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ON THE ACTIVE PRINCIPLES OF THE SPURGE FAMILY, X.[1,2] SKIN IRRITANTS, COCARCINOGENS, AND CRYPTIC COCARCINOGENS FROM THE LATEX OF THE MANCHINEEL TREE

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ABSTRACT.—From the hydrophobic fraction of the latex of *Hippomane mancinella*, weak or nonirritant mixtures of esters of polyfunctional diterpene parent alcohols of the tigliane and daphnane types were isolated belonging to the structural type of "cryptic" irritants. The cryptic factor group M'_z (tigliane type) represents 12-deoxyphorbol-13,20-diesters, and the cryptic factor groups M'_y and M'_x (daphnane type) represent mixtures of 9,13,14-orthoesters-20-esters of resiniferonol and 5β-hydroxyresiniferonol-6α,7α-oxide, respectively. All of them carry in the 20-position homologous long-chain fatty acids, ranging from C_{15} to C_{26}. They may be "activated" by mild transesterification reactions yielding corresponding irritant factor groups with free hydroxyl functions at C-20. From the hydrophilic fraction of the latex, the irritant factor M_3 (tigliane type) and the factor group M_x (daphnane type) were isolated. According to spectral data, factor M_3 represents the 13-(hexadeca-2,4,6-trienoic acid) ester of the parent 12-deoxy-5β-hydroxyphorbol-6α,7α-oxide. Factor group M_x consists of two esters inseparable by tlc (silica gel). One is identical with huratoxin; the other one represents a hexadeca-2,4,6-trienoic acid orthoester. M_x is also obtained by transesterification of M'_x and exhibits irritant and tumor-promoting activity comparable to that of TPA. Some aspects on structure activity relations are deduced from selected chemical-reaction products of factor group M_x.

The "deadly" manzanillo or manchineel tree (*Hippomane mancinella* L., plant family: Euphorbiaceae, tribe: Hippomaneae) is an evergreen tree native to Central America and the West Indies, growing along sea shores and reaching up to 40 ft in height and 2 ft in diameter. In the United States, it is restricted to the peninsula of Florida and to the Virgin Islands. It is one of the most ill-famed poisonous plants in tropical America. The early settlers especially were freightened by its poisonous properties, *e.g.*, when they tried to use the tree for timber. Soldiers training in the West Indies during the last war often suffered from bullous dermatitis and severe conjunctivitis after accidental contact with it (1). Characteristic of the tree are its round yellow-green or yellowish fruits resembling small apples ("guavas") often littering the beaches ("beach apple"). They are not offensive in odor or taste, but are very toxic. Several accidents are reported (2,3) of people who ate these fruits causing serious illness or even death. Livestock have also been affected, and cattle have suffered skin irritation from contact with the plant. The tree is known also as a source of honey, which is reported to be nontoxic (2). The leaves, twigs, and bark of the tree contain a highly irritating milky sap (latex). It produces severe inflammation and blisters on human skin and is particularly irritating to the eyes and mucous membranes (3). Caribs seem to have poisoned their arrows with the latex, but it also has been used as an ingredient in many native medicinal preparations (2,4). As a consequence of its toxic properties, the tree was almost eradicated. In the United States, it is allowed to grow in remote areas only, such as the Everglades (3,4) or the Virgin Islands National Park.

Several attempts were made to investigate the toxic principles of *H. mancinella* (5-9). Obviously more than one toxic principle is present because response to ingestion of the fruits seems not exactly comparable with that of contact with the latex (2). So far,

[1]Ninth communication, see: H. Goffa, W. Adolf, H.J. Ophenkuch, and E. Hecker, *Z. Naturforsch.*, **39b**, (in press).

[2]Part of the present work was presented at the International Congress for Research on Medicinal Plants, Munich, September 6-10, 1976; see also *loc. cit.* 17.

besides several known plant constituents, an alkaloid resembling physostigmine (6) and a "crystalline tannin" (hippomanin A) (8,9) have been isolated from leaves, wood, or fruits. Hippomanin A has been evaluated as the major toxic principle of the aqueous fraction from leaves and twigs, with the toxicity to mice ranging between 40 and 60 mg/kg when administered intraperitoneally (8). However, it seemed unlikely that either one of these products could be responsible for the high toxicity and especially for the extreme skin-irritant effects of the latex and all parts of the tree.

From different plant parts of many other toxic species of Euphorbiaceae, the typical irritant principles are well known to represent oxygenated diterpene esters of the tigliane, ingenane, or daphnane type (for recent reviews, see 10-13). Their skin-irritant activity is determined quantitatively on the mouse ear; many of them are also cocarcinogenic on the back skin of mice (14). From the chemotaxonomic point of view, therefore, it might be expected that the irritant principles of *H. mancinella* belong to a similar class of compounds. Subsequently, the isolation of the highly skin-irritant factors from the latex of *H. mancinella* will be described, as well as the elucidation of their chemical structures and their biological activities.

EXPERIMENTAL

SPECTRA.—Mass spectra were measured with a Dupont CEC-21-110 B or a VARIAN MAT 711 spectrometer; uv spectra, with a Beckmann DK2a far UV spectrometer; ir spectra, with a Perkin-Elmer spectral photometer 521; and nmr spectra, with a JEOL C 60 HL or Brüker HX 90 spectrometer. Chemical shifts refer to TMS ($\delta=0.00$ ppm) as internal standard and $CDCl_3$ as solvent.

SEPARATION METHODS.—For materials and methods of multiplicative distribution, see Hecker and Schmidt (14). For analytical and preparative tlc, Merck silica gel HF_{254} and PF_{254}, respectively, or precoated Merck tlc plates, 0.25 and 0.5 mm, were used. Spots were detected under uv light (254 nm) and by spraying with vanillin-H_2SO_4 (14). For column chromatography, Merck silica gel 0.05-0.20 mm was used, deactivated with 13% H_2O.

A Packard-Becker gas chromatograph, model 420, was used for the analysis of fatty acid methyl esters; stationary phase: 10% silicon GE SE 30 on Chromosorb W/HP, carrier gas: N_2, flow rate: 30 ml/min; detector: FID, flow rates: 25 ml/min; temperature program: 60-260°: 5° min, t >260°: isothermal. Percent-values were estimated by triangulation.

CHEMICAL REACTIONS.—Chemical reactions, which were carried out under acidic or basic conditions, were stopped by adding appropriate amounts of phosphate buffer, pH: 6.8. After removal of the organic solvent, the reaction products were extracted from the aqueous phase with EtOAc and dried over $MgSO_4$. If reactions contained pyridine, the EtOAc was additionally extracted with dilute acid (excess of 1 M HCl) and thereafter with phosphate buffer.

BIOLOGICAL ASSAYS.—Irritant doses 50 (ID_{50}) were determined on the mouse ear, and the tumor-promoting activity was assayed on the back skin of NMRI-mice according to the standard procedures (14,15).

TERMINOLOGY.—Terminology used for the compounds isolated as follows:

Factor.—irritant compound, $ID_{50}<100$ μg/ear.

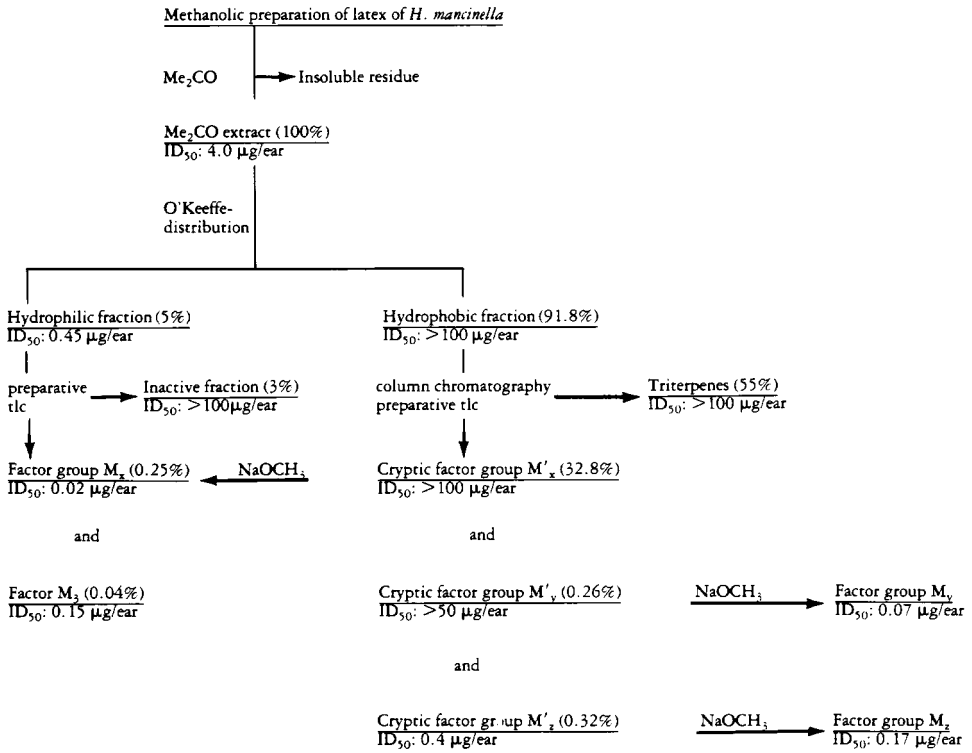
Factor group.—mixture of irritant compounds ($ID_{50}<100$ μg/ear), tlc uniform on silica gel in various solvent systems.

