A New Tumor Promoter from the Seed Oil of *Jatropha curcas* L., an Intramolecular Diester of 12-Deoxy-16-hydroxyphorbol

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**ABSTRACT**

A new type of phorbol ester, which has a macrocyclic dicarboxylic acid diester structure, was isolated from the seed oil of *Jatropha curcas* L. (Euphorbiaceae). Based on the results of spectroscopic analyses of the compound and its chemical degradation products, its structure is proposed to be an intramolecular 13,16-diester of 12-deoxy-16-hydroxyphorbol, 12-deoxy-16-hydroxyphorbol-4’-12’,14’-butadienyl]-6’-16’,18’,20’-nonatrienyl]bicyclo[3.1.0]hexane-13-(O)-2’-[carboxylate]-16-(O)-3’-[8’-butenoic-10’-ate] (DHBP). DHBP showed slightly weaker biological and biochemical activities than 12-O-tetradecanoylphorbol-13-acetate (TPA). DHBP induced ornithine decarboxylase in mouse skin (2.8 nmol CO2/min/mg protein/34 nmol application), inhibited the specific binding of [3H]TPA to a skin membrane particle fraction. This evidence showed that the tumor promoters contained in the irritant fraction were TPA-type tumor promoters, probably phorbol esters. If the tumor promoters are structurally identical to the esters of 12-deoxy-16-hydroxyphorbol which Hecker and his associates had reported, it would be interesting to determine their structures and the tumor-promoting activity of the purified compounds. We obtained 22 mg of one major compound (compound 1) from 4.5 liters of the seed oil and 9.8 kg of the seeds of *J. curcas* L. By using nuclear magnetic resonance methods and chemical degradation, the structure was elucidated to be 12-deoxy-16-hydroxyphorbol esterified with the dicarboxylic acid 4’-12’,14’-butadienyl]-6’-16’,18’,20’-nonatrienyl]bicyclo[3.1.0]hexane-2’-[carboxylic acid]-3’-[8’-butenoic acid-10’]. DHBP has a 12-membered macrocyclic dicarboxylic acid diester structure most likely between O-13 and O-16 of 12-deoxy-16-hydroxyphorbol. It represents a new type of phorbol ester. In this paper, we report the isolation, structure determination, and biochemical and biological activities, including the tumor-promoting activity of DHBP, in a two-stage carcinogenesis experiment on mouse skin.

**MATERIALS AND METHODS**

The seed oil and the seed of *J. curcas* L. were obtained from the Division of Agricultural Chemistry, Department of Agriculture, Bangkok, Thailand. DHBP used in the experiment was stored as methanol solution at −20°C in the dark. Under this condition, DHBP is stable during the experiments. The purity of DHBP was checked by HPLC. TPA and DMBA were purchased from LC Services Corp., Woburn, MA, and Sigma Chemical Co., St. Louis, MO, respectively. dl-[3H]Ornithine monohydrochloride and [20-3H]TPA were from New England Nuclear, Boston, MA. γ-[3P]ATP was obtained from Amersham, Buckinghamshire, United Kingdom. The following instruments were used: a Toyoda soda model SP8750 with a UV-8 UV detector and Shodex RI SE-51 RI detector for HPLC; a Broker WH-Z70; an AM-106; and a JEOL JNM-GX400 FT-NMR spectrometer of 1H and 13C NMR spectra; a JEOL JX 2000 mass spectrometer for mass spectra; and a Nicolet SDX FT-IR spectrometer for IR spectra.

