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⁂Short-Chain Phorbol Ester Constituents of Croton Oil[1]

GARY T. MARSHALL and **A. DOUGLAS KINGHORN,*** Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, Health Sciences Center, University of Illinois at Chicago, Chicago, IL 60612

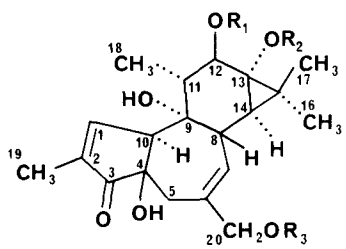
ABSTRACT

Five phorbol diesters, three 4-deoxy-4α-phorbol diesters, two phorbol monoesters, and one 4-deoxy-4α-phorbol monoester were isolated from a commercial sample of croton oil and characterized spectroscopically. Their purification was achieved using combinations of droplet countercurrent chromatography, low-pressure column chromatography over phase-bonded silica gel, and preparative thin layer chromatography. All of these isolates were shown to possess short-chain ester functionalities, with 12-*O*-tiglylphorbol-13-isobutyrate, 12-*O*-(2-methyl)butyrylphorbol-13-isobutyrate, 12-*O*-(2-methyl)butyrylphorbol-13-acetate, 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-isobutyrate, 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-acetate, 12-*O*-(2-methyl)butyryl-4-deoxy-4α-phorbol-13-acetate, phorbol-12-tiglate and 4-deoxy-4α-phorbol-13-acetate being new compounds.

INTRODUCTION

The isolation of pure biologically active constituents from the potent skin-irritating and tumor-promoting seed oil of *Croton tiglium* L. (croton oil) was achieved for the first time by 2 independent groups in the mid-1960s (1,2). Hecker and coworkers eventually isolated 11 diesters of the parent diterpene alcohol, phorbol, that were extractable from croton oil into hydrophilic organic solvents, with a further 3 compounds being obtained on the hydrolysis of a mixture of phorbol triesters present in a lipophilic solvent extract of croton oil (3). These compounds were termed A factors and B factors, respectively, depending on if the longer of 2 acyl moieties present in each molecule was affixed to the C12 or C13 position of phorbol (3). More recently, high pressure liquid chromatography (HPLC) has shown that additional diesters of the phorbol type are present in croton oil, and 3 such diterpenes with short-chain ester functionalities at both C12 and C13 were detected (4). The most abundant croton oil phorbol diester, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, phorbol myristate acetate), is now widely used in biochemical experiments as a standard tumor-promoting agent (5).

In the present communication, we wish to report the isolation and characterization of 7 short-chain esters of phorbol and 4 short-chain esters of 4-deoxy-4α-phorbol from croton oil. These new croton oil constituents were separated from a number of less polar, long-chain ester phorbol analogs, of known structure, primarily as a result of applying a refinement of a droplet countercurrent chromatographic (DCCC) solvent system that was developed in this laboratory for the isolation of phorbol and 4α-phorbol from hydrolyzed croton oil (6). Compounds generated in the present study were characterized spectroscopically, and the relative positions of ester substitution were assigned after hydrolysis and partial synthesis experiments were carried out.



Compound	R_1	R_2	R_3
I	Tiglate	Isobutyrate	H
II	2-Methylbutyrate	Isobutyrate	H
IV	Tiglate	H	H
V	Acetate	Acetate	H
VI	Tiglate	Acetate	H
VII	2-Methylbutyrate	Acetate	H
X	H	Acetate	H
XII	Acetate	Acetate	Acetate
XIV	2-Methylbutyrate	H	H
XVII	Acetate	H	H
XVIII	H	H	H

Fig. 1.

EXPERIMENTAL

Apparatus

Instrumentation used to measure optical rotations, ultraviolet (UV), infrared (IR) and 360 MHz ^{1}H nuclear magnetic resonance (NMR) spectra, and low-resolution mass spectra (MS) has been described previously (7). DCCC was

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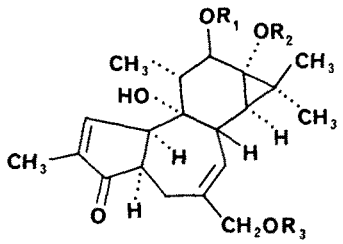
*To whom correspondence should be addressed.