Cryptic factor group.—mixture of nonirritant ($ID_{50}>100$ μg/ear) or weakly irritant compounds ($ID_{50}<100$ μg/ear), tlc uniform on silica gel in various solvent systems, affording a factor group upon treatment with $NaOCH_3$-MeOH.

PLANT MATERIAL.—Methanol preparations of latex of *H. mancinella* were collected by E.H. on several islands around St. John, U.S. Virgin Islands, in 1968 and 1969, in a manner described in principle elsewhere (16). Valuable assistance during collection and authentication of the plant by Mr. I.G. Koblick, Mr. M.E. Stephey, Dr. A.E. Dammann, and Dr. E.L. Towle from the U.S. Virgin Island Ecological Research Station and the Caribbean Research Institute, St. John, is gratefully acknowledged.

EXTRACTION AND FRACTIONATION PROCEDURES.—Of the latex preparation, 100 g was extracted several times for 24 h with 500 ml of Me_2CO under N_2. After filtration from the insoluble residue (25.7 g) and evaporation of the solvent, 71 g of a white, Me_2CO extract was obtained, ID_{50}: 4.0 μg/ear. By

O'Keeffe distribution of 10 g of the Me_2CO extract using petroleum ether-MeOH-H_2O (15:10:0.5) (v=50 ml/50 ml; z=9 elements), 0.5 g of a hydrophilic fraction (ID_{50}: 0.45 μg/ear) and 9.2 g of a hydrophobic fraction (ID_{50}>100 μg/ear) were obtained. (See Scheme 1.)



SCHEME 1. Extraction and fractionation procedure of the latex from *Hippomane mancinella* including transesterification reactions of isolated cryptic factor groups.

ISOLATION OF CRYPTIC FACTOR GROUPS, FACTOR GROUPS, AND FACTORS.—*Hydrophobic fraction.*—Column chromatography of the hydrophobic fraction (6.3 g) eluting with petroleum ether-Et_2O (2:1) followed by tlc of the nonpolar fractions in petroleum ether-Et_2O (3:1) and $CHCl_3$-EtOH (100:0.5) afforded 22 mg of the *cryptic factor group* M'_z, Rf: 0.18 [$CHCl_3$-EtOH (100:0.5)], stain: reddish brown, ID_{50}: 0.4 μg/ear, and 18 mg of the *cryptic factor group* M'_y, Rf: 0.25 [$CHCl_3$-EtOH (100: 0.5)], stain: grey, ID_{50}>50 μg/ear.

By tlc of the hydrophobic fraction (900 mg) in petroleum ether-Et_2O (2:1), 322 mg of the *cryptic factor group* M'_x was obtained, Rf: 0.16 [Et_2O-petroleum ether (1:1)], stain: grey to black, ID_{50}>100 μg/ear.

Cryptic factor group M'_z.—ms *m/z* 986/960, 958/932, 930/904, 902/876, 874/848, 846/818, 329, 312, 294, 233; ir (CH_2Cl_2) 3550, 3380 (OH), 1725, 1700 (CO), 1625, 1608 cm^{-1} (C=C); uv (hexane) λ max 230, sh 238, sh 258, 267, 279, 308.5 nm; pmr 1-H: 7.62 (m); 20-H_2: 4.45 (s); 10-H: 3.25 (m); 8-H: 3.0 (m); 5-H_2: 2.42 (s); 19-H_3: 1.78 (m); OH (exchangeable with D_2O): 5.33, 2.3 ppm; acid moieties: 1 olefinic H: 7.25 (dd); 5-6 olefinic H (incl. 7-H): 5.5-6.5; appr. $(CH_2)_{23}$: 1.28 ppm.

Cryptic factor group M'_y.—ms *m/z* 984/958, 956/930, 928/902, 900/874, 872/846, 844/818, 816/790, 396, 368, 328, 310, 233, 207; ir (CH_2Cl_2) 3550, 3400 (OH), 1725, 1703 (CO), 1660, 1628, 1608 cm^{-1} (C=C); uv (hexane) λ max 230, sh 240, 257, 268, 280, 303 nm; pmr 1-H: 7.62 (m); 16-H_2: 5.04 (s), 4.91 (s, br.); 20-H_2; 4.55 (s); 14-H: 4.38 (d, J=2.5 Hz); 8-H, 10-H: 3.2 (m); 19-H_3: 1.82 (m); 17-H_3: 1.78 (s); OH (exchangeable with D_2O): 2.45; acid moieties: 1 olefinic H: 6.75 (dd); 5 olefinic H (incl. 7-H): 5.5-6.4; appr. $(CH_2)_{24}$: 1.28 ppm; decoupling experiments prove sequence of 8-H, 14-H and 1-H, 10-H, 19-H_3.

Cryptic factor group M'_x.—ms *m/z* 988/962, 960/934, 932/906, 904/878, 876/850, 848/822, 396, 368, 342, 325; ir (CH_2Cl_2) 3510 (OH), 1732, 1690 (CO), 1628 cm^{-1} (C=C); uv (hexane) λ max sh 248, 258, 268.5, 279.5, sh 316 nm; pmr 1-H: 7.60 (m); 16-H_2: 5.01 (s) and 4.89 (s); 14-H: 4.40 (d, J=2.5 Hz); 20-H_2: 4.30±0.46 (J_{AB}=12 Hz); 5-H: 4.24 (s); 10-H: 3.84 (m); 7-H: 3.33 (s); 8-H: 2.93 (d, J=2.5 Hz); 17-H_3, 19-H_3: 1.80 (s, br.); OH (exchangeable with D_2O): 3.78, and 3.89 ppm; acid moieties: 1

olefinic H: 6.7 (dd); 4 olefinic H: 5.5-6.3; -CH_2-CO, CH_2-C=C: 2.0-2.4; $(CH_2)_{22-24}$: 1.25, CH_3: 0.87 ppm (pert. t.).

Hydrophilic fraction.—tlc of 1.0 g of the hydrophilic fraction in Et_2O-petroleum ether (3:1) yielded 48 mg of the *factor group* M_x, Rf: 0.16 [Et_2O-petroleum ether (4:1)], stain: grey to black, ID_{50}: 0.02 μg/ear, and 8 mg of the *factor* M_3, Rf: 0.13 (Et_2O-petroleum ether (4:1)], stain: dark brown, ID_{50}: 0.15 μg/ear.

Factor group M_x.—ms 610 (M^+), 584 (M^+), 579, 561, 553, 535, 360, 342, 317, 233 and 207; ir (CH_2Cl_2) 3520 (OH), 1690 (CO), 1630 cm^{-1} (C=C); uv (MeOH) λ max sh 232, sh 240, sh 249, 259, 268.5, 278.5, pmr (data at variance with those of the cryptic factor group M'_x): 20-H_2: 3.82 (s); OH (exchangeable with D_2O): 4.24, 3.93 and 2.5 ppm; acid moiety: $(CH_2)_{6-7}$: 1.28 ppm.

Factor M_3.—ms 612 (M^+), 594, 576, 233. For all other spectral data, see (17).

