PLANT ANTICANCER AGENTS. XXII. ISOLATION OF A PHORBOL DIESTER AND ITS $\Delta^{5,6}$-7β-HYDROPEROXIDE DERIVATIVE FROM *OSTODES PANICULATA*[1]

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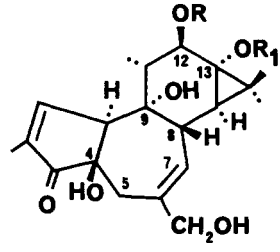
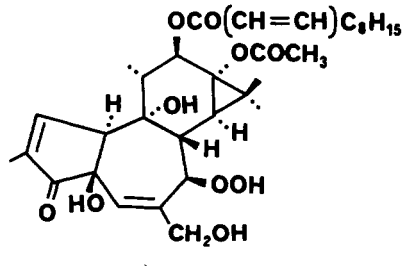
ABSTRACT.—Two novel cytotoxic compounds, 12-*O*-undecadienoylphorbol-13-acetate (**1**) and ostodin (**2**), the $\Delta^{5,6}$-7β-hydroperoxide derivative of **1**, were isolated from a chloroform extract of the stems and fruits of *Ostodes paniculata* (Euphorbiaceae). Ostodin (**2**) was shown to be an autooxidation product of **1** and, hence, probably was obtained in this investigation as an artifact.

Ostodes paniculata Blume is an arboreal species indigenous to India and Java that exudes a gum (2). While no previous phytochemical studies appear to have been performed on any members of this genus, a 50% aqueous ethanol extract of the aerial parts of *O. paniculata* has been reported to evoke hypotensive activity in the dog and antispasmodic activity on the guinea pig ileum *in vitro* (3). This extract was also found to have an LD_{50} of 17.5 mg/kg in mice, when injected intraperitoneally (3).

Our attention was turned to *O. paniculata* when a chloroform extract of the stems and fruits was found to display significant activity[2] against the P-388 lymphocytic leukemia system in cell culture. This test system was used to



	R	R_1
1	$CO(CH{=}CH)C_8H_{15}$	$COCH_3$
3	H	H



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[1]For the previous paper in this series see reference 1.

[2]Extracts, fractions and isolates were tested under the auspices of the Developmental Therapeutics Program of the National Cancer Institute (4). An isolate is considered active it shows an $ED_{50} \leq 4\mu g/ml$ in the P-388 or KB *in vitro* cell culture assays, or a T/C value of $\geq 125\%$ in the P-388 *in vivo* assay in mice.

monitor subsequent fractionation of the plant extract, which resulted in the isolation of two cytotoxic compounds, **1** and **2**.

EXPERIMENTAL[3]

PLANT MATERIAL.—Stems and fruits of *Ostodes paniculata* Blume (Euphorbiaceae) were collected in India in December, 1978, by staff members of the Economic Botany Laboratory, Agricultural Research Service, BARC-East, U.S.D.A., Beltsville, Maryland. A voucher specimen representing the collection has been deposited in the Herbarium of the National Arboretum, Washington, D.C.

EXTRACTION AND FRACTIONATION.—The air-dried, milled stems and fruits of *O. paniculata* (16.5 kg) were defatted with petroleum ether (bp 60–80°), with the marc then being extracted with methanol. After removal of the solvent, the residue was partitioned between water and chloroform. Biological activity was found to be concentrated in the chloroform extract, which was active against the P-388 lymphocytic leukemia test system both *in vivo* (T/C 134% at 125 mg/kg, ip) and *in vitro* (ED_{50} 0.09 μg/ml), but was inactive against the KB cell culture test system.

The dried chloroform extract (130 g) was chromatographed over silica gel[4] (2 kg). Elution was conducted with mixtures of petroleum ether, chloroform and methanol of increasing polarity. A total of 100 fractions (two liters each) were collected with the best activity (P-388, ED_{50} 1.6×10^{-5} μg/ml) being evident in Fraction F059, eluted from the column with chloroform-methanol (49:1). This fraction (1.75 g) was rechromatographed over silica gel[4] with chloroform and chloroform-methanol mixtures. Of the fractions obtained from this column, Fraction F080 was found to exhibit maximum P-388 activity *in vitro* (ED_{50} 5.6×10^{-6} μg/ml).

ISOLATION OF 12-*O*-UNDECADIENOYLPHORBOL-13-ACETATE (**1**) AND OSTODIN (**2**).—Fraction F080 (500 mg) was subjected to preparative tlc on silica gel GHLF[5] (250 μm, 20×20 cm plates prewashed with ethyl acetate); hexane-ethyl acetate-benzene-diethyl ether (1:3:3:1) was the developing solvent. Plates were visualized with 60% sulfuric acid and were heated at 110° for 10 min before examination. Two bands with Rf values of 0.36 and 0.46 were processed to yield compounds **1** and **2**, respectively.

