The Tumor-enhancing Principles of *Croton tiglium L.*[1]

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SUMMARY

The extraction and isolation of 2 active cocarcinogenic agents from the seed of *Croton tiglium L.* and their long-term biologic testing are described. Both materials are potent cocarcinogens at very low dosages. The biologic activity of chemically transformed derivatives of the active materials is also reported. Phorbol myristate acetate, a semisynthetic compound derived from croton resin, showed notable promoting activity. Some aspects of the chemistry of the active materials are discussed.

In our earlier reports, the fractionation, isolation, and preliminary biologic assay of 2 active tumor-enhancing (cocarcinogenic) materials labeled A and C from the seeds of *Croton tiglium L.* were summarized (10, 11). The chemical constitution of a polyhydroxyphenolic ester, m.p. 96°C, obtained by the catalytic hydrogenation of a biologically active fraction and referred to as croton resin, was also described (10).

The present report concerns long-term biologic testing of the active materials A and C, the hydrogenated polyphenolic ester (10), and derivatives of the polyhydroxy cyclic ketone, $C_{20}H_{28}O_6$, obtained by the hydrolysis of croton resin.

The isolation of the active materials A and C and the preparation of the polyhydroxy cyclic ketone and some of its derivatives are also described.

MATERIALS AND METHODS

Animals

Female Swiss mice (Millerton Research Farms, Millerton, N. Y.) were vaccinated against ectromelia and put on test at age 8 weeks. Mice were housed on sterile wood chips in stainless steel cages, 10 to a cage, and were fed Purina laboratory chow and water *ad libitum*. The animal rooms were air-conditioned and were maintained at 72°–76°F.

Procedure

The backs of the mice were clipped free of hair 2 days before the initial treatment and then as needed for the duration of the experiment. DMBA,[2] freshly recrystallized from acetone, m.p. 121°–122°C, was applied by micropipet in a single dose (150 or 300 μg) in an acetone solution (0.1 ml). This primary treatment was followed 14 days later by applications of promoters given 3 times a week. Promoter solutions were applied onto the clipped dorsal skin with a No. 5 squirrel hairbrush, with 100 mg of solution delivered per application. Mice were individually weighed before painting was started and at regular intervals thereafter. They were observed regularly, and their tumors were charted on appearance throughout the duration of the experiment. Papillomas were counted only if they persisted for 4 weeks or more. Papillomas that persisted for 4 weeks or more and then regressed were counted in the cumulative number of tumors. At death, tumors were excised and confirmed microscopically.

Extraction of Seed of *Croton tiglium L.*

Shelled croton seed (*Croton tiglium L.*, Ceylon) was crushed in a Waring Blendor and extracted exhaustively with methyl alcohol at room temperature. The solvent was distilled off in vacuum in a nitrogen atmosphere at 30°C. An oil and a crystalline precipitate separated; the crystalline residue was separated from the oil by filtration and rinsed with methyl alcohol, and the solvent was removed from the filtrate. The residue obtained from this solvent removal, whole croton seed extract, constituted 41% of the seed weight.

Fractionation of Croton Seed Extract

Solvent fractionation.—The dark-brown extract was partitioned between hexane and methyl alcohol-water (9:1). All methyl alcohol-water extracts were combined, and all hexane extracts were combined. No further work was done on the hexane extract. The methyl alcohol-water layer was filtered to remove an amorphous solid. The filtrate was evaporated to dryness in vacuum, and the residue was extracted with ether. The ether-soluble fraction was dried, filtered, and taken to dryness. The viscous brown resin was labeled *croton resin* (4) and constituted 6.8% of the whole seed extract.

Florisil chromatography.—Croton resin was chromatographed on acid-washed Florisil, and all ether eluates were combined.

Countercurrent Distribution.—The ether eluate from the Florisil chromatography was subjected to countercurrent

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[2] The abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; A and C are fractions A and C from croton resin prepared as described in this work; CR, croton resin; WSE, whole croton seed extract.