Compound	R_1	R_2	R_3
III	Tiglate	Isobutyrate	H
VIII	Tiglate	Acetate	H
IX	2-Methylbutyrate	Acetate	H
XI	H	Acetate	H
XIII	Acetate	Acetate	Acetate
XV	Tiglate	H	H
XVI	2-Methylbutyrate	H	H

Fig. 2.

performed on a Tokyo Rikakikai Model A instrument, at room temperature. Low-pressure column chromatography was undertaken with a Lobar Lichroprep RP-8 column (E. Merck, size A), and analytical and preparative thin layer chromatography (TLC) separations were carried out over 20 × 20 cm Silica gel GHLF plates (Analtech, Inc.; 0.25 mm), using chloroform/ethyl acetate (2:3, solvent 1) and ethyl acetate/methanol (10:1, solvent 2) as developing solvents. TLC plates were visualized with 60% ethanolic H_2SO_4 by heating at 110 C for 10 min.

Extraction and Fractionation of Croton Oil

Croton oil (100 g; Sigma Chemical Co., St. Louis, MO; lot 60F-0471) was dissolved in 100 mL hexane and partitioned 4 times with 50 mL aliquots of methanol/water (20:3). The aqueous-methanol layers were combined and reduced to dryness in vacuo at 40 C to afford 3% w/w of a hydrophilic residue. A portion (1.0 g) of this residue was fractionated by DCCC using as solvent system hexane/diethyl ether/1-propanol/ethanol/water (17:40:15:25:40), with the upper phase employed as mobile phase. The solute was dissolved in 5 mL each of the 2 immiscible solvent phases and was introduced in a 10 mL sample chamber. Ascending development was used for separation, at a pressure of 2-4 kg/cm^2, and fractions (120 drops each) were collected into an automatic fraction collector. Although the bulk of the croton oil phorbol esters were eluted within the first 104 DCCC fractions, the more polar short-chain phorbol esters contained in DCCC fractions 105-238 were selected for the present study.

Fractions 105-126 (69 mg) from the DCCC separation were further purified by low-pressure column chromatography, using as initial solvent water/acetonitrile/methanol (3:3:2) (50 fractions collected; CC 1-50; 80 drops per fraction). The solvent was then sequentially decreased in polarity to water/acetonitrile/methanol (1:1:1; fractions CC 51-129) and water/acetonitrile/methanol (3:3:4; fractions CC 130-150). Preparative TLC in solvent 1 of fractions CC 77-94 (R_f 0.35), CC 107-116 (R_f 0.35) and CC 122-152 (R_f 0.37) yielded, respectively, compounds I (10 mg), II (5 mg), and III (4 mg).

DCCC fractions 127-161 (68 mg) were separated further by low-pressure column chromatography, using the solvent gradient water/acetonitrile/methanol (2:2:1) (35 fractions, CC 1′-35′), water/acetonitrile/methanol (3:3:2) (fractions 36′-80′) and water/acetonitrile/methanol (1:1:1) (fractions CC 81′-110′). Compounds IV (4 mg, R_f 0.07) and V (3 mg, R_f 0.23) were obtained in pure form by preparative TLC in solvent 1 of fractions CC 1′-33′. Similar preparative TLC of fractions CC 34′-73′ afforded 24 mg of compound VI (R_f 0.25). Compound VII (6 mg) was obtained pure by combining fractions CC 48′-58′. Preparative TLC in solvent 1 of fractions CC 59′-80′ provided an additional 2 mg of compound VII (R_f 0.27) and 7 mg of compound VIII (R_f 0.40), whereas treatment in this same way of fractions CC 85′-105′ yielded an additional 4 mg of compound I and 4 mg of compound IX (R_f 0.42).