12-*O*-Undecadienoylphorbol-13-acetate (**1**, 50 mg, 0.0003%) exhibited the following physical and spectral properties: resin; $[\alpha]^{25}D$ −16.0° (c 0.5, $CHCl_3$); ir, νmax ($CHCl_3$) 3390, 2930, 2860, 1725, 1628, 1465, 1375, 1262, 1165, 1010 and 755 cm^{-1}; uv, λmax (MeOH) 230 nm (log ε 4.21); nmr, δ ($CDCl_3$) 0.91 (3H, m, terminal acyl CH_3), 0.98 (3H, d, J=6 Hz, 18-CH_3), 1.1–1.4 (10H, m, 16-, 17-CH_3 and acyl methylene H), 1.81 (3H, bd s, 19-CH_3), 2.08 (3H, s, 13-$OCOCH_3$), 2.53 (2H, s, 5-H_2), 3.23 (2H, m, 8-, 10-H), 3.99 (2H, s, 20-H_2), 5.46 (1H, d, J=10 Hz, 12-H), 5.61 (1H, m, 7-H), 5.4–6.4 (4H, m, acyl vinylic H) and 7.56 (1H, bd s, 1-H); ms (20 eV) m/z 570 (M^+, 2%) 510 (5), 403 (6), 390 (5), 389 (18), 370 (6), 329 (22), 328 (67), 311 (25), 310 (83), 309 (11), 292 (13), 282 (13), 227 (18), 210 (14), 173 (17), 139 (10), 125 (13), 123 (11) and 83 (100). Mass measurement: found 570.3189, calc. for $C_{33}H_{46}O_8$, 570.3189; found 328.1673, calc. for $C_{20}H_{24}O_4$, 328.1673.

Ostodin (**2**, 30 mg, 0.00018%) exhibited the following physical and spectral properties: resin; $[\alpha]^{25}D$+21.4° (c 0.14, $CHCl_3$); ir, νmax ($CHCl_3$) 3380, 2920, 2850, 1730, 1690, 1470, 1395, 1275, 1175, 1125, 1000 and 775 cm^{-1}; uv, λmax (MeOH) 230 nm (log ε 4.14); nmr, δ ($CDCl_3$) 0.93 (3H, d, J=6 Hz, 18-CH_3), 1.1–1.4 (10H, m, 16-, 17-CH_3 and acyl methylene H), 1.80 (3H, bd s, 19-CH_3), 2.10 (3H, s, 13-$OCOCH_3$), 3.26 (1H, m, 10-H), 3.79 (1H, d, J=5 Hz, 8-H), 4.21 (1H, d, J=5 Hz, 7-H), 4.33 (2H, s, 20-H_2), 5.43 (1H, d, J=10 Hz, 12-H), 6.01 (1H, bd s, exchangeable with D_2O, -OH), 6.93 (1H, s, 5-H) and 7.64 (1H, bd s, 1-H); ms, m/z 602 (M^+, 0.3%), 584 (0.5), 542 (2), 524 (1), 403 (6), 402 (5), 385 (2), 360 (2), 342 (7), 341 (7), 325 (80), 324 (95), 255 (95), 239 (37), 175 (47), 149 (42), 129 (42), 109 (47), 85 (53), 83 (53), 71 (84) and 69 (100). Mass measurement: found 602.3098, calc. for $C_{33}H_{46}O_{10}$, 602.3091; found, 360.1571, calc. for $C_{20}H_{24}O_6$, 360.1573.

On addition of ferrous thiocyanate reagent (5) to a sample of **2** applied to Whatman filter paper, an immediate intense brown red color was produced. Under similar conditions, the test was negative with compound **1**.

CHARACTERIZATION OF **1**.—(a) Diterpene moiety.—A sample of **1** (5 mg) was treated with 0.5 *M* KOH in methanol (1 ml) for 1 hr at room temperature. Water (5 ml) was added, and the mixture was extracted with 3×5 ml aliquots of methylene chloride. Following drying (Na_2SO_4) and filtration, the organic phase was evaporated to afford a residue (2.7 mg), identified as phorbol (3) by co-tlc on silica gel GHLF[5] by comparison with an authentic sample (6): Rf,

[3]Optical rotations were determined on a Perkin-Elmer 241 polarimeter. The uv spectra were obtained with a Beckman model DB-G spectrophotometer. The ir spectra were measured on a Beckman model 18-A spectrophotometer, with polystyrene calibration at 1601 cm^{-1}. Proton nmr spectra were recorded in $CDCl_3$ on a Varian T-60A instrument with a Nicolet TT-7 Fourier Transform attachment. Tetramethylsilane was used as the internal standard, and chemical shifts are reported in δ (ppm). Low-resolution mass spectra were obtained with either a Varian MAT-112S double focusing spectrometer or an AEI MS 902 double focusing spectrometer. Combined gc/ms was performed on a Varian MAT-112S mass spectrometer, equipped with a Varian 166 data system, linked to a Varian 1440 model gas chromatograph. Mass spectrometers were operated at 70 eV, unless stated otherwise in the text.

