H- and C-Nmr Assignments of Phyllanthin and Hypophyllanthin: Lignans That Enhance Cytotoxic Responses with Cultured Multidrug-Resistant Cells

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^{1}H- AND ^{13}C-NMR ASSIGNMENTS OF PHYLLANTHIN AND HYPOPHYLLANTHIN: LIGNANS THAT ENHANCE CYTOTOXIC RESPONSES WITH CULTURED MULTIDRUG-RESISTANT CELLS

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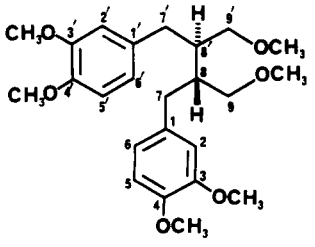
ABSTRACT.—Complete ^{1}H-nmr data and unambiguous assignments of the ^{13}C-nmr spectra of phyllanthin [**1**] and hypophyllanthin [**2**] were obtained through extensive nmr studies, including homonuclear COSY, homonuclear decoupling, APT, HETCOR, nOe difference, selective INEPT, and COLOC experiments. The absolute configuration of hypophyllanthin [**2**] was determined by cd. Neither of these lignans demonstrated significant cytotoxic activity when evaluated with a battery of cultured mammalian cells, but both were found to enhance the cytotoxic response mediated by vinblastine with multidrug-resistant KB cells. In addition, **1** was found to displace the binding of vinblastine with membrane vesicles derived from this cell line, suggesting an interaction with the P-glycoprotein.

Phyllanthus amarus Schum. & Thonn. (Euphorbiaceae) has been traditionally used for the treatment of jaundice and other diseases (1). Although the anti-hepatotoxic potential of the plant has been controversial (1–3), the major chemical components were known to be phyllanthin [**1**] and hypophyllanthin [**2**] (2). The structure of phyllanthin [**1**] was determined on the basis of the 60 MHz ^{1}H-nmr spectrum (4,5), whereas that of hypophyllanthin was proposed and revised several times (4–10). We report herein the first complete ^{1}H-nmr data and unambiguous assignments of the ^{13}C-nmr spectra of both phyllanthin [**1**] and hypophyllanthin [**2**], using combinations of 1D and 2D nmr techniques, and a determination of the absolute configuration of hypophyllanthin [**2**].



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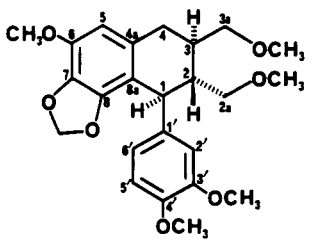

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In addition, the cytotoxic potential and ability to reverse multi-drug resistance by these compounds are reported.

RESULTS AND DISCUSSION

The physical and spectral properties of phyllanthin [**1**] isolated in this study (mp, uv, ir, ^{1}H nmr, and $[\alpha]^{20}$D) were identical with those reported earlier (5). Complete ^{1}H- and unambiguous ^{13}C-nmr assignments of **1** were readily obtained through analysis of the homonuclear COSY, NOESY, APT (11), HETCOR (12), and COLOC (13) spectra, as shown in Table 1. The structure of hypophyllanthin [**2**], however, has posed

TABLE 1. ^{1}H- and ^{13}C-nmr Assignments of Phyllanthin [**1**].[a]

Position	^{1}H	^{13}C
1 (1′)		133.6
2 (2′)	6.59 (d, 1.8)	112.2
3 (3′)		148.7
4 (4′)		147.1
5 (5′)	6.73 (d, 8.1)	111.0
6 (6′)	6.61 (dd, 8.1, 1.8)	121.0
7 (7′)	2.59 (dd, 13.8, 7.3) 2.66 (dd, 13.8, 7.5)	34.9
8 (8′)	2.01 (m)	40.7
9 (9′)	3.25 (dd, 13.8, 7.8) 3.28 (dd, 13.8, 5.4)	72.8
3 (3′)-OMe	3.78 (s)	55.9
4 (4′)-OMe	3.82 (s)	55.7
9 (9′)-OMe	3.27 (s)	58.7

[a]Recorded in $CDCl_3$; chemical shift values are reported as δ (ppm) from internal TMS at 300 MHz for ^{1}H and 75.6 MHz for ^{13}C; signal multiplicity and coupling constants (Hz) are shown in parentheses.