CHARACTERIZATION OF CRYPTIC FACTOR GROUPS, FACTOR GROUPS, AND FACTORS BY ACETYLATION AND TRANSESTERIFICATION REACTIONS.—*Transesterification of* M'_z, *affording a parent alcohol, factor group* M_z (**1,2**) *and fatty acid methyl esters.*—Of the cryptic factor group M'_z, 32 mg was treated with 0.01 M $NaOCH_3$-MeOH (12 ml). After 4 h, 4 ml of Me_2CO was added to dissolve precipitated material. The reaction was stopped after 8 h. Tlc in Et_2O-petroleum ether-Me_2CO (1:1:1) yielded 10 mg of a nonpolar fraction (Rf: 0.7-1.0), 5 mg of the factor group M_z (**1,2**) (Rf: 0.57), ID_{50}: 0.17 μg/ear, and 8 mg of a polar product [Rf: 0.18 in Et_2O-petroleum ether-Me_2CO (1:1:2)]. The latter was treated with pyridine (1 ml) and Ac_2O (0.5 ml) for 15 h. After usual work up, 8 mg of the acetylation product **3** was obtained: ms *m/z* 414 (M^+-H_2O), 372, 357, 354, 330, 329, 312, 294; ir (KBr) 3420 (OH), 1735, 1710 (CO), 1625 cm^{-1} (C=C); pmr 1-H: 7.62 (m); 7-H: 5.71 (d, J=6 Hz); 20-H_2: 4.46 (s); 10-H: 3.28 (m); 8-H: 3.0 (m); 5-H_2: 2.45 (s); 19-H_3: 1.8 (m); 16-H_3, 17-H_3: 1.09 (s) and 1.22 ppm (s); OH (exchangeable with D_2O): 5.35 and 2.3 ppm; 2 acetyl: 2.06 and 2.09 ppm.

Factor group M_z (**1,2**).—ms *m/z* 580 (M^+), 562, 554 (M^+), 536, 494, 468, 312, 294, 233, 207; ir (CH_2Cl_2) 3380 (OH), 1730, 1690 (CO), 1610 cm^{-1} (C=C); uv (MeOH) λ: 194, λ max sh 230, 240, sh 270, 309 nm; pmr 1-H: 7.6 (m); 20-H_2: 4.0 (s); 10-H: 3.28 (m); 8-H: 3.0 (m); 5-H_2: 2.5 (s); 19-H_3: 1.80 (m); 16-H_3, 17-H_3: 1.06 (s), and 1.20 ppm (s); OH (exchangeable with D_2O): 5.8 and 2.2 ppm; acid moieties: 1 olefinic H: 7.28 (dd); 5 olefinic H (incl. 7-H): 5.5-6.3 ppm.

The nonpolar fraction with Rf values between 0.7 and 1.0 was recognized as a mixture of compounds (10 mg, ms *m/z* 410, 382, 354), and further resolved by glc and identified by cochromatography with authentic samples as follows (peaks<2.5% were neglected): methyl hexacosanoate (26.6%), tetracosanoate (20.8%), docosanoate (7.5%), eicosanoate (6.3%), octadecanoate (15.5%), hexadecanoate (6.7%), and one unidentified compound (17.1%) eluting between methyl pentadecanoate and hexadecanoate.

Acetylation of M_3 (**4**), *affording the diacetate* **5**.—Factor M_3 (15 mg) was treated with pyridine (2 ml) and Ac_2O (1 ml). After 15 h at room temperature, usual work-up and purification by tlc [Et_2O-petroleum ether (1:1)] 8 mg of **5** was obtained, Rf: 0.42 [Et_2O-petroleum ether (2:1); ms *m/z* 696 (M^+), 233; pmr 1-H: 7.65 (m); 5-H: 5.56 (s); 20-H_2: 4.15±0.59 (J_{AB}=12 Hz); 10-H: 4.08 (m); 7-H: 3.16 (s); 8-H: 2.93 (d, J=8 Hz); 19-H_3: 1.76 (m); 16-H_3: 1.12 and 0.9 ppm; OH (exchangeable with D_2O): 2.94 ppm; acid moieties: 1 olefinic H: 7.3 (dd); 5 olefinic H: 5.6-6.7, 2 acetyl: 2.21 and 2.04 ppm.

Transesterification of M'_y, *affording factor group* M_y (**6,7**) *and fatty acid methyl esters.*—To 20 mg of the cryptic factor group M'_y 0.01 M $NaOCH_3$-MeOH (8 ml) was added. Seven hours later the reaction was stopped, and the reaction products were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:1:1). Besides 5 mg of a nonpolar fraction (Rf: 0.7-0.9), 3.5 mg of the factor group M_y (**6,7**) was obtained (Rf: 0.45), ID_{50}: 0.07 μg/ear.

Factor group M_y (**6,7**).—ms *m/z* 578 (M^+) and 552 (M^+), 328, 310, 233, 207; ir (CH_2Cl_2) 3600, 3550, 3430 (OH); 1705 (CO); 1660, 1630 cm^{-1} (C=C); uv (MeOH) λ max 232, 259, 269, 280, sh 320; pmr 1-H: 7.6 (m), 16-H_2: 5.04 (s), 4.91 (s, br.); 14-H: 4.38 (d, J=2.5 Hz); 20-H_2: 4.09 (s); 8-H, 10-H: 3.2 (m); 17-H_3, 19-H_3: 1.8 (m); 18-H_3: 1.15 (d, J=7 Hz); OH (exchangeable with D_2O): 2.4 ppm; acid moieties: 1 olefinic H: 6.73 (dd, J_1=10 Hz, J_2=16 Hz); appr. 5 olefinic H (incl. 7-H): 5.5-6.3; appr. $(CH_2)_{7-8}$: 1.28 ppm.

The nonpolar fraction with Rf values between 0.7 and 0.9 was recognized as a mixture of compounds (5 mg, ms *m/z* 410, 382, 354) and further resolved by glc and identified by cochromatography with authentic samples as follows (peaks<2% were neglected): methyl hexacosanoate (40.7%), tetracosanoate (31.6%), docosanoate (8.6%), eicosanoate (6.5%), octadecanoate (6.3%), hexadecanoate (3.6%), and pentadecanoate (2.7%, no reference compound available).

Acetylation of M'_x.—Cryptic factor group M'_x (66 mg) was acetylated (1 ml pyridine and 2 ml Ac_2O). The reaction was stopped after 15 h, and the acetylated M'_x [70 mg, Rf: 0.22 in Et_2O petroleum ether (1:1)] was isolated as usual; ms *m/z* 1030/1004, 1002/976, 974/948, 946/920, 918/892, 780, 752; uv (hexane) λ max 240, 258.5, 269.5 and 280.5 nm; pmr differences to spectrum of M'_x: 5-H: 5.50 (s); acetyl: 2.15 ppm.

Transesterification of M'_x, affording factor group M_x (**8,9**) *and fatty acid methyl esters.*—Cryptic factor group M'_x (100 mg) was dissolved in 3-4 ml of Me_2CO and 0.01 M $NaOCH_3$-MeOH (5 ml) was added. The reaction was stopped after 90 min. Tlc in Et_2O-petroleum ether (4:1) afforded 45 mg of tlc uniform factors exhibiting the same Rf value, ID_{50} value, and identical spectral data as factor group M_x (**8,9**) isolated from the hydrophilic fraction. Moreover, the nonpolar fraction with Rf values between 0.7 and 1.0 was recognized as a mixture of compounds (18 mg, ms *m/z* 410, 382, 354, 326, 298) and further resolved by glc and identified by cochromatography with authenic samples. Quantitative analysis of the chromatogram (peaks<2% were neglected) amounted to: methyl hexacosanoate (31.2%), tetracosanoate (29.9%), docosanoate (8.0%), eicosanoate (9.8%), octadecanoate (12.4%), and hexadecanoate (8.7%). M'_x (22 mg) was treated with 3 ml of a 0.3% $HClO_4$ in MeOH. The reaction was stopped 24 h later. By tlc, a factor group was obtained exhibiting Rf value, ID_{50}, and spectroscopic properties identical with those of M_x.

Acetylation of M_x, affording the diacetates **10,11**.—Factor group M_x (54 mg) was treated with pyridine (1 ml) and Ac_2O (1 ml) for 20 h to yield after tlc [petroleum ether-EtOAc (2:1)] 45 mg of products **10,11** [Rf: 0.47 in petroleum ether-EtOAc (1:1)]; ms *m/z* 694 (M^+), 668 (M^+); pmr differences to the spectrum of factor group M_x: 5-H: 5.52; 20-H_2: 4.12±0.52 (J_{AB}: 12 Hz); 2 acetyl: 2.18 and 2.0 ppm.