[4]E. Merck, Darmstadt, W. Germany.

[5]Analtech, Inc., Newark, Delaware.

ethyl acetate-methanol (10:1), 0.40; chloroform-methanol (4:1), 0.70; ms, *m/z* M^+ not observed, 328 (3%), 310 (2), 282 (2), 267 (2), 233 (6), 216 (16), 215 (18), 187 (9), 173 (7), 159 (11), 149 (12), 121 (11), 109 (22), 107 (14), 95 (15), 91 (20) and 83 (100).

(b) Acyl moiety.—A sample of **1** (10 mg) was treated with 0.5 *M* KOH in methanol (2 ml) for 1 hr at room temperature. The reaction mixture was then acidified to pH 4 with dilute HCl, and the fatty acid portion extracted with diethyl ether. Solvent was removed under nitrogen, and the residue (3 mg) was hydrogenated (5% Pd/C) in ethanol, methylated with diazomethane[6] and analyzed by gc/ms[7]. Methyl undecanoate produced from **1** exhibited identical data when compared with an authentic sample prepared from undecanoic acid[8]: R_t, 6.6 min; ms, *m/z*, 200 (M^+, 6%), 169 (11), 157 (11), 143 (13), 129 (6), 101 (10), 88 (8), 87 (90), 75 (21) and 74 (100).

AUTOOXIDATION OF **1** TO **2**.—12-*O*-Undecadienoylphorbol-13-acetate (**1**), when stored in the dark under nitrogen at 4° for a week, showed evidence of decomposition to **2** by tlc examination. Purification by preparative tlc gave 1 mg of this artifact from 5 mg of the original compound, **1**. This decomposition product and the isolate, **2**, exhibited indistinguishable mass spectra.

The decomposition of **1** to **2** as the major product occurred even more rapidly at room temperature with exposure to light and air.

STRUCTURE ELUCIDATION OF 12-*O*-UNDECADIENOYLPHORBOL-13-ACETATE (**1**) AND OSTODIN (**2**).—12-*O*-Undecadienoylphorbol-13-acetate (**1**) exhibited spectral properties (uv, ir, pmr, ms) typical of a phorbol 12,13-diester (7–9). The structure of the diterpene component of **1** as phorbol (**3**) was confirmed by alkaline hydrolysis of the parent compound, and direct comparison with an authentic sample of **3**. Two acylating moieties with molecular weights of 59 and 181 were found to be present in the molecule of **1** by analysis of its ms and pmr data. The long-chain substituent was assigned to C–12 because a fragment ion at *m/z* 389 in the ms of **1** indicated the loss of an acyloxy radical rather than the loss of a complete acid from the molecular ion, an effect noted previously in phorbol 12,13-diesters (8,9). In contrast, as indicated by the fragment ion at *m/z* 510, the C–13 acetate substituent was eliminated as an acid. The presence of a straight-chain 11-carbon acid substituent at C–12 in **1** was confirmed by gc/ms comparison of the product of hydrolysis, hydrogenation and methylation of **1** with an authentic sample of methyl undecanoate.

Assignment of the positions and stereochemistry of the two double bonds of the C–12 acyl substituent of **1** has not been finalized, due to the small amount of this unstable compound available. It was possible to rule out the occurrence of a conjugated diene system situated $\alpha,\beta,\gamma,\delta$- to the carboxyl function since a uv maximum was observed at 230 nm, and not at around 254 nm, as might be expected (10). In this series, two phorbol diesters with a long-chain C–12 acid substituent containing a conjugated diene system $\alpha\beta$- to the carboxyl group displayed a uv maximum at 262 nm (11,12). However, since olefinic signals assignable to the undecadienoate moiety of **1** were observed in the pmr spectrum downfield of δ 6.00, one of the double bonds must be $\alpha\beta$- to the carboxyl, with the second double bond at least two carbons away from the β-carbon (13). Since many significant pmr signals of this acid substituent were obscured by overlapping signals from the diterpene portion of **1**, details of the precise nature of the C–12 acyl group could not be assigned with any degree of confidence.

