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Cocarcinogens of the diterpene ester type from *Croton flavens* L. and esophageal cancer in Curaçao

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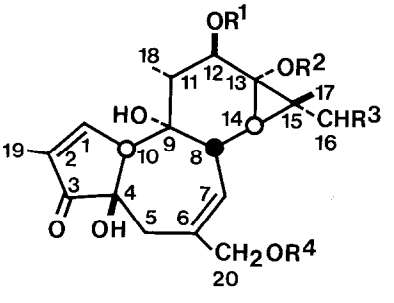
Summary. From the roots of *Croton flavens* L., 3 highly irritant and tumor promoting Croton factors $\mathbf{F}_1$–$\mathbf{F}_3$ and the corresponding 3 cryptic Croton factors $\mathbf{F}'_1$–$\mathbf{F}'_3$ were isolated and characterized as novel esters of 16-hydroxy- and 4-deoxy-16-hydroxyphorbol, respectively. These findings suggest that tumor promoters of the phorbol ester type, ingested through the widespread and frequent use of *Croton flavens* according to local habits, may be causally related to the well recognized high rate of esophageal cancer on Curaçao.

On the island of Curaçao and in other areas of Central America, the roots of *Croton flavens* L. are chewed for their 'stimulating' effect, and the fresh young leaves and tips of twigs are used to prepare 'bush tea', a popular beverage and folk remedy. To relieve oral inflammation, the leaves of this plant are held in the mouth; also they serve as an insect repellent and as a detergent[1]. It was suspected that the widespread use of this plant might be causally related to the exceptionally high rate of esophageal cancer on Curaçao[2]. In assays of extracts of *C. flavens* for solitary carcinogenic[3,4] activity by s.c. injections (rats), and by application into the upper gastrointestinal tract (mice and hamsters), negative results were obtained[5,6]. Therefore, and because of the botanical relationship of *Croton flavens* L. to *Croton tiglium* L., wellknown source of the highly irritant and cocarcinogenic (i.e. tumor promoting[3,4]) croton oil factors[7], we investigated the plant for its possible contents of irritants and cocarcinogens.

Croton factors from roots. The methanol extract from roots of *Croton flavens* L. is an irritant as measured by the irritant dose 50 (ID_{50}) on the ear of mice (for details of the assay, Hecker et al.[7]). By solvent extraction and subsequent O'Keeffe distributions[7] in different solvent systems, essentially all the irritant activity of the extract is concentrated in the 'irritant fraction'. Multistage Craig distribution[7] of this fraction yields the Croton factors $\mathbf{F}_1$, $\mathbf{F}_2$, and the cryptic types (Hecker[4,7]) $\mathbf{F}'_1$, $\mathbf{F}'_2$ and $\mathbf{F}'_3$, all of remarkable irritant activity (see structural formulae and table).

Esters of 16-hydroxyphorbol (**1**). Croton factor $\mathbf{F}_1$: IR (KBr): 3408, 3340 (OH), 1750, 1730, 1715 (C=O), 1638 cm^{-1} (C=C); UV (CH_3OH): λ_{max}: 196, 231, 334 nm (ε_{max}: 12410, 5000, 130); NMR: δ_{TMS}, $CDCl_3$, 7.55, m, 1-H; 5.65, d, J = 5 Hz, 7-H; 5.38, d, J = 10 Hz, 12-H; 3.98, s, 20-H_2; 3.81, s, 16-H_2; 3,34, t, J = 5 Hz, 8-H; 3.18, m, 10-H; 2.38, m, 5-H_2, partially superimposed on 11-H; 2.28, m, 11-H, part. superimp. on 5-H_2; 1.76, m, 19-H_3; 0,88, d, J = 6 Hz, 18-H_3, part. superimp. on the methyl group of hexadecanoic acid; 2.09, s, OAc; 5.43, 4.11, 3.18, 2.2–2.5 (OH).

