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STUDIES ON INHIBITORS OF SKIN TUMOR PROMOTION, IX.[1]
NEOLIGNANS FROM *MAGNOLIA OFFICINALIS*

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ABSTRACT.—Three neolignans, known as magnolol [1], honokiol [2] and the new monoterpenylmagnolol [3], were isolated from the bark of *Magnolia officinalis* as inhibitors of Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). The structure of 3 was determined from 2D nmr spectral data and difference nOe experiments. The MeOH extract of this plant and magnolol exhibited remarkable inhibitory effects on mouse skin tumor promotion in an in vivo two stage carcinogenesis test. This investigation indicates that these neolignans and the extract might be valuable antitumor promoters.

The bark of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae) has been used in Chinese and Japanese folk medicine for the treatment of bronchitis and emphysema (1), and this crude drug is one of the important components of the Kampo prescriptions. Previously, we have reported that several natural products showed strong inhibitory activities on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA), which is known as a strong tumor promoter (2–6). On the other hand, many compounds that inhibit EBV-EA induction by tumor promoters have been shown to act as inhibitors of tumor promotion in vivo (7–10).

As a result of our continuing search among medicinal plants for novel, naturally occurring potential antitumor promoters, the MeOH extract of the bark of *M. officinalis* was found to show significant inhibitory effects on EBV-EA activation on Raji cells (11). Bioassay-directed fractionation of the active extract led to the isolation and characterization of three neolignans, magnolol [1], honokiol [2], and monoterpenylmagnolol [3], as inhibitory principles of EBV-EA activation. In this paper, we report the structure elucidation of compound 3 and results of the assay on inhibitory effects of these neolignans on EBV-EA activation and in vivo two stage carcinogenesis of mouse skin (12).

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

3

4
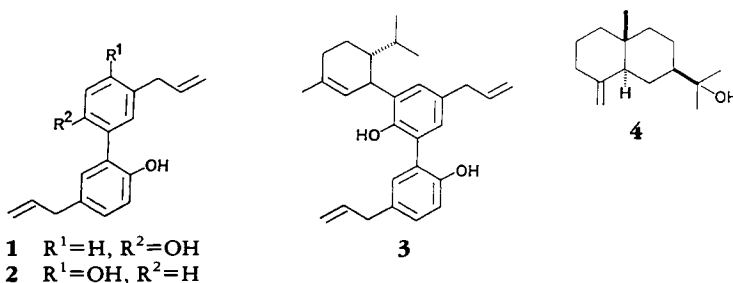
[1]For Part VIII, see Takasaki *et al.* (6).

Actions of this crude drug and constituents of this plant on the central nervous system have been reported (13, 14), but studies on their inhibitory effects on EBV-EA activation and skin tumor promotion have not been published thus far.

RESULTS AND DISCUSSION

As shown in Table 1, the Et_2O extract obtained from the original MeOH extract of bark of *M. officinalis* showed significant inhibitory effects on EBV-EA activation even at a low dose (100% inhibition of activation at 100 μg/ml and 39.3% inhibition of activation even at 1 μg/ml). These inhibitory effects are higher than those of *Wisteria brachybotrys* reported by Konoshima *et al.* (2). On the other hand, the $CHCl_3$, EtOAc, *n*-BuOH, and H_2O extracts obtained from the original MeOH extract exhibited lower inhibitory effects than *n*-hexane and Et_2O extracts. We carried out bioassay-directed fractionation of the Et_2O extract and purification of the most active fraction. Magnolol {**1**}, honokiol {**2**}, compound **3**, and β-eudesmol {**4**} were isolated by repeated cc and flash chromatography. Compound **3**, $C_{28}H_{34}O_2$, showed ir absorption bands (3300, 1650, and 1615 cm^{-1}) similar to those of compounds **1** and **2**. The comparison of ^{13}C-nmr and ^1H-nmr spectra of **3** with those of compound **1** suggested that the compound **3** was monoterpenylmagnolol. The assignments of -CH, -CH$_2$, -CH$_3$, and olefinic carbon resonances were made in the ^{13}C-DEPT experiment, and the assignments of proton

TABLE 1.   Relative Ratio of EBV-EA Activation with Respect to Positive Control[a]
(100%) in Presence of Extracts of *Magnolia officinalis*.