Further resolution of DCCC fractions 217-238 by low-pressure column chromatography, using isocratic elution in water/acetonitrile/methanol (3:2:1), generated pure compounds X (4 mg) and XI (9 mg) from combined fractions CC 15″-18″ and CC 19″-22″, respectively.

Characterization of Isolates I-XI

12-*O*-Tiglylphorbol-13-isobutyrate (I, 14 mg, 0.042% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +37° (c 0.1, $CHCl_3$); UV (EtOH, nm) 225 (log ε 3.94); IR (neat, cm^{-1}) 3418, 2979, 1706, 1260, 1160, 1135, 1078; ^{1}H-NMR, see Table I; MS m/z (rel. int.) 516 (M^+, 2), 498 (0.5), 473 (5), 428 (2), 417 (8), 410 (3), 398 (2), 328 (38), 310 (42), 292 (9), 217 (20), 173 (14), 83 (100); mass measurement, found 516.2722, calc. for $C_{29}H_{40}O_8$, 516.2723.

12-*O*-(2-Methyl)butyrylphorbol-13-isobutyrate (II, 5 mg, 0.015% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +37° (c 0.1, $CHCl_3$); UV (EtOH, nm) 227 (log ε 3.77); IR (neat, cm^{-1}) 3406, 2970, 1712, 1688, 1378, 1263, 1159, 1077; ^{1}H-NMR, see Table I; MS m/z (rel. int.) 518 (M^+, 1), 500 (1), 475 (3), 417 (11), 412 (5), 398 (2), 328 (53), 310 (51), 292 (10), 282 (11), 227 (11), 173 (18), 83 (100); mass measurement, found 518.2882, calc. for $C_{29}H_{42}O_8$, 518.2877.

12-*O*-Tiglyl-4-deoxy-4α-phorbol-13-isobutyrate (III, 4 mg, 0.012% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ −37° (c 0.1, $CHCl_3$); UV (EtOH, nm) 234 (log ε 3.95); IR (neat, cm^{-1}) 3417, 2925, 1715, 1388, 1256, 1155, 1136, 1072; ^{1}H-NMR, see Table II; MS m/z (rel. int.) 500 (M^+, 2), 482 (3), 457 (7), 412 (9), 401 (6), 400 (7), 394 (4), 382 (5), 312 (70), 294 (100), 283 (14), 279 (22), 269 (14), 251 (15), 229 (26), 216 (28), 199 (28), 83 (84); mass measurement, found 500.2775, calc. for $C_{29}H_{40}O_7$, 500.2774.

Phorbol-12-tiglate (IV, 4 mg, 0.012% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +35° (c 0.1, $CHCl_3$); UV (EtOH, nm) 231 (log ε 3.20); IR (neat, cm^{-1}) 3420, 2922, 1708, 1380, 1285, 1264, 1150, 1076; ^{1}H-NMR, see Table I; MS m/z (rel. int.) 446 (M^+, 1), 428 (0.5), 410 (1), 346 (3), 328 (28), 310 (26), 300 (8), 295 (5), 282 (5), 215 (15), 208 (20), 179 (35), 121 (65), 100 (47), 83 (100).

12-*O*-Acetylphorbol-13-acetate (V, 3 mg, 0.009% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +5° (c 0.1, $CHCl_3$); UV (EtOH, nm) 227 (log ε 3.36); IR (neat, cm^{-1}) 3410, 2923, 1712, 1378, 1262, 1080, 1021; ^{1}H-NMR, see Table I; MS m/z (rel. int.) 448 (M^+, 1), 430 (2), 389 (4), 388 (3), 370 (9), 346 (9), 328 (59), 310 (66), 294 (17), 282 (18), 228 (28), 227 (28), 216 (55), 125 (44), 83 (100).

