Antimalarial Naphthoquinones from *Nepenthes thorelii*

Kittisak Likhitwitayawuid[1,*], Rawiwun Kaewamatawong[1,3], Nijsiri Ruangrungsi[1], and Jerapan Krungkrai[2]

[1] Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand
[2] Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand
[3] Present adress: Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand

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Abstract: **Roots of *Nepenthes thorelii* yielded plumbagin, 2-methylnaphthazarin, octadecyl caffeate, isoshinanolone, and droserone. In addition, seven derivatives were prepared from plumbagin. Each of these natural and semisynthetic compounds was evaluated for *in vitro* antimalarial potential.**

Key words: ***Nepenthes thorelii*, Nepenthaceae, naphthoquinone, antimalarial activity.**

Introduction

Plants of the family Nepenthaceae are commonly found in several parts of Tropical Asia and northern Australia (1). The family consists of only one genus, *Nepenthes* (2). In Malaysia a decoction of the stem of *N. ampullaria* has been used traditionally to treat malaria (3); however, in Thailand no medicinal use has been claimed for any plant in this genus. Earlier phytochemical reports on the genus revealed the presence of naphthoquinones, steroids, triterpenoids, and long-chain hydrocarbons (4–6), but no study has yet appeared on the antiplasmodial activity of these plants. *N. thorelii* Lec., native to Thailand (7), has not been previously studied either chemically or biologically. A chloroform extract of the roots of this plant exhibited a significant inhibitory effect on the growth of *Plasmodium falciparum* (IC_{50} 10 μg/ml) in our screening of plants for antimalarial potential, as a part of our continuing efforts to discover natural products possessing antimalarial activity (8–12). A follow-up investigation of this extract was then undertaken to determine the chemical(s) responsible for the activity.

Materials and Methods

Instrumentation

UV spectra were obtained on a Milton Roy Spectronic 3000 Array spectrometer. IR spectra were measured on a Perkin Elmer FT-IR 1760x spectrometer. Optical rotations were recorded on a Perkin Elmer 341 polarimeter. EI mass spectra were determined on a Kratos Profile or a Finnigan MAT INCOS 50 mass spectrometer. ^{1}H- (500 MHz) and ^{13}C-NMR (125 MHz) spectra were recorded on a JEOL JMN-A 500 spectrometer. HMQC and HMBC spectra were obtained at 500.00/125.69 MHz using standard programs from the JEOL library: $^nJ_{C-H}$ = 7 Hz was used for HMBC experiments.

Plant materials

The roots of *N. thorelii* were collected from Chumporn province, in June 1994. A voucher specimen (NR-062537) is deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Extraction and isolation procedures