Croton factors $\mathbf{F}'_1$ and $\mathbf{F}'_3$: According to their NMR-spectra, $\mathbf{F}'_1$ and $\mathbf{F}'_3$ are esters of (**1**). Their molecular ions (table) are heavier than that of $\mathbf{F}_1$ and they differ in their NMR-spectra from $\mathbf{F}_1$ mainly in the position of the signal for 20-H_2. It is shifted to lower field: δ_{TMS}, $CDCl_3$, 4.47, s ($\mathbf{F}'_1$); 4.42, s ($\mathbf{F}'_3$), while the position of 16-H_2 remains practically unchanged: δ_{TMS}, $CDCl_3$, 3.81, s ($\mathbf{F}'_1$); 3.89, s ($\mathbf{F}'_3$). Further data: $\mathbf{F}'_1$: IR (CH_2Cl_2): 3560, 3405 (OH), 1745, 1735, 1725, 1715 (C=O), 1635 cm^{-1} (C=C); UV (CH_3OH): λ_{max}: 205 (sh), 232 (sh), 335 nm (ε_{max}: 11870, 4960, 110). $\mathbf{F}'_3$: IR (CH_2Cl_2): 3550, 3400 (OH), 1725, 1705 (C=O), 1625 cm^{-1} (C=C); UV (CH_3OH): λ_{max}: 196, 230 (sh) nm (ε_{max}: 12340, 4700).



1 $R^1 = R^2 = R^4$: H; R^3: HOH

$\mathbf{A}_1$ R^1: $CO(CH_2)_{12}CH_3$; R^2: $COCH_3$; R^3: H_2; R^4: H
$\mathbf{A}_4$ R^1: $CO(CH_2)_{14}CH_3$; R^2: $COCH_3$; R^3: H_2; R^4: H
$\mathbf{F}_1$ R^1: $CO(CH_2)_{14}CH_3$; R^2: $COCH_3$; R^3: HOH; R^4: H
$\mathbf{F}'_1$ R^1: $CO(CH_2)_{14}CH_3$; R^2: $COCH_3$; R^3: HOH; R^4: $CO(CH_2)_8CH_3$
$\mathbf{F}_3$ R^1: $CO(CH_2)_{12}CH_3$; R^2: $COCH_3$; R^3: HOH; R^4: H
$\mathbf{F}'_3$ R^1: $CO(CH_2)_{12}CH_3$; R^2: $COCH_3$; R^3: HOH; R^4: $CO(CH_2)_8CH_3$

2 R^1: $CO(CH_2)_{14}CH_3$; R^2: $COCH_3$; R^3: O; R^4: $CO(CH_2)_8CH_3$
3 R^1: $CO(CH_2)_{14}CH_3$; $R^2 = R^4$: H; R^3: HOH
4 $R^1 = R^2$: H; R^3: HOH; R^4: $CO(CH_2)_8CH_3$

By treatment of F_1' with CrO_3/pyridine/dichloromethane[8] (with pyridine in excess) the aldehyde **2** is obtained (yield: 63.4%), 16-H: δ_{TMS}, $CDCl_3$, 9.67, s. The chemical shift of 7-H in the aldehyde: δ_{TMS}, $CDCl_3$, 5.67, d, remains practically unchanged as compared to F_1': δ_{TMS}, $CDCl_3$, 5.65, d, 7-H, and is quite different from that in 12-O-tetradecanoyl-20-deoxy-20-oxophorbol-13-acetate[9]: δ_{TMS}, $CDCl_3$, 6.78, d. This proves that 16-OH of F_1' was oxidized indicating that F_1' is a 20-ester of F_1.

By acid catalyzed transesterification[7] of F_1' and F_3', respectively, with 0.2% $HClO_4/CH_3OH$ (48 h, room temp.) crystalline main products are obtained, differing in mol. ions and m.p. and showing practically identical R_f and NMR-data. They are F_1 (from F_1') as identified by spectral comparison, TLC, and mixed melting point and 12-O-tetradecanoyl-16-hydroxyphorbol-13-acetate (from F_3'), which was not isolated but is called croton factor F_3 by analogy (table).

