65, 1H, s (8.50)			8.72, 1H, s (8.53)
2^1	12.1 (11.8)	3.44, 3H, s (3.32)	142.0 (^3J), 136.2 (^3J), 131.8 (^2J)		3.45, 3H, s (3.37)
3^1	129.4 (128.3)	8.03, 1H, dd, J = 18, 11.5 (7.85)		6.32, 6.21	8.02, 1H, dd, J = 17.5, 11.5 (7.95)
3^2	122.9 (121.8)	6.32, 1H, d, J = 18; 6.21, 1H, d, J = 11.5 (6.12/6.04)		8.03	6.41, 1H, d, J = 17.5; 6.19, 1H, d, J = 11.5 (6.34, 6.21)
7^1	11.3 (10.7)	3.27, 3H, s (3.15)	155.4 (^3J), 145.2 (^3J), 136.3 (^2J)		11.20, 1H, s (11.0)
8^1	19.5 (19.0)	(3.48)	17.5		(3.85)
8^2	17.5 (17.1)	1.72, 3H, t, J = 7.5 (1.60)	145.2 (^3J), 19.5 (^2J)	3.73, 2H, t, J = 7.0	1.73, 3H, t, J = 7.0 (1.73)
12^1	12.3 (11.8)	3.75, 3H, s (3.62)	172.4, 151.0, 145.2, 137.8 (^3J), 129.1 (^2J), 126.9 (^3J)		3.61, 3H, s (3.63)
13^1	191.9 (189.0)				
13^2	88.9 (64.5)	(6.22)			6.29, s (6.25)
13^3	172.4 (168.9)				
13^4	53.5 (52.6)	3.63, 3H, s (3.88)	172.4 (^3J)		3.89, 3H, s (3.93)
17^1	31.4 (31.0)	2.54–2.62, 1H, m			2.42–2.58, 2H, m (2.45–2.85, m)
17^1-CH_3	22.6	0.85, 3H, dt, J = 19.5, 6.5			
17^2	31.1 (29.8)	2.22–2.38, 2H, m			2.20–2.28, 2H, m (2.15–2.45, m)
17^3	174.0 (172.6)				
17^4	51.8 (51.4)	3.66, 3H, s (3.57)	174.0		
18^1	22.7 (22.8)	1.61, 3H, d, J = 7.5 (1.82)	172.8 (^3J), 50.3 (^2J)	4.50, 1H, q, J = 7.5 (4.40)	1.63, 3H, d, J = 8.0 (1.85)
P1					4.32, 1H, dd, J = 14.0, 7.0; 4.14, 1H, dd, J = 15.0, 7.0 (4.39, 4.22)[c]
P2					5.24, t, J = 7.0 (4.91)[c]
$P3^1$, 7^1, 11^1					1.59, 1.60, 1.62 (1.48)[c]
P4					1.87, t, J = 7.5 (1.89)
P5 -P20					(0.74–0.92 br; 1.00–1.40, br (0.70–0.70, m)

[a] Katz and Brown (1983).
[b] Oba *et al*. (1997).
[c] Sobolev *et al*. (2005).

signal assignments of C-8^2 and C-18^1 were interchanged based on HSQC data. The proton signals at δ 4.50 ppm and δ 3.73 ppm were assigned to positions H-18 and CH_2-8^1, respectively, because of cross-peaks in the COSY spectrum between δ 1.61 ppm (H-18^1)/δ 4.50 ppm and δ 1.72 ppm (H-8^2)/δ 3.73 ppm. There were also HMBC cross-peaks between δ 22.7 ppm (C-18^1)/δ 4.50 ppm and δ 17.5 ppm (C-8^2)/δ 3.73 ppm. Further analysis of cross-peaks present in the HMBC spectrum led to the assignment of carbon resonances to the quaternary carbon atoms.

There were, however, differences in the peaks of **2** to those cited in the literature for methyl pheophorbide a (Katz and Brown, 1983). The multiplets at δ 2.20–2.40 ppm (H-17^2) and δ 2.40–2.60 ppm (H-17^1) integrated to 2H and 1H, respectively, while the signal at δ 4.50 ppm (H-18) is splitted into a quartet by H-18^1 with no further splitting by H-17. Signals at δ 64.5 ppm (C-13^2) and δ 6.22 ppm (H-13^2) were also absent. The presence of 13^2-OH and 17- and 17^1-CH_3 moieties (Fig. 1) was therefore proposed based on new signals at δ 88.9 ppm, δ 29.7 ppm/δ 1.26 ppm (3H, s), δ 22.6 ppm/δ 0.85 ppm (3H, dt, J = 19.5, 6.5 Hz). There was an HMBC cross-peak between δ 0.85 ppm and δ 29.7 ppm (^4J). Compound **2** has the molecular formula $C_{38}H_{42}N_4O_6$ with m/z 651.1283 ($[M+H]^+$) (calcd. 650.3104 $[M]^+$, 651.3182 $[M+H]^+$).