An ethanol extract was prepared by maceration of the roots of *N. thorelii* (1.3 kg) with ethanol (3 × 7 l, three days each). This extract was then partitioned between chloroform and water. The water fraction was disregarded (IC_{50} 20 μg/ml) whereas the chloroform fraction (IC_{50} 10 μg/ml) was selected for further study. The chloroform fraction (18 g) was separated by silica gel (1 kg) column chromatography, eluted with a petroleum ether/EtOAc gradient system (10 : 0, 9 : 1, 8 : 2, 7 : 3, 6 : 4, 1 : 1, 4 : 6, 3 : 7, 2 : 8, 1 : 9, and 0 : 10; 500 ml each). The eluates were examined by TLC (silica gel, pet. ether/EtOAc, 1 : 1). Fractions with similar chromatographic pattern were combined to give 8 fractions (fractions I–VIII). Fraction II [3.2 g, pet. ether/EtOAc, 9 : 1 (500 ml) and pet. ether/EtOAc (250 ml)] after recrystallization from pet. ether/acetone (1 : 1) gave 1.2 g of plumbagin (**1**) (R_f 0.43, silica gel, pet. ether/acetone 1 : 1). Fraction III (1.9 g, pet. ether/EtOAc, 8 : 2, 500 ml) was rechromatographed over a Sephadex LH-20 (1 × 80 cm) column (MeOH). Fractions 5–7 from this column (MeOH, 140 ml) were purified by preparative thin-layer chromatography (silica gel, toluene/EtOAc, 95 : 5) to give 2 mg of 2-methylnaphthazarin (**2**) (R_f 0.31, silica gel, toluene/EtOAc, 95 : 5). Chromatographic separation of fraction IV (1.3 g, pet. ether/EtOAc, 7 : 3, 500 ml) on a silica gel (100 g) column with $CHCl_3$/EtOAc gradient elution (9 : 1, 8 : 2, 3 : 2, 2 : 3, 2 : 8, and 1 : 9, 200 ml each) gave two fractions. The more polar fraction ($CHCl_3$/EtOAc, 2 : 8, 100 ml) was purified by preparative TLC (silica gel, pet. ether/EtOAc, 8 : 2) to yield 25 mg of octadecyl caffeate (**3**) (R_f 0.28 silica gel, pet. ether/EtOAc, 8 : 2). The less polar fraction ($CHCl_3$/EtOAc, 3 : 2, 200 ml) gave 14 mg of isoshinanolone (**4**) after purification by preparative TLC (R_f 0.36 silica gel, pet. ether/EtOAc, 8 : 2). Fraction VII (60 mg, pet. ether/EtOAc, 4 : 6, 500 ml) was separated on a silica gel (3 g) column eluted with mixtures of petroleum ether/EtOAc in a polarity-gradient

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manner (9 : 1, 8 : 2, 7 : 3, 6 : 4, 4 : 6, 3 : 7, 8 : 2 and 1 : 9,; 100 ml each). Fractions 28 – 38 from this column (pet. ether/EtOAc, 3 : 7, 8 : 2 and 1 : 9; 100 ml each) were combined and further separated by column chromatography (silica gel, pet. ether/acetone, 9 : 1, 8 : 2, 7.5 : 2.5, 7 : 3, 6 : 4 and 3 : 7; 50 ml each). Fractions eluted with pet. ether/acetone 7.5 : 2.5 (50 ml) were pooled and evaporated to dryness to give 7 mg of droserone (**5**) (R_f 0.32 silica gel, pet. ether/acetone 7 : 3).

Chemical transformations of **1**

Methylation of **1**: To a solution of **1** (750 mg) in $CHCl_3$ (10 ml), silver(I) oxide (3 g) and iodomethane (15 ml) were added. The reaction mixture was stirred overnight, and then filtered. The filtrate was evaporated to dryness to give 760 mg of **6**.

Epoxidation of **6**: Hydrogen peroxide (1 ml) was dissolved in 5 % w/v sodium carbonate solution (7 ml). This solution was then added to a warmed solution of **6** (700 mg) in MeOH (10 ml). The mixture was rigorously shaken for 1 min and subsequently extracted with $CHCl_3$ (70 ml). The $CHCl_3$ fraction was dried over anhydrous Na_2SO_4 and evaporated to dryness to give **7** (517 mg).

Preparation of **8**: 1 ml of sulfuric acid was added to **7** (400 mg). After the mixture turned red, it was cooled in ice bath, and water was then added until yellow precipitates occurred. The precipitates were collected and dried to give 242 mg of **8**.

Acetylation of **8**: Compound **8** (20 mg) was dissolved in 1 ml of Ac_2O/pyridine (1 : 1) solution and stirred at room temperature overnight. Then, the mixture was poured into water and extracted with $CHCl_3$. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness to give 20 mg of **9**.

Reaction of **8** *with iodomethane:* To a solution of **8** (70 mg) in $CHCl_3$ (5 ml), silver(I) oxide (400 mg) and iodomethane (10 ml) were added. The reaction mixture was stirred overnight, and then filtered. The filtrate was evaporated to dryness to give a residue which was further purified by preparative thin-layer chromatography (silica gel, petroleum ether – ethyl acetate; 7 : 3) to give **10** (12 mg) and **11** (7 mg).