12-*O*-Tiglylphorbol-13-acetate (VI, 24 mg, 0.072% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +49° (c 0.09, $CHCl_3$); UV (EtOH, nm) 228 (log ε 4.01); IR (neat, cm^{-1}) 3425, 2922, 1712, 1378, 1260, 1130, 1075; ^{1}H-NMR, see Table I; MS m/z (rel. int.) 488 (M^+, 2), 470 (1), 446 (2), 428 (2), 410 (2), 389 (4), 328 (26), 310 (29), 292 (6), 282 (6), 227 (8), 187 (6), 124 (6), 83 (100). Compound VI, the most abundant short-chain phorbol diester isolated in this

study, has previously been identified as a croton oil constituent by HPLC (4).

12-*O*-(2-Methyl)butyrylphorbol-13-acetate (VII, 8 mg, 0.024% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +37° (c 0.1, $CHCl_3$); UV (EtOH, nm) 230 (log ε 3.74); IR (neat, cm^{-1}) 3418, 2968, 1722, 1712, 1377, 1262, 1076; ^{1}H-NMR, see Table I; MS m/z (rel. int.) 490 (M^+, 1), 472 (1), 430 (2), 412 (4), 389 (5), 370 (4), 328 (41), 310 (49), 282 (11), 217 (19), 112 (15), 85 (56), 83 (100); mass measurement, found 490.2567, calc. for $C_{27}H_{38}O_8$, 490.2564.

12-*O*-Tiglyl-4-deoxy-4α-phorbol-13-acetate (VIII, 7 mg, 0.021% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ −9° (c 0.1, $CHCl_3$); UV (EtOH, nm) 225 (log ε 3.87); IR (neat, cm^{-1}) 3418, 2923, 1712, 1479, 1265, 1135, 1055; ^{1}H-NMR, see Table II; MS m/z (rel. int.) 472 (M^+, 3), 454 (3), 412 (5), 394 (4), 373 (3), 372 (4), 354 (4), 312 (39), 294 (58), 282 (7), 216 (17), 125 (16), 83 (100); mass measurement, found 472.2465, calc. for $C_{27}H_{36}O_7$, 472.2459.

12-*O*-(2-Methyl)butyryl-4-deoxy-4α-phorbol-13-acetate (IX, 4 mg, 0.012% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ −1° (c 0.1, $CHCl_3$); UV (EtOH, nm) 228 (log ε 3.29); IR (neat, cm^{-1}) 3428, 2925, 1725, 1708, 1378, 1251, 1150; ^{1}H-NMR, see Table II; MS m/z (rel. int.) 474 (M^+, 3), 456 (7), 414 (14), 396 (11), 373 (10), 372 (8), 354 (9), 330 (8), 312 (82), 294 (100), 283 (12), 229 (22), 216 (47), 125 (49), 83 (99); mass measurement, found 474.2615, calc. for $C_{27}H_{38}O_7$, 474.2617.

Phorbol-13-acetate (X, 4 mg, 0.012% w/w) exhibited the following data: resin, $[\alpha]_D^{25}$ +6° (c 0.1, $CHCl_3$); UV (EtOH, nm) 230 (log ε 3.46); IR (neat, cm^{-1}) 3373, 2927, 1700, 1378, 1263, 1133, 1053; ^{1}H-NMR, see Table I; MS, m/z (rel. int.) 406 (M^+, 1), 388 (1), 370 (1), 346 (6), 328 (24), 310 (23), 282 (11), 233 (41), 216 (99), 215 (90), 83 (100).

4-Deoxy-4α-phorbol-13-acetate (XI, 9 mg, 0.027% w/w) exhibited the following data: $[\alpha]_D^{25}$ −7° (c 0.1, $CHCl_3$); UV (EtOH, nm) 234 (log ε 3.86); IR (neat, cm^{-1}) 3418, 2945, 1717, 1696, 1376, 1263, 1252, 1130, 1052; ^{1}H-NMR, see

TABLE I

^{1}H-NMR Data of Phorbol Esters[a]