Base catalyzed transesterification of the factors F_1, F_1' and F_3' by means of 10^{-2} M $NaOCH_3/CH_3OH$ (5 h, room temp.) yields **1** and a mixture of the methylester of the acid moieties, respectively. Their long chain methyl ester components are resolved by gaschromatography and identified as methylhexadecanoate (from F_1), methylhexadecanoate and methyldecanoate (from F_1'), methyltetradecanoate and methyldecanoate (from F_3'). Further evidence for the presence of these acids are the fragment ions 404 ((M^+) 660–256 (hexadecanoic acid)) for F_1, 642 and 386 ((M^+) 814–172 (decanoic acid) –256) for F_1' and 614 and 386 ((M^+) 786–172–228 (tetradecanoic acid)) for F_3'. The presence of acetate is demonstrated by the fragment ions 600 ((M^+) 660–60) for F_1, 754 ((M^+) 814–60) for F_1' and 726 ((M^+) 786–60) for F_3'.

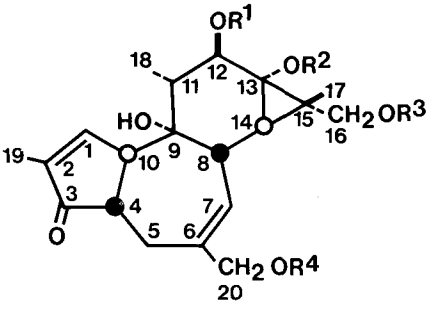
Using $5 \cdot 10^{-3}$ M $NaOCH_3/CH_3OH$ at about −20 °C F_1 (60 h) and F_1' (48 h) are converted to monoesters, F_1 to 16-hydroxyphorbol-12-hexadecanoate (**3**), ($M^+ - H_2O$) 600 and F_1' to 16-hydroxyphorbol-20-decanoate (**4**), (M^+) 534. NMR: **3**: δ_{TMS}, d_5-pyridine, 6.18, d, J = 10 Hz, 12-H; **4**: δ_{TMS}, d_5-pyridine, 4.68, s, 20-H_2.

The formation of the 20-decanoate (**4**), as one of the products of hydrolysis of F_1', is surprizing because in case of 12,13,20-triacylates of phorbol[7] 12-acylates are obtained under mild basic conditions[10]. However, the free hydroxyl function at C-16 in 16-hydroxyphorbol-12,13,20-triesters might activate the transesterification of the ester group at C-12β in a similar manner as the 9α-hydroxyl activates 13α-ester groups in phorbol[10,11]. Apparently, and at variance with phorbol-12,13,20-triesters, in 16-hydroxyphorbolesters the ester function at C-20 is not the most reactive one to be hydrolyzed.

These results altogether prove that F_1 is the 12-hexadecanoate-13-acetate of **1** and that F_1' and F_3' are 20-decanoates of F_1 and F_3. F_1 is identical with an authentic specimen of 12-O-hexadecanoyl-16-hydroxyphorbol-13-acetate isolated recently by Okuda et al.[12] from fruitshells of *Aleurites fordii* Hemsl. (mixed m.p.[13] 177–178 °C). These structures (the irritants and cocarcinogens (F_1, F_3) and their cryptic forms (F_1', F_3')) are reminiscent of those occurring as phorbolesters in croton oil[7]. In particular, croton factors F_1 and F_3 may be considered 16-hydroxylated derivatives of the croton oil factors A_4 and A_1 (TPA), respectively.

Esters of 4-deoxy-16-hydroxyphorbol (**5**). Croton factors F_2 and F_2': in comparison to F_1 and F_1', respectively, they show m/e of the mol. ions 16 units lower (table), but similar fragmentation patterns. Treatment with 10^{-2} M $NaOCH_3/CH_3OH$ and subsequent gaschromatography of the methylester fraction yields methylhexadecanoate from F_2 and methylhexadecanoate together with methyldecanoate from F_2'.