Sample	Concentration[b]		
	100	10	1
MeOH	0.0[c] (10)[d]	0.0 (60)	90.4 (>80)
n-Hexane	—[e] (0)	0.0 (60)	75.4 (>80)
Et_2O	—[e] (0)	0.0 (60)	60.7 (>80)
$CHCl_3$	—[e] (0)	54.7 (70)	100.0 (>80)
EtOAc	17.8 (70)	48.9 (>80)	100.0 (>80)
n-BuOH	—[e] (0)	82.1 (>80)	100.0 (>80)
H_2O	46.4 (60)	70.7 (>80)	100.0 (>80)

[a]TPA 20 ng/ml = 32 pM.
[b]μg/ml.
[c]Values represent percentages relative to the positive control value (100%).
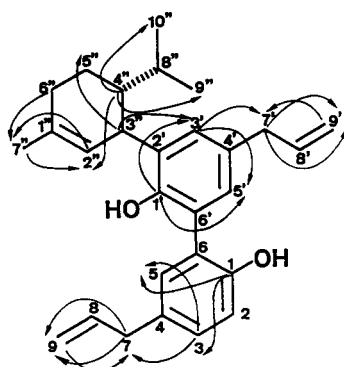[d]Values in parentheses are viability percentages of Raji cells.
[e]Not detected.

FIGURE 1.   Correlation ($^{13}C→^1H$) in the ^1H-^{13}C long-range COSY spectrum of **3**.

signals were carried out using ^{1}H-^{1}H and ^{1}H-^{13}C COSY spectra of **3** as shown in Table 2. Further, we measured the ^{1}H-^{13}C long range COSY in order to confirm the connectivities of the partial structure. As shown in Figure 1, methine carbon at δ 42.16 (C-3″) is correlated with the proton at δ 6.95 (H-3′), aromatic carbon at δ 149.20 (C-1′) is correlated with protons at δ 6.95 (H-3′) and 6.92 (H-5′), and aromatic carbon at δ 151.46 (C-1) is correlated with protons at δ 7.08 (H-5) and 7.11 (H-3). Some other significant long range ^{1}H-^{13}C correlations are indicated by arrows in Figure 1. The difference nOe's of compound **3** were measured in order to confirm the stereochemistry of **3**. Irradiation at H-4″ (δ 1.60) enhanced the signal intensity of the aromatic proton (H-3′, δ 6.95), and irradiation at the methyl proton signal (δ 0.85) enhanced the signal intensities of the methylene protons (δ 3.35) and the aromatic proton (δ 6.95). Irradiation at the methyl proton signal (7″-Me, δ 1.74) enhanced the signal intensities of the methylene proton (6″-CH$_2$, δ 2.09) and the olefinic proton (H-2″, δ 5.44) (Figure 2). Some other significant nOe results are indicated by arrows in Figure 2. From these results, it was concluded that the structure of the monoterpenyl compound **3** is as shown by the formula **3**. [This compound was reported by Dr. S. Yahara *et al.*, at the 29th annual meeting of the Japanese Society of Pharmacognosy (1982), but the structure, including the physicochemical data, has not been published.]

Three neolignans **1**, **2**, and **3** and β-eudesmol [**4**] were tested utilizing the short term in vitro assay of EBV-EA activation. Their inhibitory effects on activation and viabilities of Raji cells are shown in Table 3. The lignans **1**, **2**, and **3** exhibited remarkable inhibitory effects on EBV-EA activation and preserved high viabilities of Raji cells. Especially, compound **1** exhibited a most significant inhibitory activity (about 70% inhibition at a 1×10^2 mol ratio of inhibitor/TPA and 40% inhibition even at a 1×10 mol ratio) among these neolignans. Honokiol [**2**] and monoterpenylmagnolol [**3**] exhibited strong inhibitory activities at high doses, 1×10^3 and 5×10^2 mol ratios. On the other hand, β-eudesmol (major monoterpene from *M. officinalis*) and α-phellandrene (a part of the structure of **3**) exhibited lower inhibitory effects than the neolignans. In our experiments the inhibitory activities of **1** and **2** were more than 10 times

TABLE 2. ^{13}C and ^{1}H Assignments for Compound **3**.