FURTHER CHEMICAL REACTIONS WITH FACTOR GROUP M_x.—*Catalytic hydrogenation of M_x.*—(a) *Pd/charcoal, affording* **12,13**: 117 mg of factor group M_x was dissolved in 10 ml of EtOH, and 20 mg of Pd/charcoal was added and stirred under H_2. The hydrogenation reaction was stopped after 24 h by filtration from the catalyst. The hydrogenation products were purified by tlc in Et_2O-petroleum ether-Me_2CO (1:1:1) to yield 60 mg of compounds **12,13** (Rf: 0.35); ID_{50}: 0.028 μg/ear; ms *m/z* 589 (M^+-31), 561 (M^+-31), 577 (M^+-43), 571, 549 (M^+-43), 543, 533, 364, 321; ir (CH_2Cl_2) 3550 (OH), 1745 cm^{-1} (CO); uv (MeOH) λ max 305 nm; pmr 14-H: 4.27 (d, J=2.5 Hz); 5-H: 4.06 (s); 20-H_2: 3.77±0.04 (J_{AB}: 13 Hz); 7-H: 3.40 (s); 10-H: 2.84 (dd, J_1=5 Hz, J_2=13 Hz); 8-H: 2.72 (d, J=2.5 Hz); appr. $(CH_2)_{12}$: 1.25 ppm. (b) *PtO_2, affording* **14,15** *and* **16,17**: 180 mg of factor group M_x was dissolved in EtOAc (20 ml) and hydrogenated after adding 50 mg of PtO_2. The reaction was stopped after 6.5 h by filtration from the catalyst. After separation in EtOAc-petroleum ether (2:1) compounds **14,15** [24 mg, Rf: 0.36 in EtOAc-petroleum ether (3:1), ID_{50}: 0.035 μg/ear] and **16,17** [18 mg, Rf: 0.18 in EtOAc-petroleum ether (3:1), ID_{50}: 0.71 μg/ear] were isolated. Additional material (15 mg) still consisted of several compounds that were not further investigated.

Compounds **14** and **15**: ms *m/z* 618 (M^+), 590 (M^+), 587 (M^+-31), 569, 559 (M^+-31), 541; ir (CH_2Cl_2) 3520 (OH), 1690 (CO), 1625 cm^{-1} (C=C); uv (MeOH) λ max 243, 334 nm; pmr 1-H: 7.6 (m); 14-H: 4.31 (d, J=2.5 Hz); 5-H: 4.22 (s); 20-H_2: 3.80 (s); 10-H: 3.72 (m); 7-H: 3.45 (s); 8-H: 2.78 (d, J=2.5 Hz); CH_2-CO: 2.38 (t); 19-H_3: 1.80 (m); 18-H_3: 1.13 (d, J=7 Hz); 16-H_3, 17-H_3: 0.96 and 0.90 ppm; OH (exchangeable with D_2O): 2.6 ppm. Further, 82 mg of compounds **14** and **15** was obtained after hydrogenation of 300 mg of factor group M_x in 30 ml EtOH with 50 mg PtO_2 for 2 h and purification, as described above.

Compounds **16** and **17**: ms *m/z* 591 (M^+-31), 573, 563 (M^+-31); ir (CH_2Cl_2) 3600, 3520 cm^{-1} (OH); uv (MeOH) λ max 230 nm; pmr 5-H: 4.38 (s, br.); 14-H: 4.29 (d, J=2.5 Hz); 20-H_2: 3.80±0.06 (J_{AB}=12 Hz); 3-H: 3.5 (d, J=9.5 Hz); 7-H: 3.40 (s); 8-H: 2.85 (d, J=2.5 Hz); 16-H_3, 17-H_3: 0.97 and 0.90 ppm.

Acetylation of compounds **16,17** *affording* **18,19**.—Of compounds **16,17**, 5 mg was acetylated by treating with pyridine (2 ml) and Ac_2O (1 ml). The reaction product (7 mg) was purified by tlc in EtOAc-petroleum ether (1:1) to yield 4 mg of products **18,19** (Rf: 0.4); ms *m/z* 748 (M^+), 730, 720 (M^+), 705, 702, 688, 675, 647, 611, 573, 492, 477, 449; ir (CH_2Cl_2) 3560 (OH), 1735 cm^{-1} (CO); pmr 5-H: 5.50 (s); 3-H: 4.3 (d, J=10 Hz); 14-H: 4.23 (d, J=3 Hz); 20-H_2: 4.01±0.11 (J_{AB}=12 Hz); 7-H: 3.28 (s); 8-H: 2.84 (d, J=3 Hz); 18-H_3: 1.18 (d); 19-H_3: 0.9 ppm (d); acetyl: 2.06 ppm (9H); decoupling experiments prove sequences: 14-H, 8-H; 2-H (1.8 ppm), 3-H, 19-H_3; 11-H (2.45 ppm), 18-H_3.

Acidic cleavage of **14,15**, *affording* **20,21** *and* **22,23**.—Compounds **14,15** (80 mg) were dissolved in 9 ml of EtOH, and 4 ml of 2 M aqueous HCl were added. After refluxing for 2 h, the reaction was stopped by adding 50 ml of phosphate buffer, pH 6.8. The reaction products, two major spots with higher and lower Rf values than the starting material, were separated by tlc in Et_2O-petroleum ether (2:1). Besides 27 mg of compounds **20,21** (Rf: 0.23, ID_{50}: 2.2 μg/ear), 8 mg of compounds **22,23** was obtained (Rf: 0.42, ID_{50}>50 μg/ear).

Compounds **20,21**: ms 654/656 (intensity ratio 3:1, M^+), 626/628 (intensity ratio 3:1, M^+) 636, 618, 608, 589; ir (CH_2Cl_2) 3520, 3450 (OH), 1685 (CO), 1625 cm^{-1} (C=C); pmr 1-H: 7.60 (m); 14-H: 4.8 (s, br.); 7-H: 4.75 (d, J=10 Hz); 20-H_2: 4.13±0.28 (J_{AB}=14 Hz); 5-H: 3.9 (s); 10-H: 2.9 (m); 19-H_3: 1.80 ppm.

Acetylation of compounds **20,21**, *affording* **24,25**.—Of compounds **20,21**, 25 mg was acetylated upon treatment with pyridine (2 ml) and Ac_2O (1 ml) for 15 h. After separation in petroleum ether-EtOAc (1:1), 24 mg of the acetylation products **24,25** was obtained [Rf: 0.20 in petroleum ether-EtOAc (2:1)]; ms *m/z*

738/740 (M^+) and 710/712 (M^+); ir (CH_2Cl_2) 3540 (OH), 1740, 1705 (CO), 1635 cm^{-1} (C=C); pmr 1-H: 7.55 (m); 5-H: 5.13 (s); 20-H_2: 4.85±0.06 (J_{AB}=12 Hz); 14-H: 4.78 (d, J=2-3 Hz); 7-H: 4.81 (d, J=10 Hz); 10-H: 3.10 (m); 11-H: 2.65 (m); 8-H: 2.34 (dd, J_1=3 Hz, J_2=10 Hz); 19-H_3: 1.80 (m); 18-H_3: 1.08 (d, J=7 Hz); 16-H_3, 17-H_3: appr. 0.95 ppm, (2s), OH (exchangeable with D_2O): 3.60 and 3.02 ppm, 2 acetyl: 2.13 and 2.20 ppm; decoupling experiments prove sequences: 7-H, 8-H, 14-H; 11-H, 18-H_3; 10-H, 1-H, 19-H_3; 15-H (1.85 ppm), 16-H_3, 17-H_3.

Compounds **22,23**: ms *m/z* 654/656 (intensity ratio 3:1, M^+-H_2O) and 626/628 (intensity ratio 3:1, M^+-H_2O), 636/638, 618, 611, 600, 583, 399, 380, 362, 345, 327, 319, 301, 290; ir (CH_2Cl_2) 3520, 3420 (OH), 1725, 1683 (CO), 1625 cm^{-1} (C=C); pmr 1-H: 7.71 (m), 14-H: 5.4 (d, J=2.5 Hz); 7-H: 4.42 (s); 5-H: 4.21 (s); 20-H_2: 4.02±0.06, (J_{AB}=12 Hz); 10-H: 3.68 (m); 8-H: 3.48 (d, J=2.5 Hz); CH_2-CO: 2.45 (t); 19-H_3: 1.84 (m); OH (exchangeable with D_2O): 5.04, 4.62 and 3.58 ppm; decoupling experiments prove sequences: 10-H, 1-H, 19-H_3, and 14-H, 8-H.

Acetylation of compounds **22,23**, *affording* **26,27**.—9 Mg of compounds **22,23** was acetylated upon treatment with pyridine (1 ml) and Ac_2O (0.5 ml). The reaction was stopped after 15 h, and the acetylation products were purified in petroleum ether-EtOAc (2:1) to yield 8 mg of products **26,27** (Rf: 0.27); ms *m/z* 738/740 (M^+), 710/712 (M^+); pmr characteristic differences to the spectrum of compounds **22,23**: 5-H: 5.16 (s); 20-H_2: 4.35±0.37 (J_{AB}=12 Hz); 10-H: 3.83 (m); 2 acetyl: 2.28 and 2.22 ppm.