Compound	III	VIII	IX	XI
H-1	7.065 s	7.060 s	7.059 s	7.058 s
H-4	2.794 m	2.790 m	2.791 m	2.781 m
H-5α	3.480 d[b] (2.4)	3.440 dd (2.7, 15.7)	3.433 d[b] (15.5)	3.319 dd (2.5, 15.6)
H-5β	2.419 dd (4.6, 15.6)	2.490 dd (5.2, 15.5)	2.495 dd (4.6, 16.1)	2.521 dd (5.0, 15.7)
H-7	5.130 s	5.123 s	5.118 s	5.142 s
H-8	2.381 m	2.385 m	2.366 m	2.377 m
H-10	3.500 m	3.508 m	3.500 m	3.496 m
H-12	5.517 d (10.3)	5.534 d (10.4)	5.476 d (10.5)	4.040 d (9.8)
H-14	0.771 d (5.0)	0.807 d (5.0)	0.801 d (4.8)	0.780 d (5.3)
H-16	1.248 s[c]	1.236 s[c]	1.205 s[c]	1.220 s[c]
H-17	1.187 s[c]	1.178 s[c]	1.167 s[c]	1.193 s[c]
H-18	1.083 d (6.3)	1.083 d (5.3)	1.087 d (5.9)	1.287 d (6.4)
H-19	1.790 s	1.787 s	1.787 s	1.779 s
H-20	3.905, 4.023 ABq (J_{AB} = 11.1)	3.891, 4.009 ABq (J_{AB} = 12.4)	3.888, 4.005 ABq (J_{AB} = 12.4)	3.899, 4.003 ABq (J_{AB} = 12.8)
Ester Chain	Tiglate 1.839 d (6.7)	Tiglate 1.836 d (6.9)	2-Methylbutyrate 0.959 t (7.6)	Acetate 2.093 s
	1.878 m	1.878 m	1.207 d (6.9)	
	6.881 m	6.887 m		
	Isobutyrate 1.141 d (7.0)	Acetate 2.069 s	Acetate 2.059 s	

[a]Spectra were run in $CDCl_3$ and Me_4Si was used as the internal standard. Values are recorded in parts per million relative to Me_4Si; coupling constants (hertz) are quoted in parentheses. Multiplicity is designated as follows: s, singlet, d, doublet; t, triplet; q, quartet; m, multiplet whose center is given: AB, AB system, b, broad.

[b]Obscured by other signals.

[c]Assignment interchangeable.

TABLE II

^{1}H-NMR Data of 4-Deoxy-4α-phorbol Esters[a]

Compound	I	II	IV	V	VI	VII	X	XVII[b]
H-1	7.597 bs	7.603 bs	7.590 bs	7.580 bs	7.589 bs	7.596 bs	7.575 bs	7.582 bs
H-5	2.497, 2.555 ABq (J_{AB} = 18.5)	2.484, 2.547 ABq (J_{AB} = 18.7)	2.464, 2.541 ABq (J_{AB} = 18.3)	2.491, 2.579 ABq (J_{AB} = 19.2)	2.486, 2.568 ABq (J_{AB} = 19.4)	2.493, 2.556 ABq (J_{AB} = 18.9)	2.450, 2.526 ABq (J_{AB} = 18.4)	2.456, 2.549 ABq (J_{AB} = 18.1)
H-7	5.702 m	5.700 m	5.646 m	5.671 m	5.688 m	5.676 m	5.639 m	5.632 m
H-8	3.259 m	3.262 m	3.095 m	3.237 m	3.253 m	3.248 m	3.174 m	3.091 m
H-10	3.259 m	3.262 m	3.187 m	3.237 m	3.253 m	3.248 m	3.174 m	3.176 m
H-12	5.452 d (10.4)	5.427 d (10.4)	4.857 d (9.8)	5.380 d (10.3)	5.457 d (10.3)	5.419 d (10.5)	3.980 d (9.2)	4.823 d (9.6)
H-14	1.046 d (5.4)	1.044 d (4.7)	0.920 d (5.8)	1.092 d (5.1)	1.095 d (5.2)	1.093 d (4.9)	c	0.938 d (6.2)
H-16	1.285 s	1.257 s	1.187 s	1.241 s	1.271 s	1.246 s	1.254 s	1.191 s
H-17	1.220 s	1.211 s	1.030 s	1.221 s	1.208 s	1.203 s	1.224 s	1.051 s
H-18	0.888 m	0.895 m	1.050 d (6.5)	0.891 m	0.891 m	0.896 m	1.062 m	1.019 d (6.3)
H-19	1.764 bs	1.775 bs	1.760 bs	1.760 bs	1.760 bs	1.771 bs	1.778 bs	1.788 bs
H-20	3.996, 4.045 ABq (J_{AB} = 12.6)	4.002, 4.045 ABq (J_{AB} = 12.7)	4.013, 4.052 ABq (J_{AB} = 12.5)	3.985, 4.040 ABq (J_{AB} = 13.1)	3.988, 4.041 ABq (J_{AB} = 12.9)	3.999, 4.044 ABq (J_{AB} = 13.4)	3.990, 4.038 ABq (J_{AB} = 12.5)	3.971, 4.026 ABq (J_{AB} = 13.3)
Ester Chain	Tiglate	2-Methylbutyrate	Tiglate	Acetate	Tiglate	2-Methylbutyrate	Acetate	Acetate
	1.798 d (7.3)	0.926 t (7.4)	1.779[c]	2.082 s	1.797 d (7.0)	0.924 t (7.1)	2.122 s	2.098 s
	1.832 m	1.179 d (7.0)	1.800 m	2.094 s	1.833 m	1.162 d (6.9)		
	6.836 m		6.887 m		6.843 m			
	Isobutyrate	Isobutyrate			Acetate	Acetate		
	1.161 d (7.0)	1.158 d (7.8)			2.099 s	2.093 s		