Reaction of **7** *with hydrochloric acid:* Compound **7** (70 mg) was dissolved in 5 % HCl in MeOH solution (20 ml). The mixture was refluxed for 2 h. The reaction mixture was then passed through an Amberlite IRA-900 ion exchange column. The obtained eluant was evaporated to dryness and subsequently separated by column chromatography (silica gel, petroleum ether – ethyl acetate gradient) to give **12** (12 mg), along with **8** (14 mg) and **10** (10 mg).

Physical properties

Plumbagin (**1**): EIMS, UV, IR, and ^{1}H- and ^{13}C-NMR data as described in (13 – 15).

2-Methylnapthazarin (**2**): EIMS, UV, IR and ^{1}H-NMR as described in (16); ^{13}C-NMR see Table **2**.

Isoshinanolone (**4**): $[\alpha]_D^{25}$: +20.2° (*c* 0.2, $CHCl_3$), UV, IR, ^{1}H- and ^{13}C-NMR and EIMS as described in (13, 17).

Droserone (**5**): EIMS, UV, IR and ^{1}H-NMR as described in (18).

5-Methoxy-2-methyl-1,4-naphthoquinone (**6**): EIMS, IR and ^{1}H- and ^{13}C-NMR as described in (14).

Octadecyl caffeate (**3**): EIMS and ^{1}H-NMR as described in (17). ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 14.3 (C-18″), 22.9, 26.3, 29.2, 29.6, 29.8, 29.9, 30.0, 32.1 (C-3″ – C-17″), 29.6 (C-2″), 64.4 (C-1″), 115.1 (C-2), 115.8 (C-2′), 116.7 (C-6′), 122.0 (C-5′), 126.9 (C-1′), 145.8 (C-3), 147.7 (C-4′), 150.5 (C-3′), 167.6 (C-1).

5-Methoxy-2-methyl-1,4-naphthoquinone-2,3-epoxide (**7**): white powder; $[\alpha]_D^{25}$: 0° (*c* 0.4, $CHCl_3$); IR (KBr): ν_{max} = 2947, 2922, 1690, 1588, 1451, 1344 – 1255 cm^{-1}; EIMS: *m/z* (%) = 218 (100), 203 (32), 189 (29), 175 (44), 161 (34), 147 (23), 135 (35), 119 (30), 91 (21), 76 (61), 63 (32), 50 (29), 43 (92), 32 (18); ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.71 (3H, s, 2-CH_3), 3.83 (1H, s, H-3), 3.95 (3H, s, 5-OCH_3), 7.27 (1H, *J* = 0.9, 7.9 Hz, H-6), 7.56 (1H, dd, *J* = 0.9, 7.6 Hz, H-8), 7.64 (1H, dd, *J* = 7.6, 7.6 Hz, H-7); ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 14.4 (2-CH_3), 56.4 (5-OCH_3), 61.5 (C-2), 61.6 (C-3), 117.6 (C-6), 119.5 (C-8), 120.5 (C-4a), 134.3 (C-8a), 135.0 (C-7), 158.9 (C-5), 191.2 (C-4), 192.9 (C-1).

3-Hydroxy-5-methoxy-2-methyl-1,4-naphthoquinone (**8**): UV and ^{1}H-NMR as described in (20); EIMS: *m/z* (%) = 218 (100), 200 (7), 189 (10), 172 (16), 147 (14), 131 (14), 105 (13), 91 (12), 84 (18), 76 (29), 65 (15), 55 (30), 43 (35), 32 (50); ^{13}C-NMR see Table **2**.