**Isolation of Compound 1** from Seed Oil and Seeds of *J. curcas* L.

The seed oil of *J. curcas* L. (4.6 liters) was extracted with methanol. The methanol extract was washed with hexane and saturated sodium bicarbonate. Chromatography of the resultant methanol extract on Florisil with hexane and methylene chloride-methanol mixtures followed by flash chromatography of the irritant fraction on Florisil with hexane-ethyl ether mixtures and 50% acetone in toluene gave the active fraction, which was further purified by HPLC on a TSK gel 410 KG column (Toyoda Soda, Tokyo, Japan) with 60–90% methanol in water, then on a Hibar LiChrosorb RP-18 column (Merck, Darmstadt, West Germany)

1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; COSY, correlation spectroscopy; DMBA, 7,12-dimethylbenz(a)anthracene; HPLC, high performance liquid chromatography; LAH, lithium aluminum hydride; MS, mass spectroscopy; NMR, nuclear magnetic resonance; ODCC, ornithine decarboxylase; DHBP, 12-deoxy-16-hydroxyphorbol esterified with a dicarboxylic acid 4’-12’,14’-butadienyl]-6’-16’,18’,20’-nonatrienyl]bicyclo[3.1.0]hexane-2’-[carboxylic acid]-3’-[8’-butenoic acid-10’]. TBP, 12-O-tetradeca-2’-cis-6’-trans-8’-tetrainoylethoxyphorbol-13-acetate; s, singlet; d, doublet; t, triplet; m, multiplet; br s, broadened singlet; br d, broadened doublet; dd, doublet of doublets; ddd, doublet of doublet of doublets; td, triplet of doublets; t, triplet of quartets.

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with 75% acetonitrile in water to obtain compound 1 (3 mg) as an amorphous powder. Compound 1 (19 mg) was also isolated from the seeds (9.8 kg) of J. curcas L. The seeds were ground and extracted with hexane, methanol, and methanol:chloroform (1:1) successively. Each extract was combined and purified by the same procedure as previously described. Compound 1, MS (negative fast atom bombardment) m/z: 709 (M-1), 692 (M-18), 641 (M-69), 391 (M-319), 277 (M-433).

Methanolation of Compound 1. The solution of compound 1 (7 mg) was made in 1 ml of 0.2% methanolic sodium methoxide and allowed to stand at room temperature for 2 h. The reaction product, obtained by standard procedure, was chromatographed on Florisil with toluene-acetone to yield two products, compound 2 (5 mg) and compound 3 (0.5 mg). Compound 2, 1H NMR (400 MHz, CDCl3 + D2O) ð: 7.54, s, H-1, 2.52, d, J = 19.2 Hz, 2.43, d, J = 19.2 Hz, H2-5 5.53, br s, H-7, 2.92, m, H-8, 3.16, br s, H-10, 2.05, m, H-11, 1.98, m, 1.64, m, H-12, 0.91, d, J = 5.4 Hz, H-14, 4.28, d, J = 11.2 Hz, 4.21, d, J = 11.2 Hz, H-16, 1.05, s, H-17, 0.99, d, J = 6.3 Hz, H-18, 1.80, dd, J = 1.6 Hz, H-19, 4.03, d, J = 12.9 Hz, 3.97, d, J = 12.9 Hz, H-20, 1.75, m, H-1 -' 3.08, d, J = 8.3 Hz, H-2', 2.35, m, H-3', 3.47, m, H-4', 1.80, m, H-5', 1.70, m, H-6', 5.25, dd, J = 8.9, 15.2 Hz, H-8', 5.49, td, J = 7.7, 15.4 Hz, H-9', 2.93, m, H-10', 5.31, t, J = 10.5 Hz, H-12', 6.03, t, J = 11.0 Hz, H-13', 6.58, td, J = 10.5, 16.8 Hz, H-14', 5.17, d, J = 9.6, 15.1 Hz, H-19', 6.08, m, H-20', 5.72, td, J = 7.2, 14.4 Hz, H-21', 2.09, td, J = 7.2, H-22', 1.42, t, J = 7.2 Hz, H-23', 0.91, t, J = 7.3 Hz, H-24', 3.53, s, CH3O.