[a] Spectra were run in $CDCl_3$ and Me_4Si was used as the internal standard. Values are recorded in parts per million relative to Me_4Si; coupling constants (hertz) are quoted in parentheses. Multiplicity is designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet whose center is given; AB, AB system; b, broad.

[b] Semi-synthetic phorbol ester.

[c] Obscured by other signals.

Table II; MS m/z (rel. int.) 390 (M^+, 1), 372 (10), 354 (2), 330 (7), 312 (29), 294 (39), 217 (53), 216 (48), 200 (64), 199 (69), 125 (28), 83 (100); mass measurement, found 372.1923, calc. for $C_{22}H_{28}O_5$, 372.1937.

Complete Hydrolysis and Acetylation of Compounds I-XI

Samples (0.2-0.3 mg) of compounds I-XI were hydrolyzed with 0.5 M KOH in methanol for 1 hr at room temperature. Following overnight acetylation (pyridine/acetic anhydride, 2:1, 0.5 mL), and work-up, phorbol-12,13,20-triacetate (XII) was produced from I, II, IV-VII, and X, and 4-deoxy-4α-phorbol-12,13,20-triacetate (XIII) was produced from III, VIII, IX, and XI. Compounds XII and XIII were identified by comparison (MS, TLC) with authentic samples obtained in our previous work (6,8,9).

Partial Hydrolysis of Phorbol Esters I, II, VI and VII

Hydrolysis of the phorbol-12,13-diesters I, II, VI, and VII was carried out using 0.1 M methanolic KOH for 1 hr and, after purification by preparative TLC in solvent 2, resulted in the generation of pure phorbol 12-monoesters. Thus, selective hydrolysis of I and VI led to a product (R_f 0.55, solvent 2) with an identical MS to IV. Similar hydrolysis and work-up of II and VII provided phorbol-12-(2-methyl)-butyrate (XIV), MS, m/z (rel. int.) 448 (M^+, 1), 430 (1), 346 (2), 328 (7), 310 (12), 292 (3), 227 (1), 125 (2), 83 (100); R_f 0.56 (solvent 2).