RESULTS

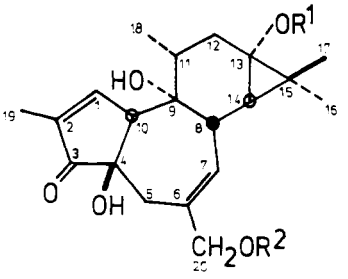
ISOLATION OF CRYPTIC FACTOR GROUPS, FACTOR GROUPS, AND FACTORS.—Treatment of a methanolic preparation of the latex of *H. mancinella* with Me_2CO yields an irritant extract. By O'Keeffe-distribution, the practically nonirritant hydrophobic fraction is separated from the highly irritant hydrophilic fraction, the former representing more than 90% of the Me_2CO extract. (See Scheme 1.)

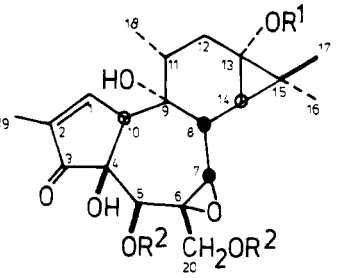
The major portion of the hydrophobic fraction consists of irritant inactive triterpenes staining bluish-red on tlc with vanillin-H_2SO_4, as revealed by column chromatography and/or preparative tlc. Furthermore, three different but tlc-uniform groups of compounds may be separated, staining brownish or grey to black. They exhibit very weak (M'_z) or practically no irritant activity (M'_x and M'_y). However, they may be activated upon treatment with sodium methoxide in MeOH, affording the irritant factor groups M_x, M_y, and M_z, respectively. A similar activation of irritant inactive compounds to irritant factors was shown for the first time with material contained in the hydrophobic fraction of croton oil, the seed oil of *Croton tiglium* L. (14) and later on found also in many other Euphorbiaceae (11). The inactive compounds were classified generally as "cryptic irritants" or "cryptic factors" (11,14). In analogy, the groups of compounds isolated from the hydrophobic fraction are named cryptic factor groups M'_x, M'_y and M'_z. M'_x is isolated in a hundred fold higher yield than that of M'_y or M'_z.

Factor group M_x is also obtained from the hydrophilic fraction; however, it is in a much lower yield compared with the amount of M'_x present in the hydrophobic fraction. Moreover, preparative tlc of the hydrophilic fraction affords factor M_3 and an inactive fraction that was not further investigated.

CHARACTERIZATION OF CRYPTIC FACTOR GROUPS, FACTOR GROUPS AND FACTORS.—*Tigliane derivatives*.—The pmr data of the cryptic factor group M'_z are indicative for a mixture of 13,20-diesters of 12-deoxyphorbol as isolated, for example, from *Euphorbia triangularis* (18) (Figure 1). Whereas the 13,20-diesters isolated from that source represent 20-acetates with relatively short-chain carboxylic acids in position 13 of 12-deoxyphorbol, detailed analyses of mass and uv spectral data of M'_z indicate presence of mixtures of saturated and unsaturated long-chain fatty acid esters. Indeed, reaction of factor group M'_z with sodium methoxide affords a mixture of saturated, even-numbered fatty acid methyl esters ranging from C_{16} to C_{26} (plus one unidentified compound) as analyzed by glc together with a mixture of two 12-deoxyphorbol-13-monoesters (factor group M_z, Scheme 1), not separable by tlc (silica gel). The chemical shift of 20-H_2 in the pmr spectrum of M_z (4.0 ppm) indicates a free primary hydroxyl group at

C-20, whereas signals for olefinic protons, as well as the uv data, prove the presence of unsaturated acid moieties. The parent ions in the mass spectrum (*m/z* 580 and 554) together with the fragment ions at *m/z* 233 and 207 indicative for acyl cations suggest the presence of a mixture of 12-deoxyphorbol-13-tetradeca-2,4-dienoate and 12-deoxyphorbol-13-hexadeca-2,4,6-trienoate (**1**,**2**, factor group M_z). Besides the fatty acid methyl esters and the factor group M_z, the transesterification reaction of M'_z yields a parent alcohol which, upon acetylation, forms a diacetate; its spectral data are identical with those of authentic 12-deoxyphorbol-13,20-diacetate (**3**), (18). From the reaction products and spectral data thereof, it may be concluded that the cryptic factor group M'_z consists of a mixture of 20-hexadecanoate, 20-octadecanoate, 20-eicosanoate, 20-docosanoate, 20-tetracosanoate, and 20-hexacosanoate of **1**,**2**.





M'_z: R^1=CO(CH=$CH_{2,3}$)$(CH_2)_8$ CH_3
R^2=CO$(CH_2)_n CH_3$
n=14,16,18,20,22,24

1,**2** (M_z): R^1=CO(CH=CH)$_{2,3}CH_3$, R^2=H

3: R^1=R^2=$COCH_3$

4 (M_3): R^1=CO(CH=CH)$_3(CH_2)_8CH_3$, R^2=H

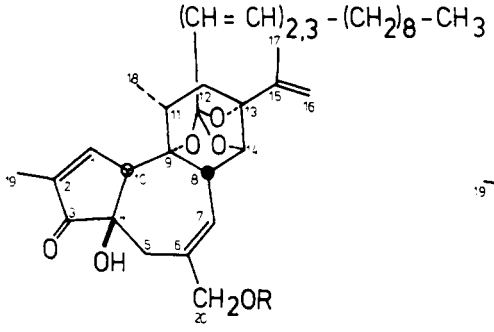
5: R^1=CO(CH=CH)$_3(CH_2)_8CH_3$, R^2=$COCH_3$

FIGURE 1. Structures of tigliane derivatives represented by factor M_3, factor group M_z, and cryptic factor group M'_z.

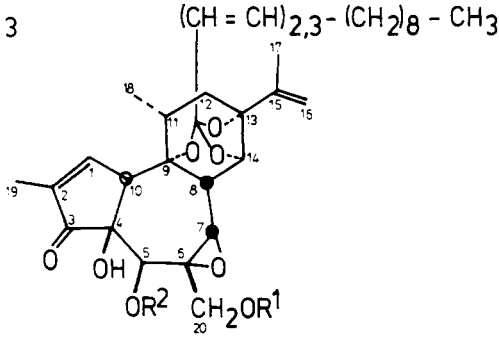
Another tigliane-type diterpene ester is factor M_3. A preliminary note of structure elucidation was reported (17). Spectral data suggest the presence of the hexadeca-2,4,6-trienoic acid ester **4**. Pmr data, including decoupling experiments, indicate functional groups as present in some daphnane derivatives, *e.g.*, huratoxin (19), simplexin (20), and baliospermin (21). Thus, the parent of factor M_3 most likely represents the 12-deoxy-5β-hydroxyphorbol-6α,7α-oxide. Acetylation of factor M_3 affords the 5,20-diacetate **5** as indicated by the downfield shifts of signals for protons 5-H (1.3 ppm) and 20-H_2 (0.35 ppm). From the acetylation reaction, the position of the ester moiety at the hydroxy group OH-13 may be deduced because a free cyclopropanol group would be esterified under the reaction conditions applied. For the new factor M_3, the trivial name mancinellin was suggested (17).