problems for natural product chemists for nearly 20 years, with a number of isomeric structures being proposed. Although the structure of **2** was deduced through X-ray crystallographic study (9) and later confirmed by synthesis (10), detailed analyses of the ^{1}H-nmr and ^{13}C-nmr spectra have never been described. Hypophyllanthin [**2**] used in this study was identified by comparison of its mp, $[\alpha]^{20}$D, ^{1}H and ^{13}C nmr, and mass spectra with those previously reported (7,8). The complete assignment of all protons, as shown in Table 2, was achieved with the aid of homonuclear COSY, homonuclear decoupling, and nOe difference experiments. In the homonuclear COSY spectrum, the aromatic proton singlet at δ 6.31 showed long-range coupling with the C-4 methylene protons at δ 2.71 and 2.79, and was therefore assigned to H-5. Moreover, H-5 displayed long-range coupling with the MeO resonance at δ 3.85, placing this MeO group at C-6. The nOe enhancements observed between H-5 and H-4β and between H-5 and 6-OMe confirmed these attributes. The H-1 resonance exhibited nOe enhancements with the doublet at δ 6.65 (J = 2.0 Hz) and the double doublet at δ 6.62 (J = 8.0, 2.0 Hz) which, therefore, were assigned to H-2′ and H-6′, respectively. As a result, the doublet at δ 6.72 (J = 8.0 Hz) must be assigned to H-5′. The MeO resonances at δ 3.78 and 3.82 were assigned to 3′-OMe and 4′-OMe, respectively, on the basis of the nOe enhancements displayed between H-2′ and 3′-OMe, and H-5′ and 4′-OMe. The resonance at δ 3.29 was assigned to 2a-OMe because of its nOe with 3′-OMe, leaving the signal at δ 3.31 to be assigned to 3a-OMe. Selective INEPT experiments (see below)

TABLE 2. ^{1}H- and ^{13}C-nmr Assignments of Hypophyllanthin [**2**].[a]

Position	^{1}H	^{13}C
1	4.07 (d, 7.8)	41.9
2	1.87 (m)	45.4
2a	3.22 (dd, 9.6, 3.5)	71.8
	3.34 (dd, 9.6, 1.0)	
3	1.94 (m)	35.9
3a	3.36 (dd, 9.6, 6.2)	75.5
	3.41 (dd, 9.6, 4.2)	
4α	2.71 (dd, 15.9, 10.8)	33.3
4β	2.79 (dd, 15.9, 5.4)	
4a		131.8
5	6.31 (s)	106.5
6		142.1
7		133.3
8		147.0
8a		115.1
1′		138.1
2′	6.65 (d, 2.0)	111.9
3′		148.6
4′		147.1
5′	6.72 (d, 8.0)	110.7
6′	6.62 (dd, 8.0, 2.0)	120.4
2a-OMe	3.29 (s)	58.9
3a-OMe	3.31 (s)	58.9
6-OMe	3.85 (s)	56.4
3′-OMe	3.78 (s)	55.8
4′-OMe	3.82 (s)	55.9
O-CH_2-O	5.63 (d, 1.4)	101.1
	5.71 (d, 1.4)	

[a]Recorded in $CDCl_3$; chemical shift values are reported as ppm from internal TMS at 300 MHz for ^{1}H and 75.6 MHz for ^{13}C; signal multiplicity and coupling constants (Hz) are shown in parentheses.

confirmed these assignments. The double doublet at δ 2.71 was assigned to H-4α since it was coupled to H-4β and showed a nOe enhancement with H-3. Other protons were assigned, as shown in Table 2, using a combination of homonuclear COSY and homonuclear decoupling techniques.