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distribution in a 200-tube (20 ml/tube) machine, with 500 transfers. The solvent for the lower phase was methyl alcohol-water-acetic acid (94.5:5.0:0.5); the upper phase was hexane. The progress of the distribution was followed by thin-layer chromatography on silica gel plates with ether used as solvent; spots were visualized by spraying with 50% sulfuric acid and brief heating with an infrared lamp. Tubes 0–16 still contained complex mixtures; Tubes 17–55 contained a mixture that gave only 2 spots on a thin-layer chromatogram; these 2 materials were labeled A and C with R_F 0.44 and 0.25, respectively. The contents of Tubes 17–55 were combined and taken to dryness; a white amorphous solid was obtained.

Separation of A and C.—A and C were separated by thick-layer chromatography on silica gel (1). The mixture of A and C, 1.42 gm, yielded 0.59 gm of A and 0.34 gm of C (recovery: 65%). Both A and C are optically active (A: $[\alpha]_D^{26}$ + 40.3°; C: $[\alpha]_D^{26}$ + 61.2°), colorless amorphous materials. Both A and C show in their infrared spectra free hydroxyl and ester carbonyl absorption.

Gas Chromatographic Determination of Fatty Acids Obtained by Hydrolysis of A and C

The materials labeled A and C were subjected to alkaline hydrolysis, and the fatty acids obtained were analyzed as their methyl esters. The acids were identified qualitatively by comparison of retention times with standard mixtures of known methyl esters. Quantitative analyses were performed by integration of the areas under the curves of the known and unknown mixtures. On hydrolysis, A yielded tiglic (6.0% of total acids), caprylic (13.4%), and capric (80.6%) acids and a trace of isobutyric acid. C yielded acetic, capric (combined 6.2%), lauric (13.1%), and myristic (80.7%) acids.

Isolation of phorbol from croton resin.—Forty gm of croton resin was dissolved in 600 ml of methyl alcohol and treated with 15 gm of barium hydroxide dissolved in methyl alcohol. The resultant suspension was refluxed for 1 hr under nitrogen and stirred overnight at room temperature. The barium soap was filtered off, the methyl alcohol removed in vacuum, and the residue extracted with ethyl alcohol. This product contained inorganic material, which was removed by chromatography on acid-washed Florisil and elution with methyl alcohol. The phorbol was crystallized from ethyl alcohol, m.p. 239°–241°C, reported m.p. 238°–240°C (3), $[\alpha]_D^{26}$ + 118° in dioxane.

Analysis.—Calculated for $C_{20}H_{28}O_6$; C, 65.99; H, 7.75; M.W., 364. Found: C, 66.16, H, 7.44; M.W. (by osmometer), 373; C—CH_3 (Kuhn-Roth), 9.6%. Ultraviolet absorption in methyl alcohol: λ_{max} 234 mμ (ϵ_{max} 5140). Phorbol gives a positive Griegee color test for a glycol and decolorizes bromine. The infrared absorption spectrum of phorbol indicates the presence of keto carbonyl with a peak of 1702 cm^{-1}. A broad bonded hydroxyl peak occurs at 3400 cm^{-1} and C=C absorption is evident at 1650 cm^{-1}.

Preparation of phorbol acetate.—Crude phorbol was acetylated with acetic anhydride-acetic acid-pyridine and chromatographed on Florisil. The ether eluate yielded an oil, which was purified by countercurrent distribution with the same solvent system and machine as that used for separation of the active materials. After 600 transfers, 4 gm of the crude acetate from the ether eluate yielded 1.2 gm of material (in Tubes 60–100), which readily crystallized from ether to give 0.5 gm of an acetate, m.p. 198°–201°C; reported (9) m.p. 198°–200°C. This product gave a single spot on a thin-layer chromatogram Silica Gel G, ether-hexane (9:1) solvent, R_F 0.50; $[\alpha]_D^{26}$ in methyl alcohol, 142°, reported $[\alpha]_D^{26}$, 138° (9). This material was identical by m.p., mixed m.p., and infrared absorption spectrum with the acetate of Thomas and Marxer (9), a sample of which was provided by Dr. A. Marxer. These workers assigned a $C_{23}H_{30}O_8$ molecular formula to their compound, but their analytical data agree well with a $C_{26}H_{34}O_9$ formula.