Daphnane derivatives.—Mass spectral analysis of the cryptic factor group M'_y suggests again the presence of mixtures of long-chain fatty acid esters, and uv and pmr spectra indicate the presence of unsaturated carboxylic acid esters. (See Figure 2 for daphnane derivatives). Furthermore, the pmr spectrum exhibits characteristic signals as present in the spectra of 12-deoxyphorbol-13,20-diesters (1-H, 8-H, 10-H, 19-H_3, 20-H_2) as well as signals characteristic for daphnane derivatives with an isopropenyl group at C-13 instead of a cyclopropane ring and a 9,13,14-orthoester function (14-H, 16-H_2, and 17-H_3). These data are in good agreement with those obtained for resiniferonol-9,13,14-orthoesters. Resiniferonol represents the parent of the highly irritant "resiniferatoxin," isolated from *Euphorbia resinifera* and *Euphorbia unispina*, (22) and of "tinyatoxin" from *Euphorbia poisonii* (23). Transesterification of M'_y with sodium methoxide affords—in analogy to cryptic factor group M'_z—a mixture of saturated

fatty acid methyl esters indicating even-numbered acids from C_{16} to C_{26} and probably also one odd-numbered acid (C_{15}) as analyzed by glc. Furthermore, the factor group M_y is obtained. Mass spectral analysis indicates the presence of the two unsaturated orthoesters **6,7** of resiniferonol, with similar fragment ions at *m/z* 207 and 233 as in the spectrum of the 12-deoxyphorbol-13-esters **1,2**. Compared to the pmr spectrum of M'_y, in the pmr spectrum of factor group M_y an upfield shift of the signal for 20-H_2 from 4.55 to 4.09 ppm is apparent, and the integration of the singlet at 1.28 ppm corresponding in the spectrum of M'_y to approximately 24 methylene groups only corresponds to 7-8 methylene groups. The absorption bands observed in the uv spectrum of M_y support the presence of two conjugated (λ max 232 nm) and three conjugated double bonds (λ max 259, 269, and 280 nm), the fine structure of the latter absorption band being characteristic for conjugated polyenes. These are compatible with unsaturated orthoester structures. Thus, factor group M_y most likely represents a mixture of resiniferonol-9,13,14-orthotetradeca-2,4-dienoate and resiniferonol-9,13,14-orthohexadeca-2,4,6-trienoate (**6,7**), and the cryptic factor group M'_y, a mixture of 20-pentadecanoate, 20-hexadecanoate, 20-octadecanoate, 20-eicosanoate, 20-docosanoate, 20-tetracosanaote, and 20-hexacosanoate of **6,7**.



M'_y: $R=CO(CH_2)_nCH_3$
$n=13,14,16,18,20,22,24$
6,7 (M_y): $R=H$



M'_x: $R^1=CO(CH_2)_nCH_3$, $R^2=H$
$n=14,16,18,20,22,24$
Acet. M'_x: $R^1=CO(CH_2)_nCH_3$, $R^2=COCH_3$
$n=14,16,18,20,22,24$
8,9 (M_x): $R^1=R^2=H$
10,11: $R^1=R^2=COCH_3$

FIGURE 2. Structures of daphnane derivatives represented by factor groups M_x and M_y and cryptic factor groups M'_x and M'_y.

Mass spectral analysis of the cryptic factor group M'_x also suggests the presence of a mixture of esters with similar long-chain fatty acid moieties such as those of M'_y and M'_z. Transesterification, according to the reactions carried out with M'_y and M'_z, affords again a mixture of fatty acid methyl esters ranging from C_{16} to C_{26} as analyzed by glc. Moreover, a factor group is obtained exhibiting identical spectral data as factor group M_x isolated from the hydrophilic fraction. Detailed analysis of spectral data [see also (17)] prove the presence of two esters of 5β-hydroxyresiniferonol-6α,7α-oxide, one being the 9,13,14-(tetradeca-2,4-dienoic acid)orthoester **8** (M^+ 584) identical with huratoxin (19), the other one representing the 9,13,14-(hexadeca-2,4,6-trienoic acid) orthoester **9** (M^+ 610). Again, the cryptic factor group M'_x represents a mixture of 20-hexadecanoate, 20-octadecanoate, 20-eicosanoate, 20-docosanoate, 20-tetracosanoate, and 20-hexacosanoate of **8,9**. Factor group M_x forms the 5,20-diacetates **10,11** upon acetylation (downfield shift of 5-H to 5.52 ppm and 20-H_2 to 4.12 ppm),

whereas M'_x reacts to 5-monoacetate (downfield shift of 5-H to 5.5 ppm). A preliminary note on structures of the cryptic factor group M'_x and factor group M_x was reported (17).

FURTHER CHEMICAL REACTIONS WITH FACTOR GROUP M_x.—Catalytic hydrogenation of factor group M_x using Pd/charcoal as catalyst affords a mixture of the compounds **12,13**. The uv spectrum indicates hydrogenation of all double bonds and the presence of one isolated carbonyl group with absorption at 305 nm. The absorption band in the ir spectrum at 1745 cm^{-1} is characteristic of a cyclopentanone group. Since a M^+-31 fragment is observed in the mass spectra of nearly all derivatives of resinioferonol-6α,7α-oxide, the fragment ions in the mass spectra of compounds **12,13** at *m/z* 589 and 561 most likely represent fragmentations of CH_2OH^+ from the assumed parent ions at *m/z* 620 and 592. Together with the pmr spectral data (signals for olefinic protons and for the allylic protons 10-H, 17-H_3, and 19-H_3 are absent), the structures **12,13** are deduced. (See Figure 3.)

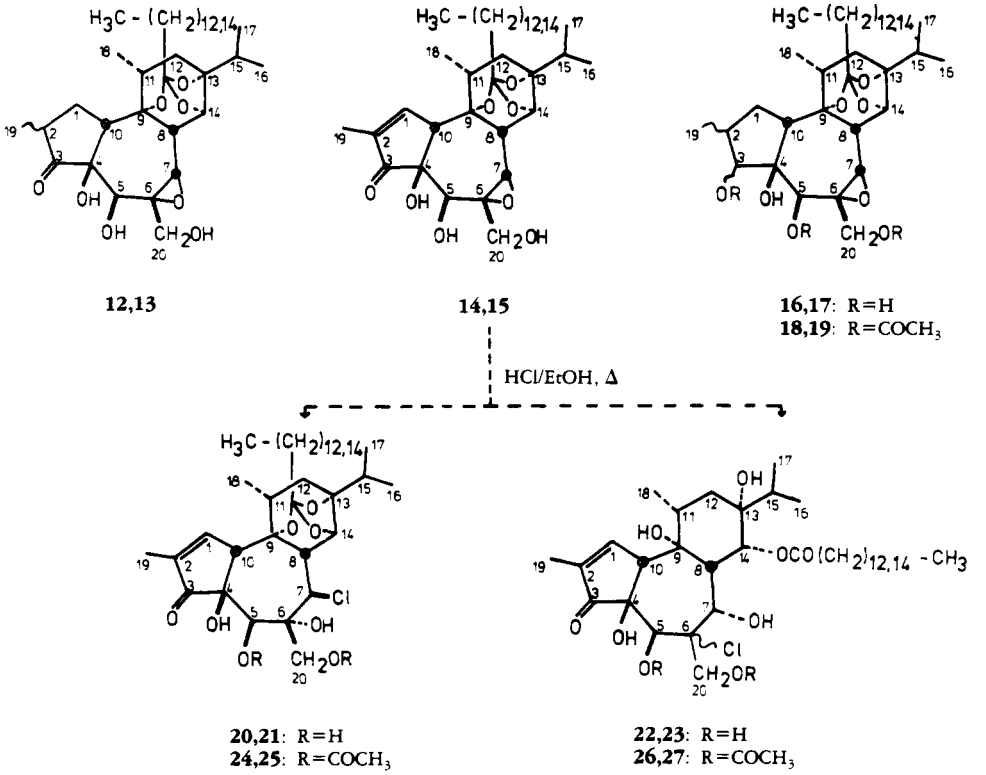


FIGURE 3. Structures of compounds **12-17** obtained upon hydrogenation of factor group M_x and derivatives **18,19** thereof and structure of compounds **20-23** obtained upon acidic hydrolysis of the hydrogenated compounds **14,15** and derivatives **24-27** thereof.

Using PtO_2 as a catalyst, hydrogenation of M_x yields the two groups of compounds, **14,15** and **16,17**, each not separable by chromatographic means. The parent ions of **14,15** at *m/z* 618 and 590, as well as vibration bands at 1690 cm^{-1} and 1625 cm^{-1} in the ir spectrum and absorption bands at 243 nm and 334 nm in the uv spectrum, suggest that the cyclopentenone-ring was not reduced by the hydrogenation procedure. The structures of **14,15** correspond to those reported for hexahydrohuratoxin, obtained by Sakata *et al.* (24) upon hydrogenation of huratoxin with PtO_2 as a catalyst. In the mass spectrum, compounds **16,17** show fragment ions corresponding to molecular

ions at *m/z* 622 and 594. In the uv spectrum, no significant absorption is apparent, and the ir spectrum does not show carbonyl-stretching vibrations. Hence, it may be assumed that in **16,17**, compared to **12,13**, the carbonyl group is reduced. Indeed, in the pmr spectrum of **16,17** at 3.5 ppm, a doublet (J=9.5 Hz) is apparent, which is shifted downfield to 4.3 ppm upon acetylation. This might represent the additional proton geminal to the hydroxyl group at carbon atom 3. Decoupling experiments prove that irradiation at 1.8 ppm (2-H) changes the doublets at 4.3 ppm (3-H) and 0.9 ppm (19-H_3) to singlets. Thus, acetylation affords triacetates **18,19**, including OH-groups at carbon atoms 3,5 and 20.