Ostodin (**2**), also isolated from the *O. paniculata* chloroform extract, exhibited a molecular ion at *m/z* 602 and was found by mass spectral elemental analysis to contain two more oxygen atoms than compound **1**. Two $\Delta^{5,6}$-7-hydroperoxide derivatives of phorbol esters have been obtained previously, although both were only partially characterized spectrally (14,15). Hence it was decided to investigate **2** for the presence of a hydroperoxide group. Ostodin (**2**) gave a positive test for this functionality with the ferrous thiocyanate reagent (5), and the observation of major fragment peaks at *m/z* 360, 342 and 324 in the mass spectrum of **2** may be rationalized as being analogous to the characteristic (8) mass spectral fragment peaks of phorbol 12,13-diesters at *m/z* 328, 310, and 292, respectively, shifted in each case, by 32 amu. However, no deuterium oxide-exchangeable signal assignable to a hydroperoxide proton at about δ 10.5 (16) was observed in the pmr spectrum of **2**.

Placement of the hydroperoxide group in compound **2** was possible following inspection of its pmr spectrum. Since signals observed for protons in the five-, six- and three-membered rings of **2** were relatively consistent in chemical shift with those in the pmr spectrum of **1**, it was concluded that the hydroperoxide function of **2** must be inserted into the seven-membered ring (ring B). The $\Delta^{6,7}$ double bond of compound **1** was translocated to the $\Delta^{5,6}$ position in **2** since the methylene proton singlet at δ 2.53 in the pmr spectrum of **1** was replaced by a vinyl proton signal at δ 6.92 in that of **2**. This being the case, the hydroperoxide group of **2** was, therefore, most likely attached to C–7 in ring B of **2**. Downfield shifts in the pmr spectrum of **2** compared with that of **1** for the C–20 methylene (4.33 *vs.* 3.99 ppm) and C–8 (3.79 *vs.* 3.23 ppm) protons are in accord with the introduction of the electronegative hydroperoxide group at C–7. The C–7 proton exhibited a chemical shift at δ 4.21, a value that compares closely with δ values for methine carbons bearing a –OOH group (16,17). Since $J_{7H,8H}=5$ Hz, it could

[6]An ethereal solution of diazomethane was generated using the Diazald Kit, manufactured by Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

[7]Gc/ms was carried out using Reoplex 400 (15%) on Chromosorb W (100–120 mesh size) as stationary phase, with the temperature programmed 120–160°, 4° min^{-1}, and the flow rate of helium 18 ml min^{-1}. The injector temperature was set at 210°, and the interface separator at 220°, and spectra were recorded with 6 sec between scans.

[8]An authentic sample of undecanoic acid was obtained from Supelco, Inc., Bellefonte, Pennsylvania.

be concluded that these protons are *trans*-oriented and that the 7-OOH function has a β-orientation in **2**. Ostodin (**2**) is, thus, the $\Delta^{5,6}$-7β-hydroperoxide of 12-*O*-undecadienoylphorbol-13-acetate (**1**). Because of the ease of conversion of **1** to **2**, ostodin (**2**) was almost certainly obtained in this investigation as an artifact.

BIOLOGICAL ACTIVITY.—12-*O*-Undecadienoylphorbol-13-acetate (**1**, NSC-353447) was highly cytotoxic in the P-388 lymphocytic leukemia test system (ED_{50} 4.5×10^{-5} μg/ml) but was inactive in this test system *in vivo* in the dose range of 0.37–0.75 mg/kg.

Ostodin (**2**, NSC-353862) exhibited an ED_{50} of 0.055 μg/ml against the P-388 test system *in vitro*.

DISCUSSION

12-*O*-Undecadienoylphorbol-13-acetate (**1**) is the most active compound against the P-388 lymphocytic leukemia cell culture system that has so far been obtained in our laboratory among representatives of the tigliane and daphnane ester classes of diterpenes (9,18,19). Unfortunately, the limited quantities of this isolate obtained did not permit its more extensive *in vivo* testing.

The rapid rate of decomposition experienced in this study of **1** to ostodin (**2**), its less cytotoxic $\Delta^{5,6}$-7β-hydroperoxide derivative, contrasts with previous observations on the standard tumor-promoting agent 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is apparently much more stable than **1** when stored in the solid form at 4° (14). Therefore, it would seem prudent to routinely store biologically active phorbol 12,13-diesters of unknown stability in dry ice, under nitrogen and protected from light, until ready for evaluation.

ACKNOWLEDGMENTS

The authors thank the Economic Botany Laboratory, Science and Education Administration, BARC-East, U.S.D.A., Beltsville, Maryland, funded by the National Cancer Institute, for the provision and identification of the plant material. This work was carried out under contract CM-97295 with the Division of Cancer Treatment, National Cancer Institute, Department of Health, Education and Welfare, Bethesda, Maryland. The authors are grateful to Dr. C. T. Che and Mr. D. D. McPherson of the University of Illinois at the Medical Center for the provision of pmr and ms data, respectively, and to Dr. M. Wani and Mr. F. Williams, Research Triangle Institute, for the high-resolution mass spectra (AEI MS 902 instrument).

S.S.H. is grateful to the Panjab University, Chandigarh, India, for the sanction of special leave from the University.

Received 3 May 1982

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