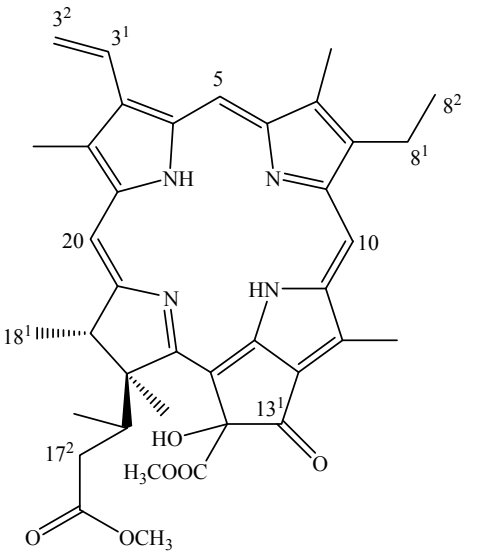

Fig. 1. Proposed chemical structure of **2**, an analogue of methyl pheophorbide a.

The ^{1}H NMR spectra of **1** and **2** were almost identical, except for the very intense signals in **1** at δ 0.70–1.50 ppm (Table II), which indicated the presence of a phytyl group (Oba *et al.*, 1997). The diagnostic peak at δ 11.20 ppm for 7^1-CHO is characteristic of pheophytin b. The ^{1}H NMR spectrum of **1** is typical of pheophytin b, which can be almost completely assigned based on δ_H and J_{H-H} values. The molecular formula of pheophytin b is $C_{55}H_{72}N_4O_6$ with m/z 885.20. The observed molecular ion peak of **1** at m/z 881.3080 (calcd. 881.5217) indicated the presence of two additional double bonds, which are, biosynthetically determined, at positions P6/P7 and P10/P11 of the phytyl group (Fig. 2).

The ^{1}H NMR spectrum identified the -O-CH_2-CH=C(CH_3)- phytyl fragment by signals at δ 4.32 ppm (1H, dd, J = 14.0, 7.0 Hz) and δ 4.14 ppm (1H, dd, J = 15.0, 7.0 Hz); δ 5.24 ppm (t, J = 7.0 Hz); and δ 1.59–1.62 ppm, respectively. The three unresolved peaks at δ 1.59 ppm, δ 1.60 ppm, and δ 1.62 ppm were assigned to $P3^1$, $P7^1$, $P11^1$; and multiplets at δ 1.90–2.10 ppm (4H) and δ 2.28–2.44 ppm (4H) to P4/P8 and P5/P9,

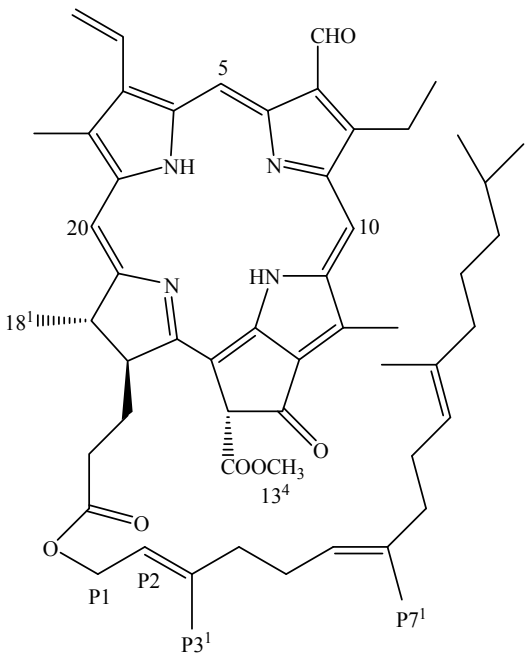

Fig. 2. Proposed chemical structure of **1**, an analogue of pheophytin b.

respectively. Triplets at δ 4.59 ppm and δ 4.87 ppm may account for P6/P10 protons.

Phorbins are thermally unstable and partially decompose under MS conditions. The appearance of their mass spectra is quite complex and depends critically on the experimental conditions used.

Experimental

General

^{1}H NMR spectra were recorded at 500 MHz and ^{13}C NMR spectra at 125 MHz in $CDCl_3$. FT-IR as neat samples using NaCl plates and normal phase vacuum liquid chromatography (NPVLC), gravity column chromatography (NPGCC), and TLC on silica gel using gradient ratios of *n*-hexane, EtOAc/*n*-hexane, and EtOH/EtOAc were conducted. Detection included spraying with I_2, vanillin/H_2SO_4, followed by heating and visualization under an UV lamp.