Carbon	Chemical shifts[a]		Carbon	Chemical shifts		Carbon	Chemical shifts	
	^{13}C[b]	^{1}H[c]		^{13}C	^{1}H		^{13}C	^{1}H
1	151.46		1′	149.20		1″	138.52	
2	116.97	6.89 (d, 8.1)	2′	131.28		2″	124.19	5.48 (br s)
3	137.63	7.11 (dd, 8.1, 2.2)	3′	137.68	6.95 (d, 2.2)	3″	42.16	3.49 (br d)
4	132.41		4′	132.67		4″	44.42	1.60 (m)
5	129.49	7.08 (d, 2.2)	5′	129.58	6.92 (d, 2.2)	5″	21.60[i]	1.41 (m)
6	125.21		6′	125.02		6″	30.25	2.09 (br s)
7	39.41[g]	3.34 (d, 4.9)[d]	7′	39.41[g]	3.35 (d, 4.9)[d]	7″	23.65	1.74 (br s)
8	137.63	5.08 (m)[e]	8′	137.68	5.10 (m)[e]	8″	27.43	1.61 (m)
9	115.59[h]	5.98 (tdd, 16.9, 10.1, 6.7)[f]	9′	115.59[h]	5.97 (tdd, 16.9, 10.1, 6.7)[f]	9″	16.53	0.89 (d, 6.6)
1-OH		5.87 (s)	1′-OH		6.09 (s)	10″	21.60[i]	0.85 (d, 6.6)

[a]Ppm relative to TMS.
[b]^{13}C chemical shifts assigned on the basis of DEPT and ^{1}H-^{13}C COSY experiments.
[c]^{1}H chemical shifts assigned on the basis of ^{1}H-^{1}H and ^{1}H-^{13}C COSY experiments. Abbreviations and values in parentheses represent coupling patterns (s: singlet, d: doublet, dd: doublet of doublet, tdd: triplet of doublet of doublet) and coupling constants (Hz).
[d,e,f]Assignments with the same superscripts may be interchanged.
[g,h,i]These signals with the same superscripts were overlapping.
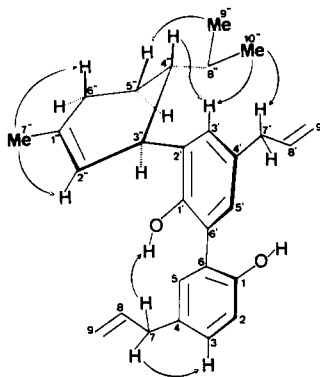
FIGURE 2.   Significant enhancement of signal intensity
by difference nOe experiments of **3**.

higher than those of retinoic and glycyrrhetinic acids, which are known as strong antitumor promoters (10,18). Furthermore, the present investigation by in vitro assay strongly suggested that these neolignans may be valuable antitumor promoters and that the inhibitory effect of the MeOH extract of *M. officinalis* on EBV-EA activation is due to the combined effects of these neolignans.

On the basis of the results of the in vitro assay, the effects of the extract of *M. officinalis* and magnolol on two stage carcinogenesis in vivo were investigated. The activities, evaluated by both the rate (%) of papilloma-bearing mice and average number of papillomas per mouse, were compared with those of a positive control. As shown in Figure 3, both the MeOH extract (50 µg) and magnolol (85 nmol), when applied continuously before each TPA treatment, delayed the formation of papillomas in mouse skin as compared with the control experiment only with TPA. Furthermore, they reduced the number of papillomas per mouse (about 40% reduction even at 20 weeks). These results suggested that magnolol and the extract of *M. officinalis* might be valuable anti-promoters in carcinogenesis. Furthermore, activity shown in the in vivo test of the MeOH extract would be a combined effects of neolignans with other phenolic compounds and/or terpenes. Studies on the details of the inhibitory mechanisms of neolignans and on inhibitory effects of these neolignans on lung cancer promotion are now in progress. The search for other active constituents and studies on the details of the combined effects by plural constituents are also in progress.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Ir spectra were measured on a Shimadzu IR-408 spectrometer. ^{1}H-nmr and ^{13}C-nmr spectra were recorded on a Varian XL-300 spectrometer in CDCl$_3$ using TMS as an internal standard. 2D nmr and difference nOe spectra were recorded on a JEOL JNM GX-400 spectrometer. Mass spectra were determined on a Hitachi M-80 mass spectrometer at 20 eV using a direct inlet system. Si gel (Merck G 60, 230 mesh) was used for column and for flash chromatography (15), and Si gel plates (Merck, 60 F-254, 0.25 mm) were used for analytical tlc. Compounds were visualized by uv light and by spraying with 10% cerium (IV) sulfate in 10% H$_2$SO$_4$ solution followed by heating.