Partial Hydrolysis of 4-Deoxy-4α-phorbol Esters III, VIII and IX

Selective hydrolysis of the 4-deoxy-4α-phorbol-12,13-diesters III, VIII and IX, was conducted in a similar manner to that described for the croton oil short-chain phorbol diesters. The major hydrolysis product obtained in this manner from compounds III and VIII was identified as 4-deoxy-4α-phorbol-12-tiglate (XV), MS, m/z (rel. int.) 430 (M^+, 1), 412 (1), 394 (1), 330 (1), 312 (1), 294 (4), 283 (16), 279 (12), 227 (13), 83 (100); R_f 0.55 (solvent 2). Compound IX, on partial hydrolysis, yielded 4-deoxy-4α-phorbol-12-(2-methyl)butyrate (XVI), MS, m/z (rel. int.) 432 (M^+, 1) 414 (1), 396 (2), 330 (4), 312 (8), 294 (9), 284 (10), 227 (3), 125 (4), 83 (100); R_f 0.56 (solvent 2).

Synthesis of Phorbol-12-acetate and Phorbol-13-acetate and Partial Hydrolysis of Phorbol Ester V

Authentic phorbol-12-acetate (XVII) was obtained from phorbol-12,13,20-triacetate (XII) by reaction with sodium methoxide, according to published conditions (10), and was purified by preparative TLC in solvent 2 (R_f 0.52). This compound exhibited MS, m/z 370 (M^+ -18-18, 1), 352 (1), 328 (4), 310 (9), 267 (4), 227 (8), 215 (8), 179 (9), 123 (10), 121 (16), 83 (100), and ^{1}H-NMR as in Table I. XVII was identical (^{1}H-NMR, MS, TLC) to the product obtained from V as a result of selective hydrolysis with 0.1 M methanolic KOH for 10 min at room temperature.

Acetic anhydride in dimethylformamide (1:5) was used to selectively acetylate phorbol (XVIII), as published previously (10), to yield phorbol-13-acetate (X). On purification by preparative TLC in solvent 2 (R_f 0.61), this product was shown to be identical to X, as isolated from croton oil, when compared by UV, IR, ^{1}H-NMR and MS.

RESULTS AND DISCUSSION

Compounds I, II, IV-VII and X were identified as esters of the tetracyclic diterpene phorbol (XVIII), as a result of an analysis of their spectroscopic (UV, IR, ^{1}H-NMR, MS) parameters (3,10). On complete hydrolysis with 0.5 M methanolic KOH, and subsequent acetylation, the known compound phorbol-12,13,20-triacetate (XII) was produced in each case. In an analogous fashion, isolates III, VIII, IX, and XI were assigned from their spectroscopic features as 4-deoxy-4α-phorbol esters (8,9,11-13), and complete hydrolysis and acetylation generated from each known 4-deoxy-4α-phorbol-12,13,20-triacetate (XIII).

Assignments of the chain length and relative position of the ester substituents in I-XI were made by observations of the ^{1}H-NMR and MS of each intact molecule, and after the generation of certain monoesters after partial hydrolysis or synthesis. Initial identifications of each short-chain ester function in I-XI were proposed from ^{1}H-NMR data observations (Tables I and II). Thus, we concluded that resonances originating from a tiglate moiety were apparent in the ^{1}H-NMR spectra of compounds I, III, IV, VI, and VIII. Similar reasoning led to the assignment of an isobutyrate functionality in compounds I-III, a 2-methylbutyrate unit in compounds II, VII and IX, and one or more acetate groups in compounds V-XI.

In numerous representatives of the phorbol- and 4-deoxyphorbol-12,13-diester series, the relative positions of ester substitution may be tentatively determined from their electron impact MS fragmentation pathways. The C12 attached ester functions are characteristically lost from the parent ion as acyloxy radicals, while the C13 affixed substituents are lost as whole acids (3,8,9). In this manner, the occurrence of prominent fragment peaks representing the loss of 99 amu from the molecular ion in diesters I, III, VI and VIII, were used to indicate that the tiglate units were affixed to C12 in each case. Other C12 attached substituents of the diester isolates were shown to be 2-methylbutyrate in compounds II, VII and IX (presence of M^+-101 fragment peak), and acetate in compound V (presence of M^+-59 fragment peak). In contrast, the loss of isobutyric acid as a whole unit (M^+-88) in the MS of compounds I-III, suggested the presence of this ester group at C13. Similarly, a C13 acetate group could be proposed for compounds V-IX.