F_2: IR (CH_2Cl_2): 3610, 3410 (OH), 1745, 1735, 1710 (C=O), 1637 cm^{-1} (C=C); UV (CH_3OH): λ_{max}: 205 (sh), 226, 310 (sh) nm (ε_{max}: 11170, 6460, 170); NMR, δ_{TMS}, 7.55, m, 1-H; 5.52, d, J = 5 Hz, 7-H, partially superimposed on 12-H; 5.4, d, J = 10 Hz, 12-H, part. superimp. on 7-H; 3.95, s, 20-H_2; 3.75, s, 16-H_2; 3.22, m, 10-H; 2.9–1.9, m, 4-H and



5 4-deoxy-16-hydroxyphorbol, $R^1 = R^2 = R^3 = R^4$: H
F_2 R^1: $CO(CH_2)_{14}CH_3$; R^2: $COCH_3$; $R^3 = R^4$: H
F_2' R^1: $CO(CH_2)_{14}CH_3$; R^2: $COCH_3$; R^3: H; R^4: $CO(CH_2)_8CH_3$
7 R^1: $CO(CH_2)_{14}CH_3$; $R^2 = R^3 = R^4$: H
9 $R^1 = R^2 = R^3$: H; R^4: $CO(CH_2)_8CH_3$
11 $R^1 = R^2 = R^3 = R^4$: $COCH_3$

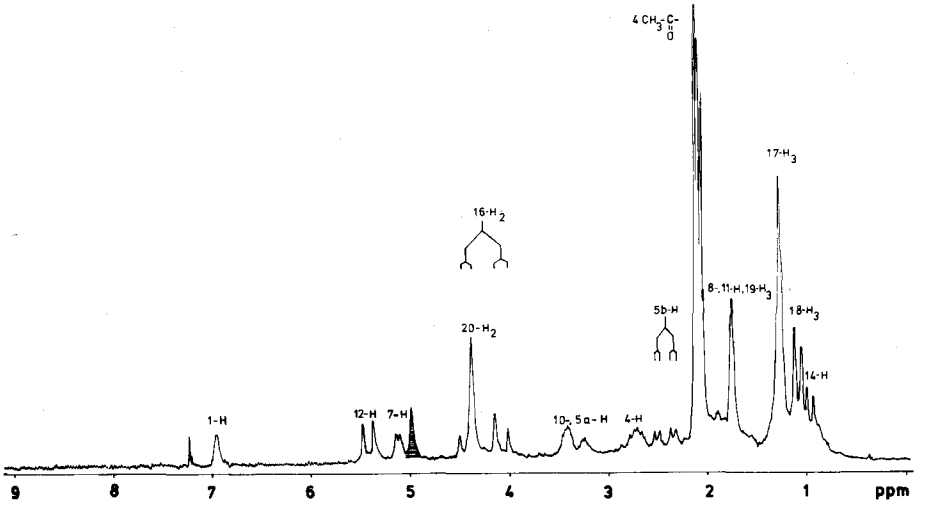


Chart 1. 90-MHz-spectrum of 4-deoxy-16-hydroxy-4α-phorbol-12,13,16,20-tetraacetate (**6**).

5-H_2, part. superimp. on 8-H; 2.52, t, J=5 Hz, 8-H, superimp. on 5-H_2; 1.73, m, 19-H_3, part. superimp. on 11-H; 1.62, m, 11-H, part. superimp. on 19-H_3; 0.87, d, J=6 Hz, 18-H_3, part. superimp. on the methyl group of hexadecanoic acid; 2.1, OAc; 5.49, 3.25 and 2.2–2.5 (OH).

The main difference in the NMR-spectrum of $\mathbf{F}_2'$ as compared to $\mathbf{F}_2$ is the chemical shift of 20-H_2: δ_{TMS}, $CDCl_3$, 4.45, s ($\mathbf{F}_2'$). $\mathbf{F}_2'$: IR (CH_2Cl_2): 3610 (OH), 1745, 1735, 1710 (C=O), 1638 cm^{-1} (C=C); UV (CH_3OH) λ_{max}: 198, 226, 310 nm (ε_{max}: 11080, 5460, 250). Acid catalyzed transesterification[7] of $\mathbf{F}_2'$ (0.2% $HClO_4/CH_3OH$, 48 h, room temp.) yields $\mathbf{F}_2$ (TLC and spectral comparison). Hence $\mathbf{F}_2'$ most probably is the 20-decanoate of $\mathbf{F}_2$.