Plant materials

Leaves of *Jatropha curcas*, three months old, were purchased from Pampanga Agricultural College in Magalang, Pampanga, Philippines and Tarlac Agricultural College in Camiling, Tarlac, Philippines; they were dried in-doors. Authentication was done by Dr. Daniel Lagunzad, Institute of Biology, University of the Philippines, Diliman, Philippines, and a voucher specimen with accession number 14593 was deposited at the Dr. Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines, Diliman, Philippines.

Extraction and purification

The air-dried leaves (3946 g) were homogenized in MeOH. The concentrated methanol extract (JM, 638.8 g, 16.2% yield) was partitioned between *n*-hexane and water (6:1, v/v). The aqueous layer was further extracted with ethyl acetate (EtOAc, 6:1, v/v). The hexane (JH) and ethyl acetate (JE) extracts were then concentrated *in vacuo* yielding 2.8% JH (111.9 g) and 0.5% JE (19.4 g), respectively. Repeated fractionation of JH (10.0731 g) by NPVLC using 400 ml each of *n*-hexane, 10% gradient ratios of EtOAc in *n*-hexane, and EtOAc gave fractions JH3 to JH11.

Repeated NPVLC of JE (19.3166 g) using 225 ml each of *n*-hexane, 10% gradient ratios of EtOAc in *n*-hexane, EtOAc, 10%, 30%, 50% EtOH/EtOAc, and EtOH yielded fractions JE1 to JE15. Subsequent bioautography of JE5 to JE8 showed anti-*S. aureus* compounds with Rf values of 0.59 and 0.48 in 40% EtOAc/*n*-hexane as developing solvent. Repeated and sequential NPVLC of fractions JE5 to JE8 and sub-fractions labeled JE5(11–15) and JE6(10–21) gave semi-pure isolates, eluted with 20% to 58% EtOAc in *n*-hexane. Repeated NPGCC of semi-pure isolates yielded isolates **1** (6.5 mg), eluted with 28% to 36% EtOAc in *n*-hexane, and **2** (40.4 mg), eluted with 38% to 54% EtOAc in *n*-hexane.

1: FT-IR: ν = 3344.57 (N-H str.), 2954.95, 2927.94 and 2858.51 (C-H str.), 1728.22 (C=O str.), 1604.77 cm^{-1} (C=C str.). – HRMS (ESI positive mode, TOF-MS, nanospray at 3200–3600v, flow rate of 0.100 μl/min): *m/z* (%) = 881.3080 $[MH]^+$ (7) (calcd. 881.5217 for $C_{55}H_{69}N_4O_6$ and 880.5139 for $C_{55}H_{68}N_4O_6$), 865.0406 (16), 677.4648 (18), 655.1171 (100), 608.3611 (36), 456.0872 (46), 439.8948 (55), 386.3028 (23), 357.8970 (18), 235.4708 (21), 126.1097 (23).

2: FT-IR: ν = 3397.57 (N-H str.), 2954.95, 2927.94 and 2858.51 (C-H str.), 1728.22 (C=O str.), 1612.49 cm^{-1} (C=C str.). – HRMS (ESI positive mode, TOF-MS, nanospray at 3200–3600v, flow rate of 0.500 μl/min): *m/z* (%) = 651.1283 $[MH]^+$ (calcd. 650.3104 for $C_{38}H_{42}N_4O_6$), 646.9548 (12), 624.6147 (48), 235.4713 (12), 198.3240 (52), 146.0711 (42), 126.1098 (12), 118.0433 (100).

Antimicrobial assays

The agar-well method was done at the Microbiological Services Laboratory, Natural Sciences Research Institute, University of the Philippines, Diliman, Philippines. The extracts were dissolved in DMSO, and three concentrations were used: 50 mg/ml, 10 mg/ml, and 1 mg/ml. Three equidistant wells were made on the inoculated plate. Exactly 200 μl of the test sample were placed in each agar hole (10 mm i.d.). Chloramphenicol (30 μg) and Canesten® (0.2 g), containing 1% clotrimazole, were used as the positive controls. The test organisms included *Escherichia coli* UPCC1195, *Mycobacterium phlei* UPCC1365, *Staphylococcus aureus* UPCC1143, *Bacillus subtilis* UPCC1295, *Candida albicans* UPCC2168, *Saccharomyces cerevisiae* UPCC2115, *Trichophyton mentagrophytes*

UPCC4193, *Pseudomonas aeruginosa* UPCC1244, and *Serratia marcescens* UPCC1357.

Bioautography was done at the Marine Natural Products Laboratory, Marine Science Institute, University of the Philippines, Diliman, Philippines. *S. aureus* was cultivated in Mueller Hinton broth for 12 h, after which an 1% v/v molten Mueller Hinton agar solution was prepared. TLC plates were overlaid with the inoculated agar and incubated at 37 °C for 10 h. Zones of inhibition were visualized by spraying with a sterile 5 mg/ml solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in phosphate buffered saline (PBS).

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