3-Acetyloxy-5-methoxy-2-methyl-1,4-naphthoquinone (**9**): yellow powder; IR (KBr): ν_{max} = 3454, 1663, 1276, 1165 cm^{-1}; EIMS: *m/z* (%) = 260 (2), 218 (85), 200 (12), 172 (12), 161 (9), 131 (6), 91 (6), 76 (15), 63 (9), 55 (14), 43 (100), 32 (7); ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 2.06 (3H, s, 2-CH_3), 2.40 (3H, s, 3-$OCOCH_3$), 4.00 (5-OCH_3), 7.28 (1H, dd, *J* = 0.9, 7.9 Hz, H-6), 7.67 (1H, dd, *J* = 7.6, 7.9 Hz, H-7), 7.79 (1H, *J* = dd, 0.9, 7.6 Hz, H-8); ^{13}C-NMR see Table **2**.

3,5-Dimethoxy-2-methyl-1,4-naphthoquinone (**10**): IR and ^{1}H-NMR as described in (21); EIMS: *m/z* (%) = 232 (100), 217 (42), 202 (13), 189 (23), 173 (22), 159 (12), 145 (10), 135 (16), 115 (20), 91 (12), 76 (43), 63 (23), 55 (20), 39 (26). ^{13}C-NMR see Table **2**.

2,2-Dimethyl-3-hydroxy-3-methoxycarbonyl-4-methoxy-1H-inden-1-one (**11**): white powder; $[\alpha]_D^{25}$: 0° (*c* 0.2, $CHCl_3$); IR (KBr): ν_{max} = 3443, 2961, 2931, 1726, 1272, 1004 cm^{-1}; EIMS: *m/z* (%) = 264 (0.1) 205 (100), 190 (9), 175 (2), 144 (3), 115 (3), 91 (4), 77 (4), 63 (2), 51 (2), 45 (6); HRFABMS: 265.1088 (M + H) calcd. for $C_{14}H_{17}O_5$ (265.1076); ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 1.12 (3H, s, 2-CH_3), 1.22 (3H, s, 2-CH_3), 3.67 (3H, s, 3-OCH_3), 3.90 (3H, s, 4-OCH_3), 7.11 (1H, d, *J* = 7.93 Hz, H-5), 7.40 (1H, dd, *J* = 0.6, 7.6 Hz, H-7), 7.48 (1H, dd, *J* = 7.6, 7.6 Hz, H-6); ^{13}C-NMR (125 MHz, $CDCl_3$): δ =18.3 (2-CH_3), 23.0 (2-CH_3), 52.9 (3-OCH_3), 55.0 (C-2), 55.8 (4-OCH_3), 82.1 (C-3), 115.8 (C-7), 116.2 (C-5), 131.5 (C-6), 137.2 (C-7a), 138.2 (C-3a), 157.0 (C-4), 174.1 ($OCOCH_3$), 206.3 (C-1).

3-Chloro-5-methoxy-2-methyl-1,4-naphthoquinone (**12**): Yellow powder; IR (KBr): ν_{max} = 3400, 2800, 1656, 1585, 1321, 1275, 1055 cm^{-1}; EIMS: *m/z* (%) = 238 (36), 236 (100), 219 (4), 201 (28), 186 (8), 171 (48), 158 (9), 143 (69), 130 (11), 115 (90), 102 (32), 91 (16), 76 (57), 63 (35), 50 (24); HREIMS: 236.0228 calcd. for $C_{12}H_9O_3Cl$ (236.0238); ^{1}H-NMR (500 MHz, $CDCl_3$): δ

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= 2.30 (3H, s, 2-CH_3), 4.02 (3H, s, 5-OCH_3), 7.34 (1H, dd, J = 0.9, 8.4 Hz, H-6), 7.68 (1H, dd, J = 7.6, 7.6 Hz, H-7), 7.77 (1H, dd, J = 0.9, 7.6 Hz, H-8); ^{13}C-NMR see Table **2**.