By transesterification of $\mathbf{F}_2$ and $\mathbf{F}_2'$ with 10^{-2} M $NaOCH_3/CH_3OH$ (5 h, room temp.) the novel parent alcohol 4-deoxy-16-hydroxy-4α-phorbol, a positional isomer of 4α-phorbol[7,14] is obtained (see **5** with 4-H in α- instead of β-position). Acetylation with pyridine/acetic anhydride results in 4-deoxy-16-hydroxy-4α-phorbol-12,13,16,20-tetraacetate (**6**): m.p. 191–192 °C, decomp.: (M^+) 532; IR (KBr): 3425 (OH), 1755, 1745, 1725, 1710 (C=O), 1635 cm^{-1} (C=C); UV (CH_3OH): λ_{max}: 194, 231 nm (ε_{max}: 11880, 6790). The NMR-spectrum (chart 1) shows a shift of the signals of 1-H, 7-H, and 8-H (δ_{TMS}, $CDCl_3$, 6.99, m; 5.15, d, J=5 Hz, and 1.9, m) to higher field and wider splitting of 5-H_2. By an INDOR experiment the connection between the A- (δ_{TMS}, $CDCl_3$, 3.35) and the B-part (δ_{TMS}, $CDCl_3$, 2.45) and their positions in the spectrum were revealed. The A part of 5-H_2 is found in the proximity of 10-H (δ_{TMS}, $CDCl_3$, 3.44) and is partially superimposed on 10-H. Similar findings were reported for 4α-phorbol- and 4-deoxy-4α-phorbol-12,13,20-triacetate[14]. Irradiation at 5a-H converts the signal of 4-H (δ_{TMS}, $CDCl_3$, 2.72, m) to a triplet (J=5 Hz) because of the remaining coupling with 5b-H and 10-H.

By treatment of $\mathbf{F}_2$ and $\mathbf{F}_2'$ with $5 \cdot 10^{-3}$ M $NaOCH_3/CH_3OH$ at −20 °C for 24 h each factor yields 1. an approximately 1:1 mixture of the native and the 4-epimeric parent alcohols inseparable by TLC and 2. a mixture of the 4-epimeric (approximate ratio 1:1) 4-deoxy-16-hydroxy-phorbol- (**7**) and -4α-phorbol-12-hexadecanoates (**8**) (M^+ 602) and in the case of $\mathbf{F}_2'$ 4-deoxy-16-hydroxy-phorbol- (**9**) and -4α-phorbol-20-decanoates (**10**) (M^+ 518). The latter mixture was separated on precoated silica gel plates (0.5 mm, dichlormethane:acetone system) and exhibits similar differences of the π-π^+ absorption in UV (CH_3OH) as observed also in phorbol/4α-phorbol[14] and in 4-deoxyphorbol/4-deoxy-4α-phorbol[15] and their derivatives. After acetylation of the mixture of the 4-epimeric parent alcohols with pyridine/acetic anhydride and separation by TLC (precoated silica gel plates, 0.5 mm, ether:hexane 4:1) 4-deoxy-16-hydroxyphorbol-12,13,16-20-tetraacetate (**11**), R_f 0.3, and its 4α-epimer (**6**), R_f 0.38 (see above, and chart 1) were obtained; **11**: (M^+) 532, NMR (see chart 2), IR (CH_2Cl_2): 3410 (OH), 1727, 1693 (C=O), 1627 cm^{-1} (C=C); UV (CH_3OH): λ_{max}: 204, 232, 304 nm (ε_{max}: 10740, 5930, 110).

Refluxing of the 4α-epimer of **5** for 4 h under conditions similar to those applied to **1** by Okuda et al.[12] (0.02 N $HClO_4$ in aq. CH_3OH) yields 4-deoxy-4α-bisdehydrophorbol, (M^+) 346, exhibiting in its NMR-spectrum the isopropenyl group δ_{TMS}, $(CD_3)_2SO$, 4.92 and 4.66, 16-H_2; 1.58, s, 17-H_3 similarly as in its analogue bisdehydrophorbol[16]. This reaction is reminiscent of the formation of crotophorbolone from 12-deoxy-16-hydroxyphorbol-esters under basic conditions[17].