The hydrogenated compounds **14,15** were hydrolyzed to two groups of compounds. The major group, **20,21**, showed a very similar pmr spectrum as reported for a bromohydrin obtained from diacetylhexahydrohuratoxin upon treatment with HBr in HOAc (24). Indeed, the mass spectrum (parent ions at 654/656 and 626/628) indicates opening of the epoxide to a chlorohydrin, and the ir spectrum exhibits only the absorption band for the cyclopentenone carbonyl group at 1685 cm^{-1}. Therefore, it may be concluded that the orthoester group was not hydrolyzed to a normal ester group. In the pmr spectrum, the signal of a proton at 4.75 ppm (7-H, d, J=10 Hz) indicates coupling of 7-H with 8-H (contrary to all daphnane-type factors with 6α,7α-oxide group) and therefore, as in the bromohydrin of hexahydrohuratoxin, the configuration of a 7β-chloro-6α-hydroxyhexahydrohuratoxin. This was also suggested by decoupling experiments of compounds **24,25** obtained after acetylation of **20,21**. Furthermore, the upfield shift of the signal for proton 10-H in the pmr spectra of both **20,21** and **24,25** to 2.9 and 3.1 ppm, respectively, demonstrates opening of the epoxide. The signal of 10-H appears in derivatives of resiniferonol-6α,7α-oxides at relatively low field (3.8 ppm) as a result of the stereochemical relationship of 10-H to the epoxide as proved by X-ray analysis of huratoxin (24).

The second group of compounds (**22,23**) is obtained in lower yield after acidic treatment of compounds **14,15**. In the mass spectrum, the ions with the highest *m/z* values appear at *m/z* 654/656 and 626/628; they correspond to those of the chlorohydrins **20,21**. However, an additional absorption band at 1725 cm^{-1} in the ir spectrum indicates cleavage of the orthoester moiety to a normal ester function. Indeed, in the pmr spectrum, the signal for proton 14-H is shifted downfield from 4.31 to 5.4 ppm, proving the presence of a 14-ester; also, a paramagnetic shift of the signal for 8-H (0.7 ppm) is in accordance with this conclusion. The ions observed in the mass spectrum, therefore, represent M^+-H_2O fragments. Since, in the pmr spectrum of **22,23**, the signal of 7-H appears as singlet at 4.42 ppm (downfield shift of 0.97 ppm) and for 8-H as the characteristic doublet coupling only with 14-H (J=2.5 Hz), it is assumed that the formation of the chlorohydrine occurred in the same manner as observed for the formation of the "polyol" obtained from huratoxin (24) with the chlorine atom entering at C-6 and the hydroxyl group at C-7 resulting from the epoxide with retention of the configuration. Obviously, the hydroxyl group in 7α-position still relates sterically to 10-H since the signal for this proton appears at a similar magnetic field as in the 6α,7α-epoxyderivatives. Acetylation of compounds **22,23** affords the diacetates **26,27**. Since only the signals for protons 20-H_2 and 5-H are shifted downfield, the secondary hydroxyl group OH-7 obviously did not react with Ac_2O in pyridine, as already reported for the "polyol" (24). Reaction of compounds **22,23** with various concentrations of sodium methoxide at various temperatures did not afford defined reaction products. Even careful treatment with 5 x 10^{-4} M sodium methoxide yielded uv inactive material which was not further characterized.

BIOLOGICAL ACTIVITIES.—*Irritant activity on the mouse ear.*—For ID_{50}^{24} values of

factors, factor groups, cryptic factor groups and synthetic compounds derived from factor group M_x, see Table 1.

TABLE 1. Irritant Doses 50 (ID_{50}^{24}) of Factors, Factor Groups, and Synthetic Derivatives of Factor Group M_x, Compared to the ID_{50}^{24} of TPA, as well as ID_{50}^{24} of Cryptic Factor Groups Compared to the ID_{50} of TPA-20-Tetradecanoate

Factors, factor groups and synthetic compounds	ID_{50}^{24} (μg/ear)	Corresponding cryptic factors/ factor groups	ID_{50}^{24} (μg/ear)
4 (M_3)	0.15		
1,2 (M_z)	0.17	M'_z	0.4
6,7 (M_y)	0.07	M'_y	> 50
8,9 (M_x)	0.02	M'_x	>100
12,13	0.028	—	—
14,15	0.035	—	—
16,17	0.71	—	—
20,21	2.2	—	—
22,23	>50	—	—
TPA	0.01	TPA-20-tetra-decanoate	0.64 (26)

Tumor-promoting activity on the back skin of mice.—Factor M_3, factor group M_x, and cryptic factor group M'_x were assayed for tumor-promoting activity. Due to the relatively low amounts of M'_y and M'_z obtained from the hydrophobic fraction (see Scheme 1), the corresponding factor groups M_y and M_z were not tested. Factor M_3 proved to be not tumor-promoting in a dose of p=20 nmoles/application (12.2 μg/application), and the cryptic factor group M'_x was inactive in a dose of p=60 μg/application (see Table 2). However, factor group M_x exhibited high tumor rates and tumor yields in the same dose as TPA (5 nmoles/application) and with one-fourth of the dose of huratoxin (see Table 2 and Figure 4). In another dose p (10 nmoles-application), factor group M_x produced comparable tumor rates but higher tumor yields than TPA; in a dose of p=2.5 nmoles/application, the tumor rates and yields were lower. In the test for solitary carcinogenity, *i.e.*, without initiation, factor group M_x did not exhibit any activity within 48 weeks of application.

In all experiments, the survival rates were higher than 90% after 20 weeks and higher than 85% after 30 weeks. In the experiments using higher promoting doses p the survival rates dropped and ranged at the end of the experiments between 50% and 60%.

In all tumor-promoting experiments with factor group M_x and TPA and huratoxin as standards among numerous benign tumors, malignant tumors were also observed. With the higher promoting doses p of factor group M_x, the number of malignant tumors was twice as high as the number observed with the standards TPA and huratoxin.

DISCUSSION

FACTORS AND MATERIALS IN THE LATEX OF *HIPPOMANE MANCINELLA*.—The hydrophobic fraction of the latex of *H. mancinella* contains complex mixtures of esters of the tigliane (M'_z) as well as of the daphnane type (M'_x and M'_y). These share certain structural similarities. In the case of the parent alcohol, 12-deoxyphorbol, two unsaturated fatty acids, *i.e.*, tetradeca-2,4-dienoic acid and hexadeca-2,4,6-trienoic acid, are attached to the hydroxyl group at C-13; in the case of the parent alcohols, resiniferonol and 5β-hydroxyresiniferonol-6α,7α-oxide, the same acids are attached to the three hy-

TABLE 2. Results of the Standardized Assays for Tumor-Promoting Activity on the Back Skin of NMRI-Mice of Materials and Factors Isolated from the Latex of *H. mancinella*

Applications[a]			Tumor Rates[b]		Tumor Yields[c]		Survival Rate	Histologic Diagnosis[d]	
								tumors in treated area	
factor/ factor group	single dose p µg (nmol)	duration of application in weeks	12 weeks	24 weeks	12 weeks	24 weeks	at 24 weeks (%)	total/mice investigated histologically	total malignant tumors
TPA	3.1 (5)	48	3/27	20/27	4/27	112/27	96	102/18	3 PEC
Huratoxin	11.7 (20)	48	15/28	23/27	58/28	76/27	96	56/21	3 PEC, 1 SPSA
M_x	6 (10)	48	21/28	23/24	95/28	164/24	86	181/25	6 PEC, 1 FISA
M_x	3 (5)	42	12/28	21/27	28/28	111/27	96	95/23	6 PEC
M_x	1.5 (2.5)	42	0/28	10/27	0/28	36/27	96	66/14	1 PEC
M_x	12.2 (20)	34	0/28	1/28	0/28	1/28	100	1/1	0
M'_x	60	48	0/28	0/28	0/28	0/28	100	—	—
M_x[e]	6 (10)	48	0/27	0/26	0/27	0/26	93	1/1	0

[a]Initiation: i=0.1 µmole of DMBA (7,12-dimethylbenz(a)anthracene); promotion: twice weekly doses p of the promoter.
[b]Average tumor rate: numbers of tumor bearing animals/number of survivors of the group in percent.
[c]Average tumor yield: number of tumors/number of survivors of the group.
[d]PEC: squamous cell carcinoma, SPSA: spindle cell sarcoma, FISA: fibrous cell carcinoma.
[e]Without initiation; following the 19th week, two tumors were observed, which disappeared after the 22nd week; following the 37th week, one tumor was registered.