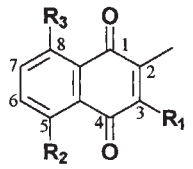
Antimalarial activity assay

Evaluation of the antimalarial activity of the extracts and pure compounds was carried out according to the previously described procedures (8–12). The parasites used in this study were from the $T_{9/94}$ line of *Plasmodium falciparum*, and were cultivated in a candle jar at 37 °C according to the method of Trager and Jensen (25). In short, 200 µl of 1.6% parasitized red blood cell suspension in complete medium were added to wells of a standard 96-well microtiter plate containing 25 µl of test compound. The plate was incubated in a candle jar at 37 °C for 24 h. Then 25 µl of [^3H(G)]-hypoxanthine solution in complete medium (25 µCi/ml) were added to each well. The microtiter plate was returned to the candle jar and incubated for 18 h at 37 °C. The incubation was terminated by harvesting the red blood cells with a Nunc 8 cell harvester. The filter strips were dried, and the filter discs were cut out and placed in vials with scintillation fluid. Radioactivity was determined with a Packard Tri-carb 2700 TR liquid scintillation counter. Each test sample was assayed in duplicate over a concentration range of 12.5–0.78 µg/ml. The known antimalarial drugs pyrimethamine and chloroquine were used as positive controls. Concentrations of both test compounds and positive controls that inhibited parasite specific incorporation of [^3H]-hypoxanthine by 50% (IC_{50}) were determined by non-linear regression analysis. Zero-drug controls defined 100% incorporation.

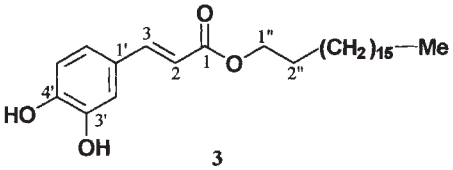
Results and Discussion

After repetitive chromatography, five pure compounds, namely plumbagin (**1**), 2-methylnaphthazarin (**2**), octadecyl caffeate (**3**), isoshinanolone (**4**), and droserone (**5**), were isolated. The structures of these compounds were determined by comparison of their spectral properties (mainly MS and ^{1}H-NMR) data with previously reported values (13–19). Although plumbagin (**1**) and droserone (**5**) have been isolated from *N. rafflesiana* (5), compounds **2–4** have not been found previously in this genus. Each of these compounds was then evaluated for its antimalarial activity against *Plasmodium falciparum*, and the IC_{50} values were calculated (Table **1**). It could be concluded that among these isolates (**1–5**), plumbagin (**1**) possessed the strongest activity and was the major component (1.2 g) responsible for the antimalarial activity of *N. thorelii*. On the other hand, only weak antiplasmodial activity was observed for compounds **2–5**. It should be noted that a number of natural and synthetic naphthoquinones, including plumbagin (**1**), have been shown to possess antimalarial activity (22–24); however, prior to this work no studies on the antiplasmodial activity of compounds **2–5** have been described.

The large amount of plumbagin (**1**) isolated in this study provided us a good opportunity to obtain additional data concerning the structural factors influencing the antiplasmodial activity of the naphthoquinones. Hence, several derivatives (**6–12**) were chemically prepared, using **1** as the first starting material (Scheme **1**). These compounds (except for **11**) are 5-methoxynaphthoquinone analogues, and all of them (**6–12**) have not been studied previously for their antimalarial activity. For compounds **9, 11** and **12**, it should also be



	R_1	R_2	R_3
1	H	OH	H
2	H	OH	OH
5	OH	OH	H
6	H	OMe	H
8	OH	OMe	H
9	OAc	OMe	H
10	OMe	OMe	H
12	Cl	OMe	H



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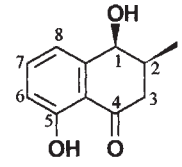

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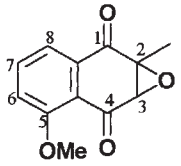