Thus, Croton factors $\mathbf{F}_2$ and $\mathbf{F}_2'$ are shown to be 12-O-hexadecanoyl-13-O-acetyl-4-deoxy-16-hydroxyphorbol and 12-O-hexadecanoyl-13-O-acetyl-4-deoxy-16-hydroxyphorbol-20-decanoate, respectively. Again, their structures are reminiscent of those of the free (in case of $\mathbf{F}_2$) and cryptic (in case of $\mathbf{F}_2'$) irritant and cocarcinogenic phorbol-12,13-

Some data of the Croton factors isolated from the roots or prepared by partial synthesis as compared to croton oil factors A_1 and A_4[7]

Factors	Yield[a] (%)	R_f-value[b]	m.p. (°C)	Mol. ion (m/e)	ID_{50}^{24} h (nM/ear)
$\mathbf{A}_1$[c]	–	0.3[d]	72	616	0.016
$\mathbf{A}_4$[e]	–	0.4[d]	–	644	0.003
$\mathbf{F}_1$	0.78	0.15[f]	177–178	660	0.008
$\mathbf{F}_2$	0.63	0.24[f]	–	644	0.016
$\mathbf{F}_3$	–[i]	0.15[f]	164–165	632	0.01
$\mathbf{F}_1'$	0.95	0.23[g]	–	814	0.17
$\mathbf{F}_2'$	0.65	0.26[g]	–	798	0.01
$\mathbf{F}_3'$	0.11	0.23[g]	–	786	0.36

[a] by weight, dry weight of methanol extract = 100%; [b] TLC on Silica Gel 'Merck HF_{254}' (chamber saturation); [c] 12-O-tetradecanoylphorbol-13-acetate (TPA); [d] dichloromethane/acetone = 3/1; [e] 12-O-hexadecanoylphorbol-13-acetate; [f] dichloromethane/acetone = 2/1; [g] diethyl ether/hexane = 4/1; [h] SD σ: 1.3, significance level α = 0.05, 24 h after administration; [i] by partial synthesis from $\mathbf{F}_3'$ (see text).

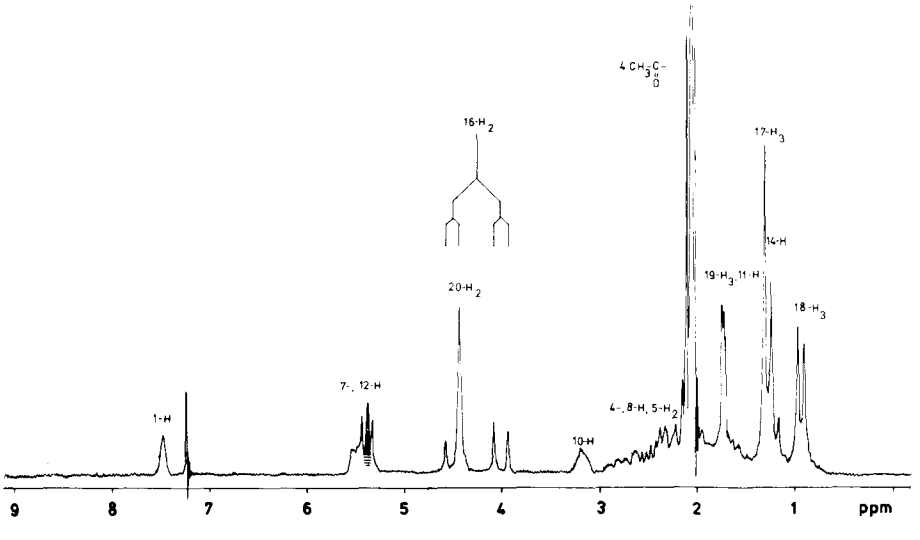


Chart 2. 90-MHz-spectrum of 4-deoxy-16-hydroxyphorbol-12,13,16,20-tetraacetate (**11**).

diesters occurring in croton oil. It is interesting to note here that in case of croton oil several esters of 4-deoxy-4α-phorbol have been shown to be present as minor constituents[7]. However, it may be argued[7] that these esters in fact are not the native constituents: they may be formed from naturally occurring (most sensitive[15,18,19]) esters of 4-deoxyphorbol by epimerization under the acidic milieu of croton oil or of the croton seeds themselves. Although the diterpene moiety of 4-deoxy-16-hydroxyphorbol esters, as for example in Croton factors $\mathbf{F}_2$ and $\mathbf{F}_2'$, is susceptible towards alcali catalyzed epimerization in 4-position, no corresponding 4α-epimeric esters were isolated from roots of *Croton flavens*.