Characterization of the Alcohol Moiety. The 1H NMR spectrum of compound 2 revealed the characteristic features of 12-deoxy-16-hydroxyphorbol esters as shown in Table 1 (10). The spectrum showed two exchangeable singlets at 5.24 and 2.26 ppm, OH-4 and OH-9, and a broadened AB quartet centered at 4.02 ppm, H-20, which suggested that the hydroxyl groups at C-4, C-9, and C-20 were free (10). The nonequivalent methylene signals ascribable to H-16 and the absence of a coupling between the H-16 and the hydroxyl proton suggested that the hydroxyl group at C-16 was esterified. Since compound 3, a methanolyis product of compound 1, was spectroscopically identical with crotophorbolone, the alcohol moiety of compound 1 was, therefore, 12-deoxy-16-hydroxyphorbol. The 13C NMR spectrum of compound 1 and its conversion into crotophorbolone with sodium methoxide also indicated the presence of an ester carbonyl group at C-13. In conclusion, the hydroxyl groups of compound 1 at C-13 and C-16 were esterified, and those at C-4, C-9, and C-20 were free.

Evidence of Macroyclic Ring. The 1H NMR spectrum of compound 2, which is another product from methanolysis of compound 1, was similar to that of compound 1, except for the additional signal due to a methoxy group and a shift of the H-12 signals to 4.28 and 4.21 ppm. These results indicated that an ester linkage at O-16 was cleaved by sodium methoxide and then a methyl ester was formed. Based on these results, compound 1 was indicated to contain a dicarboxylic acid moiety, whose two carboxyl groups were attached to O-13 and O-16 of 12-deoxy-16-hydroxyphorbol to form a macrocyclic diester ring.

Characterization of the Dicarboxylic Acid Moiety. The molecular formula of dicarboxylic acid moiety, C20H18O4, was estimated by subtracting that of the alcohol moiety, C20H20O6, from the molecular formula of compound 1. Ten unsaturations which were indicated by the molecular formula and the 13C NMR spectrum (Table 1) suggested the presence of two additional ring systems along with two carbonyls and six double bonds. The two-dimensional C/H COSY NMR spectrum of compound 1 showed assignment of protons in the dicarboxylic acid moiety and gave the correlations between C and H, as shown in Table 1. Inspection of the homonuclear COSY NMR spectrum (Fig. 1) revealed the presence of the partial structure, nona-16',18',20'-trieryl group, by following the correlation signals from the H-24' methyl to H-16' methine. The COSY
The spectrum also showed that coupling exists between the three methines, H-5', H-1', and H-6'. The interaction of the three methines indicates the presence of a cyclopropane ring. These three methines absorb at a higher magnetic field than normal methines but at a lower magnetic field than normal cyclopropane protons, since C-6' was bonded to C-16' of a conjugated triene in the nonatrienyl group. Further inspection of the COSY spectrum of compound 1 revealed the partial structure (Fig. 2A).

The 'H NMR analysis of the diol, which was obtained from compound 1 by reduction with LAH, revealed that a methine, H-2' at 2.23 ppm, and a methylene, H-10' at 2.27 ppm, coupled with hydroxymethyls at 3.38 and 3.64 ppm (center of an AB quartet), respectively. These results indicated that 2 carbonyl groups are located at C-7' and C-11'. Therefore, the 2 carbon atoms, C-1' and C-2' must be connected to each other and thus complete a 5-membered ring. The zero coupling between H-1' and H-2' indicates a nearly 90-degree dihedral angle between these protons (11). The relative stereochemistry of the other carbons of the bicyclo ring, excepting the C-6' position, was also elucidated as shown in Fig. 2B from vicinal coupling constants in the 'H NMR spectrum (Table 1). In this configuration, the calculated coupling constants from dihedral angles using the Karplus rule were in agreement with vicinal
coupling constants of 5-membered ring protons (11). Configurations of double bonds were also elucidated as shown in Fig. 2B from vicinal coupling constants of olefinic protons (Table 1). In the nuclear Overhauser effect spectroscopy spectrum of compound 1, remarkable nuclear Overhauser effect spectroscopic differences between H-2' and H-3', H-13' and H-15', H-17' and H-19', and H-19' and H-21' also supported the proposed structure of the dicarboxylic acid moiety (Fig. 2B).