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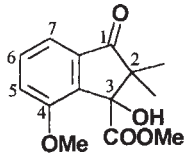

11

mentioned that prior to the present investigation neither a natural nor a synthetic product of each of these structures was known. The formation of **11** during the reaction of **8** with iodomethane in the presence of Ag_2O could be explained as follows. The reaction began with the deprotonation of 3-OH to generate an anion which then underwent *O*-methylation to furnish **10**, and *C*-alkylation at C-2 to produce an α-diketo intermediate. The C-4 carbon of this intermediate was then attacked by OH^-, leading to the migration of the aryl moiety to the C-3 carbonyl carbon (benzil-benzylic rearrangement). This resulted in the contraction of the diketo-hexacyclic structure to form a five-membered ring. Finally, methylation of the carboxyl group gave the product **11**.

Regarding their antimalarial potential (Table **1**), it was clear, however, that none of these semisynthetic products (**6–12**) demonstrated activity equal to or greater than that of their progenitor (**1**). When the IC_{50} values of all of these natural and synthetic compounds (**1–12**) were compared, it was obvious that the quinone structure was essential for the activity for these compounds, as can be seen, for example, in the activity of **1** which was diminished in **4**. This was also reflected in the loss of activity in **11** when compared with **10**. Antiplasmodial activity was observed for **7** despite its lack of a naphthoquinone nucleus. This was probably due to the presence of the epoxide functionality which was sensitive to nuceophilic attack. For the naphthoquinones in this series, the presence of an oxygen atom at C-3 seemed to attenuate their antimalarial activity, as evidenced by the IC_{50} values of compounds **8, 9** and **10** which were higher than that of compound **6**. A similar phenomenon was also observed between **5** (IC_{50} 22.06 µM) and **1** (IC_{50} 0.27 µM). Introducing a halogen atom to C-3 of the naphthoquinone nucleus such as chlorine in **12** demolished its antimalarial activity. It appeared that the presence of a heteroatom such as oxygen or chlorine at position 3 of the naphthoquinone nucleus caused the

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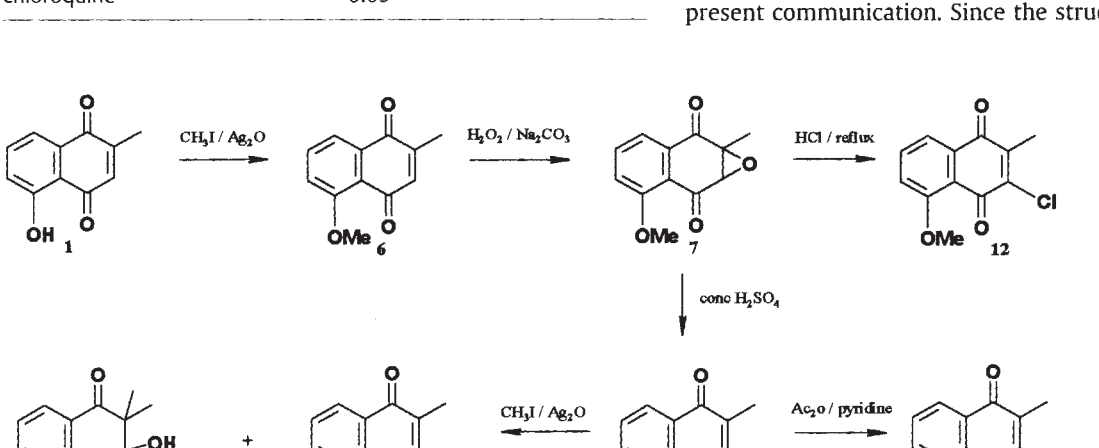
weakening or loss of the activity. The results obtained in this study should be useful for investigators interested in developing naphthoquinone analogues with antiplasmodial activity.

Table 1 Antimalarial activity of compounds **1 – 12**.