Croton factors from fruits. A preliminary investigation of the fruits of Croton flavens, each containing three seeds, yielded mixtures of croton factors exhibiting the same R_f values and similar irritant activities as the factors isolated from the roots. The presence in these mixtures of the parent alcohols 16-hydroxyphorbol (**1**) and 4-deoxy-16-hydroxyphorbol (**5**) was demonstrated by transesterification and subsequent thin layer chromatography. The contents (by weight %) of esters in this material as judged from the amounts of **1** and **5** is lower than that of the roots. According to the NMR-spectra of the mixtures of esters, the corresponding croton factors carry an acetate moiety. Further, the presence of long chain acid moieties was demonstrated by transesterification/gas chromatography.

Biological activities of some of the Croton factors described. In our semiquantitative standard assay on the back skin of mice with 7,12-dimethyl-benz(a)anthracene as initiator[7], the methanol extract of the roots and the irritant Croton factors $\mathbf{F}_1$ (16-hydroxyphorbol ester type) and $\mathbf{F}_2$ (4-deoxy-16-hydroxyphorbol ester type) were shown to exhibit cocarcinogenic in terms of tumor promoting activities comparable to that of croton oil factor $\mathbf{A}_1$ (TPA). The corresponding factors of the cryptic irritant type ($\mathbf{F}_1'$ and $\mathbf{F}_2'$) exhibited little if any cocarcinogenic activity[20]. Because of lack of material, the rest of the croton oil factors isolated was not available for assay of tumor promoting activity.

These findings strongly suggest that the exceptionally high rate of esophageal cancer on Curaçao may be causally related to cocarcinogenic phorbol derivatives ingested by the widespread and frequent use of *Croton flavens* on this island according to local habits[1,2]. Some ideas as to the origin of the initiator(s) involved have been discussed[20]. In addition to a large body of already existing indirect evidence[4,21], the present findings illustrate the role which cocarcinogens of the tumor promoter type may play in the etiology of human cancer as 2nd order carcinogenic risk factors[3,4,21].

*Dedicated to Prof. Dr H.P. Rusch, Director Wisconsin Clinical Cancer Center, Madison, Wis. USA, on occasion of his 70th birthday. We wish to thank Dr J.F. Morton, Morton Collectanea, University of Miami, Florida, USA, for kindly supplying us with the plant material.

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Isotrinervi-2β-ol. Structural isomers in the defense secretions of allopatric populations of the termite *Trinervitermes gratiosus*[1]

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Summary. The defense secretions of 3 allopatric populations of the nasute termite *Trinervitermes gratiosus* were analyzed. One population afforded a new trinervitene, isotrinervi-2β-ol, a missing link in the hypothetical biosynthesis of the trinervitenes. Populations could be readily distinguished on the basis of the chromatographic profiles of their major and minor soldier frontal gland secretions.

Nasute termite soldiers (Isoptera: Termitidae: Nasutitermitinae) eject an irritating, sticky defense secretion when provoked. Considerable progress has been made in the elucidation of the structures of the individual diterpenoid 'resins'[2], the overall chemical composition of the secretions[3], and the use of the secretions in defense[4]. Interspecific variations in defense secretion chemistry are commonplace among termites[2]. However, no examples of intraspecific variation have been reported. My investigations of 3 allopatric populations of the East African grass-feeding termite *Trinervitermes gratiosus* (Sjöstedt) demonstrate significant differences in the chemical compositions of the soldier secretions within a single species, including the production of isomeric compounds in different populations.