Analysis.—Calculated for $C_{26}H_{34}O_9$: C, 63.73; H, 6.99; M.W., 490. Found: C, 63.74; H, 6.79; M.W. (by osmometer), 467; C—CH_3, 15.13%; saponification number, 100. Ultraviolet absorption in methyl alcohol: λ_{max} 238 mμ (ϵ_{max} 8150); reported (9) λ_{max} 233 mμ (ϵ_{max} 8000), λ_{max} 324 mμ (ϵ_{max} 53). The infrared absorption spectrum shows a sharp hydroxyl band at 3430 cm^{-1}, C—H stretching at 3010 (olefinic C—H), 2950, and 2880 cm^{-1}, ester and keto carbonyl at 1740 and 1710 cm^{-1}, respectively, and carbon-carbon double bond absorption at 1640 cm^{-1}.

Crystalline esters from A.—A total of 200 mg of material A was dissolved in 10 ml of dry benzene in the presence of 120 mg of 4-chlorocarboxy-4′-nitroazobenzene (6) and 0.1 ml of dry pyridine and refluxed for 1.5 hr. The benzene solution was extracted successively with 25 ml of 0.1 N sulfuric acid, 25 ml of 1.0% sodium carbonate solution, and 25 ml of water. The benzene layer was separated, dried over anhydrous sodium sulfate, and filtered, and the solvent was removed under nitrogen on a steam bath; 188 mg of a red residue were obtained. This material was purified by chromatography on thick silica gel plates with ether-hexane (6:4) used as solvent. Two major bands appeared with R_F 0.44 (AD-1) and R_F 0.40 (AD-2). The bands were scraped off the plates separately and extracted with ether. The ether extracts were taken to dryness to give 80 mg of AD-1 and 68 mg of AD-2. These materials were crystallized from ether-hexane to give orange needles; AD-1, 65 mg, m.p. 120°–121°C; AD-2, 52 mg, m.p. 151°–152°C.

Crystalline ester from C.—A total of 230 mg of C was converted to the 4-carboxy-4′-nitroazobenzene derivative as described above for A. The product was also purified by thin-layer chromatography but gave one band only, R_F 0.37; this material was crystallized from ether-hexane to give red needles, 165 mg, m.p. 123°–124°C. This material was labeled CD. The analytical data on the 3 crystalline esters derived from A and C are given in Table 1.

Phorbol acetate from the 4-carboxy-4′-nitroazobenzene derivatives.—Twenty mg of the derivative, m.p. 151°–152°C, obtained from A and 20 mg of the similar derivative, m.p. 123°–124°C, prepared from C, were hydrolyzed with barium hydroxide in absolute methyl alcohol by refluxing for 1 hr. The methyl alcohol was removed

TABLE 1

ANALYTICAL DATA OF 4-CARBOXY-4′-NITROAZOBENZENE DERIVATIVES OF A AND C

Compound No.	AD-1	AD-2	CD
m.p.	120°–21°C	151°–52°C	123°–24°C
Molecular formula	$C_{48}H_{61}N_3O_{11}$	$C_{47}H_{59}N_3O_{11}$	$C_{49}H_{63}N_3O_{11}$
m.w.			
Calculated	856	842	870
Found			
Osmometer	875	855	883
Rast	857	836	
Analysis			
Calculated			
C	67.35	67.04	67.64
H	7.18	7.06	7.30
N	4.91	4.98	4.83
Found			
C	67.47	67.07, 66.52	67.77, 67.42, 67.39
H	7.23	6.93, 6.90	6.95, 7.21, 7.45
N	5.00	5.04, 5.11	5.28
% C—CH_3 (Kuhn-Roth)	8.38	8.88	8.40
% Active hydrogen	0.36	0.39	0.32
Saponification No.	274	219	249
Ultraviolet absorption in methyl alcohol			
λ_{max}	330[a]	330[a]	330[a]
ϵ_{max}	34,800	20,000	32,510