Conjugation Sites between 12-Deoxy-16-hydroxyphorbol and the Dicarboxylic Acid. Comparison of the 1H NMR spectrum of compound 1 with that of compound 2 revealed that an α-oriented proton, H-10, of compound 2 showed an upfield shift, which suggested that the acid moiety was located on the α side of the 12-deoxy-16-hydroxyphorbol ring and the conjugated double bonds of the acid moiety caused the deshielding effect of H-10. Conformational analysis of compound 1 using a Dreiding model indicated that the carbonyl groups at C-7' and C-11' may be connected with O-13 and O-16 of 12-deoxy-16-hydroxyphorbol, respectively. From these data, we proposed the structure of compound 1 as in Fig. 2C.

Biological and Biochemical Activity of DHPB

DHPB induced ODC in mouse skin in a dose-dependent manner. The ODC induction by DHPB linearly increased as the amount applied was increased from 1 nmol to 34 nmol (Fig. 3A). Application of 34 nmol of DHPB induced 2.8 nmol of CO2/30 min/mg of protein, while the same dose of TPA induced 5.0 nmol of CO2/30 min/mg of protein. The potency of DHPB was slightly weaker than that of TPA. DHPB inhibited the specific binding of [3H]TPA to a particulate fraction of mouse skin. The effective doses for 50% inhibition of DHPB and TPA were 17.0 and 3.0 nM, respectively (Fig. 3B). Therefore, DHPB-specific binding to the phorbol ester receptors is slightly weaker than that of TPA. DHPB activated protein kinase C in vitro in a dose-response manner similar to that of TPA (Fig. 3C). The activity of about 15 nM (10 ng/ml) of DHPB was 4 × 10^3 cpm, while that of TPA was 11 × 10^3 cpm. The dose-response curve of DHPB showed slightly weaker activity of DHPB than that of TPA. The concentrations required for 50% activation were 36.0 and 9.0 nM for DHPB and TPA, respectively.

Tumor-promoting Activity of DHPB

DHPB is more toxic than TPA. When we examined the tumor-promoting activity of DHPB using 10 μg of DHPB per application, topical application of DHPB twice a week on mouse skin killed 25% of the tested mice within 1 week. Therefore, we followed this with a second two-stage carcinogenesis experiment, using lower doses (2 and 5 μg of DHPB from week 18). One week after initiation with DMBA, 2 μg of DHPB were applied topically on mouse skin twice a week until week 17. The first tumor appeared at week 12. Because the tumor incidence of the group did not increase as expected, the amount of DHPB was increased from 2 to 5 μg per application from week 18. As Fig. 4A shows, the percentage of tumor-bearing mice increased gradually from week 20 and reached 46.7% in week 30. The average number of tumors per mouse in the group treated with DMBA plus DHPB was 0.6 (Fig. 4B). Macroscopically, all the tumors seemed to be papillomas. The group treated with DMBA alone yielded one tumor in 1 mouse of 15. The group treated with DHPB alone did not show any tumors. Thus, DHPB showed tumor-promoting activity in a two-stage carcinogenesis experiment.

DISCUSSION

We have proposed the structure of the new tumor promoter DHPB through spectroscopic analysis by using modern 1H NMR methods, such as COSY NMR, in combination with chemical degradation. The structural characteristics of DHPB are: (a) it is a 12-deoxy-16-hydroxyphorbol ester; (b) the dicarboxylic acid moiety of DHPB has a new and unusual bicyclic ring structure, 4''-[12',14'-butadienyl]-6'-[16',18',20'-nonatrienyl]-bicyclo[3.1.0]hexane-2''-[carboxylic acid]-3''-[8'-butenoic acid-10'']; (c) DHPB has a macrocyclic diester structure interlinking O-13 and O-16. Although we have determined the relative configuration of the bicyclo[3.1.0]hexane ring from the coupling constants in the 1H NMR spectrum of DHPB, it

![Fig. 3. Effects of DHPB (●) and TPA (○) on ODC induction (A), inhibition of specific [3H]TPA binding (B), and activation of protein kinase C in vitro (C).](image_url)
should be emphasized that the absolute structure is still tentative. It is worthwhile to mention that the sites of attachment of the two carbonyl groups to the 12-deoxy-16-hydroxyphorbol molecule were determined from the chemical shift of H-10 in the 1H NMR spectra of DHPB and its 13-monoester. However, we cannot exclude the other possibility that the carbonyl group at C-7′ may be attached to O-16 of the 12-deoxy-16-hydroxyphorbol and the other at C-11′ to O-13.