Unequivocal assignment of the ^{13}C-nmr spectrum of hypophyllanthin [**2**], with its numerous close chemical shifts, affords the opportunity to utilize a series of APT, HETCOR, and selective INEPT experiments, as previously described (14–17). Close examination of the APT and HETCOR spectra permitted complete assignment of all nonquaternary carbons. Selective INEPT irradiation of H-5 at δ 6.31 enhanced the signal of C-4 (δ 33.3) and three aromatic quaternary carbon resonances at δ 115.1, 133.3, and 142.1. Polarization transfer from H-4β (δ 2.79) resulted in enhancement of the resonances at δ 45.4 (C-2), 106.5 (C-5), 115.1, and 131.8. This led to the assignment of C-8a at δ 115.1 and C-4a at δ 131.8. Magnetization transfer via irradiation of either methylenedioxy proton (δ 5.64 or 5.71) enhanced the resonances at δ 133.3 and 147.0. Thus, the former signal (δ 133.3) was assigned to C-7 and the latter (δ 147.0) to C-8, leaving the resonance at δ 142.1 to be assigned to C-6. Enhancement of the signals of C-2, C-2a, C-8a, C-6′, C-4a, C-1′, and C-8 was observed when H-1 was irradiated. Polarization transfer from H-5′ (δ 6.72) enhanced the C-1′ signal and the resonance at 148.6. The latter signal should therefore be assigned to C-3′, and the resonance at δ

147.1 to C-4′. Irradiation of 2a-OMe (δ 3.29), as expected, enhanced the C-2a signal (δ 71.8), and completed the resonance assignments.

The absolute configuration of hypophyllanthin [**2**] has been suggested (10), but no conclusive spectral evidence has been reported. In our study, the cd spectrum of hypophyllanthin showed a negative couplet at 289–273 nm, indicating the aryltetralin type A structure (18). Thus, it was concluded that hypophyllanthin has the absolute configuration 1β,2α,3β (trans/trans) or 1*S*,2*R*,3*R*, consistent with the earlier proposed configuration (10).

Evaluation of the cytotoxic potential of **1** and **2** was then conducted with cultured P-388 cells and a battery of human tumor cell lines. The ED_{50} values exceeded the highest concentration tested (20 μg/ml) with tests conducted with P-388, BCA-1, HT-1080, LUC-1, MEL-2, COL-2, A-431, LNCaP, and ZR-75-1 cells (data not shown). As summarized in Table 3, however, phyllanthin [**1**] demonstrated an ED_{50} value of 9.0 μg/ml with KB-V1 cells in the absence of vinblastine, and this value was decreased to 2.1 μg/ml in the presence of vinblastine. Hypophyllanthin [**2**] did not mediate a cytotoxic response in the absence of vinblastine, but on addition of this substance, an ED_{50} value of 3.8 μg/ml was obtained. Neither compound demonstrated activity with the drug-sensitive cell line, KB-3 (Table 3).

TABLE 3. Cytotoxicity of Phyllanthin [**1**] and Hypophyllanthin [**2**] against KB-3 and KB-V1 Cells.[a]

Compound	Cell line		
	KB-3	KB-V1	
		without VLB	with VLB[b]
Phyllanthin [**1**]	>20	9.0	2.1
Hypophyllanthin [**2**]	>20	>20	3.8

[a]Data are ED_{50} values (μg/ml).
[b]At 1 μg/ml.

The nature of this effect was further studied by examining the potential of compounds **1** and **2** to inhibit the binding of radiolabelled vinblastine with membrane vesicles derived from KB-V1 cells. As illustrated in Figure 1, phyllanthin [**1**] mediated a

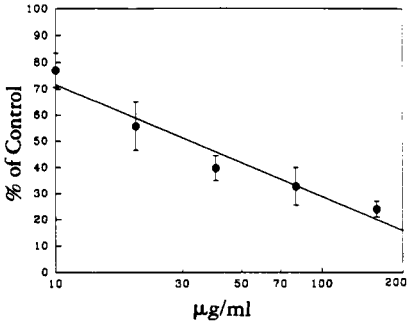

FIGURE 1. Dose-dependent inhibition of [^{3}H]-vinblastine binding with membrane vesicle preparations isolated from KB-V1 cells. Experiments were performed as described in the Experimental section, adding the indicated concentration of phyllanthin [**1**].

dose-dependent inhibition of vinblastine interaction, yielding an IC_{50} value of 28 μg/ml. Conversely, hypophyllanthin [**2**] was not active in this assay (IC_{50} >160 μg/ml) (data not shown).