[a] All 3 materials showed an inflection on the 330 mμ maximum at 345 mμ and an inflection on the short wavelength end absorption at 235 mμ.

under nitrogen; 10 ml of dry pyridine and 30 ml of acetic acid-acetic anhydride (1:1) were added, and the solutions were refluxed for 4 hr. The solvents were removed in vacuum. The residues were extracted with ether, and the ether extracts were chromatographed on acid-washed Florisil. The ether eluates were taken to dryness, and aliquots were chromatographed on silica gel plates with ether-hexane (9:1) used as solvent. Phorbol acetate, m.p. 198°–201°C, was used as a marker. The derivatives of both A and C showed spots identical in R_F with the spots of phorbol acetate.

Phorbol myristate acetate.—Phorbol was treated with acetic anhydride and pyridine at room temperatures for 24 hr, and dried in vacuum. The residue was treated with myristoyl chloride and pyridine in the same manner, and pyridine was removed in vacuum. The residue, dissolved in ether, was washed with cold sodium bicarbonate solution; the ether-soluble material was purified by thin-layer chromatography. The product was an amorphous solid but gave only 1 spot on a chromatogram; it was used as such for biologic testing.

RESULTS AND DISCUSSION

The results obtained in initiation-promotion experiments with DMBA used as initiator are summarized in Table 2. With croton resin and fractions A and C at the higher dosage level, the 1st tumors appeared at 38–51 days from the date of initiation. The high activity of the croton resin and of A and C is clearly indicated in these results. Even at the very low dosages of 0.5 μg applied twice weekly, benign and malignant tumors were obtained; the latent periods were, however, much longer with the lower dosage.

Multiple tumors were obtained in the initiation-promotion experiments with croton resin, A, and C (at the higher dosage levels); the range was 3–8 papillomas/tumor-bearing animal. Very few of these tumors regressed (0–5%).

A small number of benign tumors were obtained in control groups that had received fractions A and C at the higher dosage (5.0 μg) but not at the lower dosage (0.5 μg). In the control group in which the animals had received a single treatment with 300 μg DMBA, 3 papillomas were observed; 1 of these tumors regressed. Ten of the 20 animals in this group were accidentally given a single treatment with croton oil 6 months after the beginning of the experiment, by which time 1 animal in the group already bore a papilloma, which subsequently regressed. The 2 other papillomas appeared after the accidental croton oil treatment.

Two dosage levels, 150 and 300 μg, of the initiator, DMBA, were used in some tests listed in Table 2 to determine whether the higher dosage would effect tumor response and rate of 1st tumor appearance. From the results given in Table 2 it appears that the higher dosage does not significantly enhance tumor response.

In addition to these materials, phorbol myristate acetate, phorbol acetate, and the hydrogenated polyphenolic ester described earlier (10) were tested for biologic activity. These materials were applied at a dosage of 5 μg/application 3 times weekly and were preceded by a single treatment with 150 μg of DMBA 14 days before the beginning of promotion. After 30 weeks of testing, phorbol acetate and the hydrogenated polyphenolic ester showed no activity. However, of the 20 animals given DMBA followed by phorbol myristate acetate, 11 bore