PLANT MATERIAL AND EXTRACTION.—The bark of *M. officinalis* was collected from Jiangsu Prefecture, China and obtained from Fukuda-Ryu Co. Ltd., Japan, from whom the voucher specimens are available. Herbarium specimens are deposited in the herbarium of Kyoto Pharmaceutical University. The chopped bark of *M. officinalis* (2.2 kg) was exhaustively extracted with hot MeOH (3 liters × 3). After the solvent was removed in vacuo, a dark brown residue (340 g) remained. Extraction and fractionation of the active compounds was monitored by an in vitro short-term assay of EBV-EA activation. The active syrup was suspended in H$_2$O and extracted several times with *n*-hexane. The aqueous layer was extracted with Et$_2$O, CHCl$_3$, EtOAc, and *n*-BuOH saturated with H$_2$O, successively. Each organic layer was evaporated in vacuo to give residue (*n*-hexane 80 g, Et$_2$O 195 g, CHCl$_3$ 60 g, EtOAc 39 g, and *n*-BuOH 37 g). The results of the bioassay of these extracts are in Table 1.

TABLE 3. Relative Ratio of EBV-EA Activation with Respect to Positive Control[a] (100%) in Presence of Constituents of *Magnolia officinalis*.

Compound	Concentration[b]				
	1×10^3	5×10^2	1×10^2	10	1
Magnolol [1]	0.0±0[c] (70)[d]	0.0±0 (>80)	31.2±2.7(>80)	56.9±3.1(>80)	94.4±0(>80)
Honokiol [2]	0.0±0 (60)	18.5±3.1(>80)	69.4±2.6(>80)	90.1±0.8(>80)	100.0±0(>80)
Monoterpenylmagnolol [3] . . .	11.6±2.9 (60)	20.9±2.5(>80)	82.6±1.4(>80)	100.0±0 (>80)	100.0±0(>80)
β-Eudesmol [4]	40.1±3.8 (>80)	68.9±2.8(>80)	83.8±2.3(>80)	100.0±0 (>80)	100.0±0(>80)
α-Phellandrene	78.5±4.2 (>80)	93.7±1.7(>80)	100.0±0 (>80)	100.0±0 (>80)	100.0±0(>80)

[a]Induction by TPA at 20 ng/ml = 32 pM.
[b]Mol ratio/TPA (20 ng/ml = 32 pM/ml).
[c]Values represent relative percentages to the positive control values (100%), and averages of three determination ± SD.
[d]Values in parentheses are viability percentages of Raji cells, and in this screening test, the cell viability required for the judgment of inhibitory effect was more than 60%.
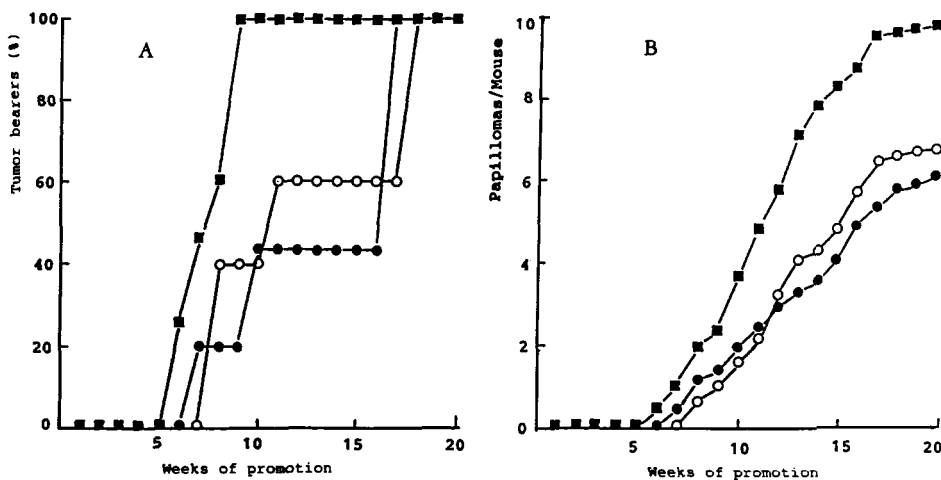
FIGURE 3.   Inhibition of TPA-induced tumor promotion by multiple application of magnolol (85 nM) and the MeOH extract of *M. officinalis* (50 μg). All mice were initiated with DMBA (390 nM) and promoted with 1.7 nM of TPA given twice weekly starting 1 week after initiation. A: Percentage of mice with papillomas. B: Average number of papillomas per mouse. ■, control TPA alone; ●, TPA + 50 μg of MeOH extract of *M. officinalis*; ○, TPA + 85 nM of magnolol.