Because phyllanthin [**1**] was not active as a cytotoxic agent with KB-3 cells, but was active with KB-V1, affinity for the P-glycoprotein may be suggested. This suggestion is further strengthened since the cytotoxic activity mediated by vinblastine with KB-V1 cells was augmented by the addition of **1** and moreover, since binding of vinblastine with KB-V1-derived membrane vesicles was displaced by **1** in a dose-dependent manner. The interaction of hypophyllanthin [**2**] with the P-glycoprotein is less clear since no appreciable activity was observed with the binding assay, but binding may be implied due to the structural similarity with compound **1** and the ability of **2** to reverse the resistance of KB-V1 cells to vinblastine. However, it appears that the interaction of compound **1** with the P-glycoprotein is greater than the interaction of compound **2**. Making use of the spectroscopic parameters described in this communication for compounds **1** and **2**, it is possible that structural regions of these molecules that are involved in the P-glycoprotein interaction can be defined.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mp's were determined on a Kofler hot plate and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. Uv spectra were obtained on a Beckman DU-7 spectrometer, and ir spectra measured on a Nicolet MX-1 FT-IR (KBr) interferometer. ^{1}H-nmr, homonuclear COSY, ^{13}C-nmr, APT, and HETCOR spectra were recorded in $CDCl_3$, with TMS as internal standard, employing a Varian XL-300 instrument. Standard Varian pulse sequences were used. The COLOC experiment was carried out at 75.6 MHz on a Varian XL-300 nmr spectrometer, using relaxation delay 1.0 sec, acquisition time 0.112 sec, $\Delta_1 = 25$ msec, $\Delta_2 = 30$ msec, 48 experiments of 64 scans, total measuring time 1 h. Selective INEPT experiments were performed at 90.8 MHz using a Nicolet NMC-360 spectrometer. Data sets of 16K covering a spectral width of 10 MHz were acquired. Proton pulse widths were calibrated by using a sample of HOAc in 10% C_6D_6 ($^1J = 6.7$ Hz) in a 5-mm nmr tube. The radio frequency field strength for the soft proton pulse was on the order of 25 Hz for these experiments. Eight Hertz was used as $^3J_{CH}$ for aromatic protons, and 6 and 3 Hz for aliphatic protons. Mass spectra were obtained with a Varian MAT 112S instrument operating at 70 eV. The cd spectrum was run in MeOH with a JASCO J-40A spectropolarimeter.

PLANT MATERIAL.—The aerial parts of *P. amarus* were collected in July 1990 in Bangkok and at Sireerukhachart Botanical Garden, Mahidol University, Nakorn Pathom province, Thailand. The plant was identified by Prof. Payow Maunwongyathi, Sireerukhachart Botanical Garden. A voucher specimen is on deposit at the Faculty of Pharmacy, Mahidol University.

EXTRACTION, FRACTIONATION, AND ISOLATION.—The dried and powdered aerial parts of *P. amarus* (3 kg) were extracted with hexane at room temperature, and the solvent was removed to afford a residue (27 g). A portion of the hexane extract (4 g) was chromatographed over Si gel and eluted with hexane, toluene, EtOAc, and CH_2Cl_2 in a polarity gradient manner. Fractions 226–231, after removal of the solvent, gave crude phyllanthin which was subsequently purified by recrystallization from petroleum ether to yield phyllanthin [**1**] (786.6 mg, 0.18%). Fractions 176–206 were combined, evaporated, and further chromatographed over Si gel, using a series of hexane/Me_2CO mixed solvents with increasing polarity, to afford crude **2** and other unidentified components. Pure hypophyllanthin [**2**] (178.4 mg, 0.04%) was obtained through recrystallization from petroleum ether.

Phyllanthin [**1**].—Mp 96° (petroleum ether); $[\alpha]^{20}D$ +15.5° ($c = 0.10$, EtOH); uv λ max (EtOH) 230 (log ε 4.30), 280 (2.15) nm; ir ν max (KBr) 3201, 2917, 1518, 1465, 1182, 1165, 965 cm^{-1}; ^{1}H and ^{13}C nmr, see Table 1; eims *m/z* (rel. int.) $[M]^+$ 418 (9), 386 (4), 203 (17), 177 (12), 151 (100), 107 (13), 45 (31).

Hypophyllanthin [**2**].—Mp 128° (petroleum ether); $[\alpha]^{20}D$ +3.8° ($c = 0.11$, MeOH); uv λ max (MeOH) 213 (log ε 4.65), 231 (4.33), 280 (3.65) nm; ir ν max (KBr) 3010, 2902, 1639, 1426, 1260, 1152, 1026, 936, 794 cm^{-1}; ^{1}H and ^{13}C nmr, see Table 2; eims *m/z* (rel. int.) $[M]^+$ 430 (23), 398 (5), 367 (5), 222 (9), 208 (15), 151 (50), 41 (100); cd (MeOH) Δε: −0.29 (289), +0.59 (273), −2.19 (244), +9.46 (231).