Biehl and Hecker (12) reported the isolation of 12-deoxy-16-hydroxyphorbol ester from the same plant, J. curcas L. The structure of their phorbol diester has polyunsaturated acid moieties at O-13 and O-16 with seven double bonds (12). Since the acid moiety of DHPB contains six double bonds, we presume that the structure of DHPB is slightly different from the phorbol ester which Biehl and Hecker found, although they did not elucidate its complete structure.

Recently another example of an Intramolecular dicarboxylic acid diester structure associated with the phorbol ester class was reported. An intramolecular dicarboxylic acid 13,16-diester of another phorbol-type parent alcohol, 12,20-dideoxy-5β,16-dihydroxyphorbol, was isolated (13) from the latex of Anthostema senegalense (Anthostema factor S3). The dicarboxylic acid contained in factor S3 is most likely generated by a secondary intramolecular Diels-Alder condensation of the two highly unsaturated original 13,16-diester groups, i.e., 13-decatetraenoate and 16-decatetraenoate. In this way, the ester groups are interlinked by a tetra-substituted cyclohexene ring involving C-4′/C-5′/C-6′/C-7′ of the 13-decatetraenoate and C-5′/C-6′ of the 16-decatetraenoate to form a dicarboxylic acid. The bicyclo[3.1.0]hexane ring of DHPB bears some analogy to the cyclohexene ring of factor S3.

DHPB was slightly less potent than TPA with respect to biological and biochemical activities, such as irritant activity (data not shown), induction of ODC in mouse skin, inhibition of specific binding of [3H]TPA to a particulate fraction of mouse skin, and activation of protein kinase C in vitro. DHPB is more toxic than TPA. The tumor-promoting activity of DHPB was weaker than that of TPA, because applications of 2.5 μg of TPA induced tumors in nearly 100% of mice initiated with DMBA by week 12, as reported previously (9). The experiment with DMBA plus DHPB resulted in 46.7% incidence of tumors by week 30. The weaker activity of DHPB might be explained by the structural differences between DHPB and TPA: (a) the alcohol moiety, i.e., 12-deoxy-16-hydroxyphorbol of DHPB and phorbol of TPA; and (b) the acid moieties, i.e., the unsaturated acid of DHPB and saturated acids of TPA.

From the results of purification, we estimate that 1 μg of DHPB may be contained in 1 ml of seed oil of J. curcas L. The HPLC analysis showed that DHPB was a major irritant principle of the irritant fraction (data not shown), of which we previously demonstrated tumor-promoting activity (2). DHPB is thought to be a major causal agent of tumor promotion by the seed oil of J. curcas L., and this calls for care in the handling.

Hecker and Marks and their associates reported the biological activities of Ti8, isolated from the plant Euphorbia tirucalli (14), and of the semisynthetic compound, 12-O-retinoylphorbol-13-acetate (15, 16). These compounds have potent irritant activity, whereas their tumor-promoting activity is very weak (17–19). The only structural difference between TPA and Ti8 is the double bonds in the acid moiety of Ti8. These double bonds might act to fix the conformation of the molecule. The molecular flexibility might increase the probability of interaction with surrounding lipid molecules. DHPB has six double bonds in the dicarboxylic acid moiety. However, the conformation of DHPB seems to be more flexible than that of Ti8 and 12-O-retinoylphorbol-13-acetate. This flexibility might account for the weak tumor-promoting activity of DHPB.

DHPB is a new tumor promoter of the phorbol ester class, containing an intramolecular macrocyclic diester moiety. Knowledge of its structure should improve our understanding of the structure-function relationships of phorbol esters.

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