TABLE 2

TUMOR PROMOTION WITH CROTON SEED EXTRACT, CROTON RESIN, AND FRACTIONS A AND C

TREATMENT[a]		MEDIAN SURVIVAL TIME (DAYS)	DAYS (FROM PRIMARY TREATMENT) TO 1ST		CUMULATIVE NO. OF MICE WITH		DAYS TO TERMINATION
Primary[b] (μg/100 mg acetone)	Secondary[c] (μg/100 mg acetone)		Tumor	Cancer	Tumor	Cancer	
DMBA (150)	CR (25)	276	49	110	17	11	Lifetime
DMBA (300)	CR (25)	254[d]	38	123	25	2	328
DMBA (300)	A (0.5)	>435	222	419	8	1	435
DMBA (300)	A (5.0)	365	51	273	19	19	435
DMBA (300)	C (0.5)	>435	104	322	9	1	435
DMBA (300)	C (5.0)	225	51	189	20	9	435
DMBA (150)	WSE (25)	>380	73	198	5	3	380
DMBA (150)	None	370			0	0	380
DMBA (300)	None	423	266		3	0	435
DMBA (150)	Acetone	304			0	0	380
None	Acetone	>435			0	0	435
None	A (0.5)	>435			0	0	435
None	A (5.0)	>435	224		1	0	435
None	C (0.5)	>435			0	0	435
None	C (5.0)	407	91		3	0	435
None	CR (25)	439	426		1	0	Lifetime
None	WSE (25)	>372			0	0	372

[a] There were 20 Swiss Millerton female mice/group except where noted.

[b] Single treatment.

[c] Three times weekly beginning 14 days after primary treatment except for A and C at 0.5 μg/application, which were treated twice weekly. Promoters were as follows: CR is croton resin; A and C are fractions A and C of this work; WSE is the whole seed extract described in this work.

[d] Thirty animals in this group.

papillomas. The 1st tumors appeared at 87 days from the date of initiation. No tumors were observed in the control group, which received only phorbol myristate acetate.

Materials A and C each constitute approximately 1% of the whole croton seed extract. The tumor response obtained with A and C at the lower dosage level, 1 μg/week, was only slightly lower than that obtained with the whole seed extract at a dosage level of 75 μg/week (see Table 2). It appears that A and C account for most of the tumor-promoting activity of the whole seed extract. However, it is likely that fractions B and D are also biologically active. These materials have not yet been obtained free from A and C and therefore have not yet been tested.

The polyphenolic ester, m.p. 96°C, described earlier (10), was obtained by catalytic hydrogenation of croton resin followed by countercurrent distribution. This material is inactive as a cocarcinogen. The present results with A and C indicate that they do not contain an aromatic nucleus but rather that they are esters of phorbol, $C_{20}H_{28}O_6$, the isolation of which is described in the present report. Phorbol has been known for many years as a component of croton oil (3–5). The polyhydroxy compound, $C_{26}H_{28}O_6$, isolated (6) from croton oil may be the same compound, although no reference to this effect was made. Hecker (6) prepared an acetate of the polyalcohol, to which the molecular formula $C_{26}H_{34}O_9$ was assigned; an m.p. for this compound was not given. This acetate is different in its optical rotation from that reported in the present work. Our triacetate is identical by m.p., mixed m.p., and infrared absorption spectrum with that prepared by Thomas and Marxer (9).

Gas chromatographic analysis of A and C gave 4 acids in each case. Saponification numbers, however, suggest only 2 or 3 ester functions in A and C. It follows that both A and C contain small amounts of closely allied materials. In addition, A yielded two 4-carboxy-4′-nitroazobenzene derivatives. These 2 derivatives and that obtained from C differ in their molecular formulae by methylenes only. Substracting the derivative moiety, 3 molecular formulae for the original materials are arrived at: $C_{34}H_{52}O_8$; $C_{35}H_{54}O_8$; and $C_{36}H_{56}O_8$. These suggest that the original materials differ only in the chain length of the esterifying acids, which accounts for the difficulties encountered in their purification.

A 4-carboxy-4′-nitroazobenzene derivative, $C_{51}H_{64\text{-}66}N_3O_{12}$, m.p. 86°–87°C, has been described earlier (6); this molecular formula was later revised to $C_{49}H_{63}N_3O_{11}$. This compound is different in its m.p. from the 3 esters of the same type prepared in the present work. From the limited amount of information available in the brief communications of Hecker and co-workers (6–8), it appears, however, that the active materials described are of the same structural type as that obtained in the present work.

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