Confirmation of these preliminary ester group position assignments in diesters I-III and V-IX was made by partial hydrolysis with weak methanolic KOH, in which the C13 ester function is known to be more susceptible to hydrolysis than the C12 substituent (3,8,9). Under such treatment, I and VI led to phorbol-12-tiglate (IV); II and VII provided phorbol-12-(2-methyl)butyrate (XIV); V afforded phorbol-12-acetate (XVII); II and VIII yielded 4-deoxy-4α-phorbol-12-tiglate (XV), and IX generated 4-deoxy-4α-phorbol-12-(2-methyl)butyrate (XVI). The identity of each monoester was checked by MS. Therefore, compounds I, II, and VII may be assigned as the new phorbol diesters 12-*O*-tiglylphorbol-13-isobutyrate, 12-*O*-(2-methyl)butyrylphorbol-13-isobutyrate, and 12-*O*-(2-methyl)butyrylphorbol-13-acetate, respectively. The remaining 2 phorbol 12,13-diesters isolated in this study were identified as the known compounds 12-*O*-acetylphorbol-13-acetate (V) and 12-*O*-tiglylphorbol-13-acetate (VI). The first of these 2 compounds is known as a semisynthetic derivative of phorbol (10), although it has not previously been obtained as a naturally occurring compound. Compound VI was recently identified as a croton oil constituent by HPLC/MS by Wagner and coworkers (4). Therefore, a total of 16 phorbol 12,13-diesters are now known to occur in croton oil. Compounds III, VIII and IX were assigned, respectively, as the new diesters 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-isobutyrate, 12-*O*-tiglyl-4-deoxy-4α-phorbol-13-acetate and 12-*O*-(2-methyl)butyryl-4-deoxy-4α-phorbol-13-acetate. Although researchers have speculated that 4-deoxy-4α-phorbol esters may occur in croton oil (3), the present study provides for the first time structural details of such compounds. The 4-deoxy-4α-

phorbol esters obtained in this investigation were probably not produced artifactually during work-up from analogous labile 4-deoxyphorbol esters because of the generally gentle nature of the isolation methods used.

Monoesters of the phorbol ester type have not been previously recorded as croton oil constituents. Compound X was shown, by direct comparison with synthetic phorbol-12-acetate (XVII) and phorbol-13-acetate (X), to be the latter compound. The characteristic ^{1}H-NMR C12 methine proton doublet occurring at δ 4.823 in the C12 esterified monoester XVII was observed at a similar resonance in the ^{1}H-NMR spectrum of IV, which was thus assigned as the new compound phorbol-12-tiglate. Isolate XI was identified as the new compound 4-deoxy-4α-phorbol-13-acetate, on the basis of the downfield nature of its C12 methine proton resonance (δ 4.040), when compared with the equivalent signal in the ^{1}H-NMR spectra of 4-deoxy-4α-phorbol monoesters containing a C12 acyl function (8,9).

Droplet countercurrent chromatography (DCCC) is a technique that is usually associated with the isolation of polar plant constituents (14), although a nonaqueous system has been used satisfactorily for the separation of several components of the essential oil of *Matricaria chamomilla* (15). Our success in applying this method as the primary isolation tool, not only for the present study on short-chain phorbol esters of croton oil, but also for ingenol and ingol esters of *Euphorbia hermentiana* latex (7,16), suggests that DCCC may well be useful for a wider range of applications in the separation of fixed and volatile oil constituents than at present.

The various phorbol and 4-deoxy-4α-phorbol short-chain esters of croton oil were obtained in this investigation in a combined yield of 0.258% w/w. The short-chain phorbol ester compounds described in this communication may be of considerable biological significance when croton oil is used in 2-stage carcinogenesis experiments because they may interfere with the tumor-promoting activity of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Several such compounds, while inactive as mouse skin tumor promoters themselves, have recently been found to drastically inhibit, in a dose-response fashion, the activity of TPA (17).

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