Compound	IC_{50} (μM)
1	0.27
2	5.79
3	11.76
4	20.83
5	22.06
6	0.89
7	2.75
8	32.11
9	123.07
10	3.88
11	56.82
12	168.07
pyrimethamine	11.29
chloroquine	0.09

With regard to the spectroscopic properties of the natural compounds (**1 – 5**), their NMR data were extensively studied in the present investigation through the use of several 2D-NMR techniques, including homonuclear COSY, NOESY, DEPT, HMQC and HMBC. Recently, a controversy concerning the ^{13}C-NMR data of plumbagin (**1**) has arisen from the discrepancies between the assignments proposed by Sankaram and co-workers (14) and those by Yue et al. (15). The HMQC and HMBC correlations of **1** observed in our experiments confirmed the assignments by Sankaram and co-workers (14). The earlier ^{13}C-NMR assignments of droserone (**5**) (18) were also revised in this work based on its HMQC and HMBC correlations. In addition, complete ^{13}C-NMR assignments were obtained in a similar manner for **2**, whose ^{13}C-NMR properties were partially studied (26). As to compound **3**, the results in this study provided its first ^{13}C-NMR report. The NMR data of the semisynthetic compounds (**6 – 12**) were also obtained in a similar fashion. In short, the earlier ^{13}C-NMR assignments of **6** (14) were confirmed. Complete ^{1}H- and ^{13}C-NMR data were obtained for compound **7**, whose synthesis had been formerly reported with no NMR data provided (27). The ^{13}C-NMR data of **8** and **10** were also described for the first time in the present communication. Since the structures of **9, 11**, and **12**



Scheme 1 Chemical transformations of plumbagin (**1**).

Table 2 ^{13}C-NMR (125 MHz) data of compounds **2, 5, 8 – 10** and **12**[a].

Position	2	5	8	9	10	12
1	185.2 s[b]	184.2 s	184.8 s	185.0 s	185.8 s	182.9 s
2	150.0 s	121.8 s	118.0 s	133.5 s	128.9 s	145.0 s
3	134.9 d	152.8 s	153.6 s	151.9 s	158.7 s	142.4 s
4	190.5 s	184.5 s	179.6 s	176.6 s	180.2 s	176.0 s
4a	115.5 s	112.9 s	116.9 s	118.5 s	119.3 s	119.2 s
5	161.4 s	161.2 s	160.0 s	160.1 s	159.5 s	160.1 s
6	124.3 d	123.2 d	116.7 d	117.8 d	117.2 d	117.9 d
7	138.0 d	137.5 d	136.2 d	135.2 d	134.7 d	135.1 d
8	135.6 s	119.6 d	119.6 d	119.4 d	118.9 d	119.7 d
8a	128.2 s	132.7 s	135.2 s	134.3 s	134.3 s	133.9 s
2-CH_3	16.6 q	8.8 q	8.4 q	9.6 q	8.9 q	14.1 q
3-$OCOCH_3$	–	–	–	167.9 s	–	–
3-$OCOCH_3$	–	–	–	20.4 q	–	–
3-OCH_3	–	–	–	–	60.9 q	–
5-OCH_3	–	–	56.5 q	56.5 q	56.4 q	56.5 q

[a] In $CDCl_3$ with TMS as internal standard.
[b] Determined by DEPT (s = C, d = CH and q = CH_3).

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had been hitherto unknown, both of their ^{1}H- and ^{13}C-NMR data were fully presented. The ^{13}C-NMR data of compounds **2, 5, 8–10** and **12** were summarized in Table **2** whereas those of compounds **3** and **7** were given in the experimental section.

Acknowledgements

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Kittisak Likhitwitayawuid, Ph. D.

Department of Pharmacognosy
Faculty of Pharmaceutical Sciences
Chulalongkorn University
Bangkok 10330
Thailand
E-mail:lkittisa@netserv.chula.ac.th
Fax: +66/255-8227

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