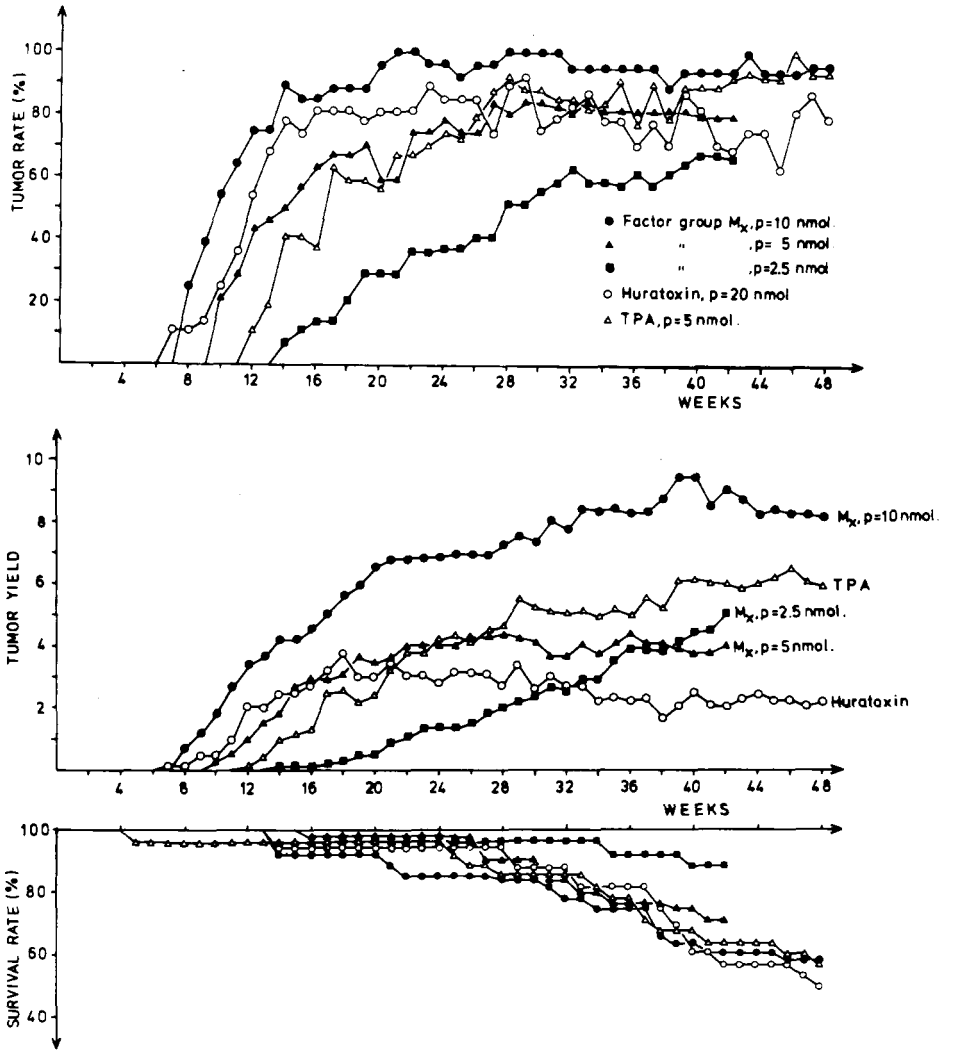


FIGURE 4. Tumor rates and tumor yields (for definition, see footnote to Table 2) of factor group M_x at various doses as compared to those of huratoxin and TPA (12-O-tetradecanoylphorbol-13-acetate).

droxyl groups at C-9, -13, and -14, forming orthoesters. Furthermore, the primary hydroxyl group at C-20 is esterified essentially with the even-numbered, unbranched fatty acids from C_{16} to C_{26}. Hence, the structural type of cryptic irritants and cocarcinogens (11) is represented in all of these esters. This may be demonstrated by exposure of the cryptic irritants to mild acidic or else basic conditions, which affords the (more) active factor groups M_x, M_y and M_z (see Scheme 1). All cryptic factor groups could not be separated by conventional tlc methods, but initial attempts to achieve a further resolution into individual compounds by hplc demonstrated that this approach is feasible in principle.

The hydrophilic fraction of the latex contains as a major constituent factor group M_x, consisting of the two unsaturated 9,13,14-fatty acid orthoesters **8,9** with 5β-hydroxyresiniferonol-6α,7α-oxide as the parent alcohol. The structure of one of these esters is identical with that of huratoxin, the toxic constituent of the sap of *Hura crepitans*; the other one is as yet unknown. Moreover, a factor M_3 was isolated representing a 13-

(hexadeca-2,4,6-trienoic acid) ester of the parent alcohol 12-deoxy-5β-hydroxyphorbol-6α,7α-oxide.

The presence of all these factors and cryptic factors in the latex of *H. mancinella* is also interesting from the biogenetic point of view. The oxygenation of the two tigliane derivatives, 12-deoxyphorbol (M'_z) and 12-deoxy-5β-hydroxyphorbol-6α,7α-oxide (M_3), is also present in the daphnane derivatives, resiniferonol (M'_y), and 5β-hydroxyresiniferonol-6α,7α-oxide (M'_x). Thus, it appears likely that the biogenetic formation of the daphnane derivatives occurs via tigliane-type precursors by the opening of the cyclopropanol ring [see also (12)].

SOME ASPECTS ON STRUCTURE ACTIVITY RELATIONSHIP.—*Irritant activity.*—Factor group M_x represents the most active irritant principle present in the latex of *H. mancinella*, exhibiting an ID_{50} comparable to that of TPA. Factor M_3 only shows moderate irritant activity. Cryptic irritants esterified in the 20-position with long-chain fatty acids exhibit low irritant activity (M'_z, tigliane type) or are practically nonirritant (M'_x and M'_y, daphnane type). (See Table 1.)

The only exception from the structural type of cryptic irritants in the daphnane series seems to be represented by resiniferatoxin and tyniatoxin, the extremely irritant factors of *E. resinifera*, *E. poisonii*, and *E. unispina* (22,23,25). Resiniferatoxin carries, *e.g.*, in position 20, a (4-hydroxy-3-methoxyphenyl)acetic acid group. However, resiniferatoxin already exhibits its maximal irritant activity 2-5 h after application, whereas all irritant esters isolated from *H. mancinella* show their highest irritant activity 24 h after application.

From the products of the catalytic hydrogenation, it may be concluded that neither the 1,2-double bond (**12,13**) nor the 15,16-double bond (**12,13** and **14,15**) is necessary for irritant activity; also, the double bonds in the acid moieties are not contributing to the irritant activity. It is interesting to note that in the case of the phorbol esters (*e.g.*, TPA), the 1,2-double bond is essential for the biological activity (26). On the other hand, 1α-alkyldaphnane derivatives, lacking the double bond between C-atoms 1 and 2, exhibit high irritant (and tumor-promoting) activity (27).

Reduction of the carbonyl group at C-3 of compounds **12,13**, where all double bonds are hydrogenated, leads to a 20-fold decrease of the irritant activity (compounds **16,17**). However, hydrolytic opening of the 6α,7α-epoxide by addition of a chlorine atom at C-7 (**20,21**) reduces the activity by a factor of nearly 100. Opening of the orthoester moiety to a 14-tetradecanoate/14-hexadecanoate of 6-chloro-15,16-dihydro-5,7-dihydroxyresiniferonol (**22,23**) leads to a complete loss of the irritant activity. A similar effect was observed with the formation of resiniferatoxin from proresiniferatoxin, where the generation of an orthoester from a 14-ester increases the irritant activity by a factor of 1000 (22).

Tumor-promoting activity.—As may be seen in a series of dose-response experiments, factor group M_x exhibits tumor-promoting activity comparable to that of the strong tumor-promoter TPA, whereas M'_x is inactive as a promoter. Thus, the cryptic irritants present in M'_x proved also to be cryptic promoters. They may easily be converted to free promoters either by chemical transesterifications or by enzymatic reactions (11). The free promoters do not show any solitary carcinogenic effect, but with higher tumor-promoting doses, a relatively high number of malignant tumors were observed. Hence, due to the presence of free promoters and also to the high amount of cryptic promoters (32% of the acetone extract of the latex), intensive or chronic contact with the plant *H. mancinella* should be avoided.

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