CYTOTOXICITY ASSAYS.—Using procedures described previously (19), the cytotoxic potentials of

phyllanthin [**1**] and hypophyllanthin [**2**] were determined using the following human cell lines: BCA-1 (breast), COL-1 (colon), LUC-1 (lung), MEL-2 (melanoma), HT-1080 (sarcoma), A-431 (squamous cell carcinoma), LNCaP (prostate), and ZR-75-1 (breast). Tests were also conducted with cultured P-388 cells. Briefly, various concentrations of the test compounds (dissolved in 10 μl of 10% DMSO) were transferred to 96-well plates, and 190 μl of cell suspensions were added. The plates were then incubated for 2–4 days at 37° (100% humidity with a 5% CO_2 atmosphere in air). At the end of the incubation period, 50 μl of cold 50% trichloroacetic acid (TCA) was layered on top of the growth medium in each well. The cultures were incubated at 4° for 1 h and then washed 5 times with tap H_2O to remove TCA. Plates were air-dried and stained with sulforhodamine B solution. Stained cultures were washed with 1% HOAc, air-dried, and treated with 10 mM Tris base, and the optical density was determined at 515 nm using an ELISA plate reader. Relative to controls, the percent growth of compound-treated cells was calculated.

In addition to the cells lines listed above, similar studies were conducted with KB-3 and (drug-resistant) KB-V1 cell cultures. To investigate the effect of phyllanthin [**1**] and hypophyllanthin [**2**] on reversing multidrug-resistance, KB-V1 cells were treated with different concentrations of compounds in the presence or absence of 1 μg/ml vinblastine. This concentration is lethal with KB-3 cells, but does not affect the growth of KB-V1 cells.

MEMBRANE VESICLE PREPARATION.—Cell membrane vesicles were prepared from KB-V1 cells essentially by the procedure of Cornwell *et al.* (20). Cells were grown to approximately 80% confluence and rinsed with phosphate buffered saline (PBS). PBS containing 2 mM EDTA and 1% aprotinin (Sigma) was then added, and the cells were incubated at room temperature for 10 min. The cells were harvested by gentle aspiration with a serological pipet, collected by centrifugation, suspended in 0.25 M sucrose buffer (0.25 M sucrose, 0.2 mM $CaCl_2$, 1 mM EDTA, 0.01 M Tris-HCl, pH 7.5), and homogenized using a Polytron (2500 rpm, 30 sec). Following this procedure, less then 5% of the cells remained intact. The homogenate was then diluted with 4 volumes of 0.025 M sucrose solution (0.025 M sucrose, 0.01 M Tris-HCl, pH 7.5) and centrifuged (1000 × *g*, 10 min). The supernatant was layered over a 35% sucrose cushion (35% sucrose, w/v, 1 mM EDTA, 0.01 M Tris-HCl, pH 7.5) and centrifuged (16,000 × *g*, 30 min). The interface (about 5 ml) was collected, diluted with 4 volumes of 0.25 M sucrose containing 0.01 M Tris-HCl, pH 7.5, and centrifuged (100,000 × *g*, 1 h). The vesicle pellet was suspended in PBS containing 1 mM phenylmethylsulfonyl fluoride, using a 27-gauge needle, and stored at −80°. Protein concentration of the membrane vesicle preparation was determined by the method of Bradford (21).

VINBLASTINE BINDING ASSAY.—Experiments were performed using 96-well plates. Reaction mixtures (100 μl) were prepared containing 40 μg membrane vesicle protein in transport buffer (0.125 M sucrose, 0.5 mM ATP, 5 mM $MgCl_2$, 0.01 M Tris-HCl, pH 7.5) containing 0.16 μM ^{3}H-vinblastine (16 Ci/mmol, Amersham) and various concentrations of compounds **1** or **2**. The mixture was incubated at room temperature for 20 min, and samples were collected on glass fiber filters (prewashed with transport buffer containing 3% bovine serum albumin) using a semi-automatic cell harvester (Skatron). Nonspecific binding was determined by adding unlabeled vinblastine (final concentration 1 mM) to reaction mixtures, followed by filtration. Radioactivity was determined by liquid scintillation counting, and the results were expressed as a percentage after correcting for nonspecific binding.

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