ISOLATION OF COMPOUNDS **1–4**.—The Et_2O extract (5 g) was fractionated by cc on Si gel [solvent $MeOH$-$CHCl_3$ (3:97)] to afford 28 fractions. These fractions were subjected to the abovementioned assay. Purification of the most active fractions was carried out by repeated flash chromatography [adsorbent Si gel (Merck, Kieselgel 60, 230 mesh); solvent $CHCl_3$; pressure 1.4 kg/cm^2 (N_2 gas flow)] to afford magnolol [**1**] (180 mg, 2.06% of dried MeOH extract), honokiol [**2**] (85 mg, 0.97%), monoterpenylmagnolol [**3**] (20 mg, 0.23%), and β-eudesmol [**4**] (70 mg, 0.80%). Compounds **1**, **2** and **4** were identified by comparison with previously reported data (16,17).

Compound **3**.—Colorless oil, $[\alpha]D$ − 138.0° (c = 1.03, $CHCl_3$); ir ($CHCl_3$) cm^{-1} 3300 (OH), 1615 (phenyl), 1650 (vinyl); ^1H nmr and ^{13}C nmr see Table 3; eims m/z $[M]^+$ 402 (base peak), $[M - C_3H_7]^+$ 359 (22%), $[M - C_5H_{10}]^+$ 332 (due to retro Diels-Alder cleavage of monoterpenyl moiety, 20%), $[332 - C_3H_5]^+$ 291 (53%). *Anal.* found C 83.50%, H 8.59%; calcd for $C_{28}H_{34}O_2$, C 83.54%, H 8.51%.

CHEMICALS.—TPA and DMBA were obtained from Sigma Chemical, U.S.A., and α-phellandrene was purchased from Tokyo Kasei, Japan.

BIOLOGICAL ACTIVITIES IN VITRO.—The inhibition of EBA-EA activation was assayed using the EBV genome-carrying lymphoblastoid cell (Raji), which was cultivated in RMPI 1640 medium. The indicator cells were incubated at 37° for 48 h in 1 ml of the medium containing *n*-butyric acid (4 mM, co-inducer), TPA (20 ng/ml, 32 pM), and a known amount of test compound in DMSO. Smears were made from the cell suspension. The activated cells were stained by high titer EBV-positive sera from nasopharyngeal carcinoma (NPC) patients and detected by a conventional indirect immunofluorescence technique. In each assay, at least 500 cells were counted and the experiments were repeated twice. The average EA induction was compared to that of positive control experiments with *n*-butyric acid (4 mM) and TPA (32 pM), in which EA induction was ordinarily around 30%.

TWO-STAGE CARCINOGENESIS TEST IN VIVO.—Female ICR mice (7 weeks old) were obtained from Shizuoka Laboratory Animal Center, Shizuoka, Japan. Each group of 15 mice was housed 5 mice per cage and given H_2O ad libitum. The back of each mouse was shaved with surgical clippers. The mice were initiated with dimethylbenz[*a*]anthracene (DMBA, 100 μg, 390 nM) in Me_2CO. One week after initiation, they were promoted twice a week by application of TPA (1 μg, 1.7 nM) in Me_2CO. One hour before each TPA treatment, the mice were treated with sample (85 nM or 50 μg, respectively) in Me_2CO. The incidence of papillomas was observed weekly for 20 weeks.

ACKNOWLEDGMENTS

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