Journal of Natural Products
Vol. 51, No. 4, pp. 662-674, Jul-Aug 1988

IRRITANT PRINCIPLES OF THE MEZEREON FAMILY (THYMELAEACEAE), V.[1] NEW SKIN IRRITANTS AND TUMOR PROMOTERS OF THE DAPHNANE AND 1α-ALKYLDAPHNANE TYPE FROM *SYNAPTOLEPIS KIRKII* AND *SYNAPTOLEPIS RETUSA*

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ABSTRACT.—Seventeen mostly new, skin irritant diterpene esters (DTE) of the daphnane and 1α-alkyldaphnane types were isolated from roots of *Synaptolepis kirkii* and *Synaptolepis retusa*. The parent alcohols of the daphnane types are shown to be 5β-hydroxyresiniferonol-6α,7α-oxide [**1**] and 5β,12β-dihydroxyresiniferonol-6α,7α-oxide [**2**]. Ten of the daphnane types are 9,13,14-orthoesters and three are conventional esters involving tertiary or secondary hydroxyl groups at C-13 or C-14, respectively. The latter may be considered immediate precursors of corresponding orthoesters. The four 1α-alkyldaphnane types are intramolecular 9,13,14-ortho-(2-hexadecenoic acid)-esters in which, formally, the second to last C atom of the orthoester moiety is linked covalently to C-1α of the diterpene parent alcohols **1** or **2**. Thus, in the new structure, a macrocyclic ring bridges the α side of the diterpene moiety in an "ansa" type manner.

The irritancies on the mouse ear of the DTE obtained cover a wide range ($I^{24} = 0.05$–670 nmole^{-1}). Some of them are considerably more irritant than the daphnane type standard simplexin. Structure/activity investigations reveal that an ester group instead of a free hydroxyl group at C-20 ("cryptic types"), or presence of a hydroxy or an acetoxy group in position 12 diminishes the irritancies of the daphnane types isolated, similar to what is known in corresponding tigliane types. In the standardized initiation/promotion protocol on the back skin of mice, some of the irritant DTE exhibit tumor-promoting activities higher than that of simplexin.

Species of the plant family Euphorbiaceae often contain irritant and tumor-promoting diterpene esters (DTE) of polyfunctionalized tigliane, ingenane, and daphnane structures (1–4). In recent years from the plant family Thymelaeaceae also an increasing number of irritant and tumor-promoting diterpene esters of the tigliane, daphnane, and the 1α-alkyldaphnane types were isolated (2,3,5–8, see also 23–25). Plant preparations from species of both of these families are used in ethnomedicines. For example, in East Africa roots of *Synaptolepis kirkii* Oliv. (Thymelaeaceae) are used in treatment of epilepsy and as an antidote for snake bites (9), but also they are known to produce emesis (10). In West Africa *Synaptolepis retusa* H.H.W. Pearson is utilized as a component of arrow poison (11). Sometimes these plants are used also for primitive treatment of cancer (9). Therefore, the antineoplastic activity of a number of diterpene esters of tigliane, ingenane, and daphnane types has been investigated (12,22). More specifically, from plant parts of Thymelaeaceae species some daphnane- or 1α-alkyldaphnane-type orthoesters with antileukemic activity were isolated (13,14). In addition, many of them exhibit, or are suspected to exhibit, de facto tumor-promoting activity in mouse skin (2,3). Thus, when utilized for therapeutic purposes they may be considered potentially as "iatrogenic" risk factors of cancer (3,13,14).

We have found that MeOH extracts from roots of both *Synaptolepis* spp. mentioned above exhibit irritant activity, and we reported briefly on the 1α-alkyldaphnane-type structure of *Synaptolepis* factor K_1 (15). We now present the results of the investigation

[1]For previous communication, see Adolf *et al.* (8); the topic of the series is slightly modified beginning with the present communication.

of both species as to their putative irritant and possibly tumor-promoting constituents including certain structure/activity relationships.

RESULTS AND DISCUSSION

The dry residues of MeOH extracts from roots of both *S. kirkii* and *S. retusa* were partitioned between EtOAc and H_2O. Filtration of the EtOAc extracts through Si gel removed the very polar constituents. Filtrates were subjected to multiple Craig distributions and fractions were combined into sections guided by tlc (Table 1). From sections with an $ID_{50}^{24} \leq 20$ μg/ear, by a combination of tlc (SiO_2) and hplc (reversed-phase columns), 17 irritant *Synaptolepis* factors[2] were isolated. For an overview covering yields, R_f values, molecular ions, and structures, see Table 1 and Figures 1–3.

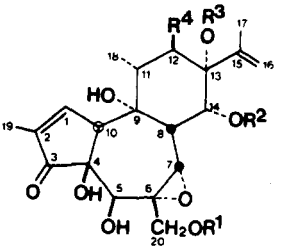
TABLE 1. *Synaptolepis* Factors of Roots of *Synaptolepis kirkii* and *Synaptolepis retusa* Isolated from Sections of Craig Distributions: Yields, R_f-Values, Molecular Ions, and Assigned Structures.

Synaptolepis	*S. kirkii*		*S. retusa*		R_f[b]	Molecular ion (*m/z*)	Structure
	Combined fractions	Yield[a] (%)	Combined fractions	Yield[a] (%)			
K_5	71–105	0.009	—	—	0.21	528	**3**
K_6	251–320	0.023	—	—	0.26	598	**4**
K_8	501–600	0.004	551–650	0.011	0.32	616	**5**
K_7	371–470	0.063	371–470	0.22	0.31	614	**6**
K_7'	1751–2000	0.017	1750–1900	—[c]	0.74	852	**7**
R_1	—	—	71–130	0.034	0.19	614[d]	**8**
R_2	—	—	71–130	0.018	0.07	614[d]	**9**
R_1'	—	—	651–750	0.04	0.59	870	**10**
K_3	21–70	0.048	—	—	0.13[e]	584	**13**
K_4	21–70	0.065	—	—	0.13[e]	586	**14**
K_3'	900–1020	0.011	—	—	0.63	822	**15**
K_4'						824	**16**
R_3	—	—	161–190	0.019	0.17	642	**17**
K_1	161–250	0.16	191–300	0.64	0.23	614	**18**
K_1'	1751–2000	0.021	1750–1900	—[f]	0.73	852	**19**
K_2	106–130	0.021	—	—	0.20	672	**20**
R_4	—	—	71–130	0.012	0.07	630	**21**

[a]Yields refer to the EtOAc extract = 100%.
[b]SiO_2 in Et_2O-petroleum ether-Me_2CO (2:2:1) if not indicated otherwise.
[c]Isolated as **6** after partial transesterification of the section.
[d]$[M - H_2O]^+$.
[e]RP-18 in MeOH-H_2O (90:10); **13** R_f 0.40; **14** R_f 0.34.
[f]Isolated as **18** after partial transesterification of the section.

DAPHNANE-TYPE DERIVATIVES.—*Esters of 5β-hydroxyresiniferonol-6α,7α-oxide* [**1**].—The ^{1}H-nmr spectral data of **3** and **4** were very similar to those of huratoxin, a piscicidal constituent of *Hura crepitans* (17). The mass spectrum of **3** ($[M]^+$ at *m/z* 528, intense fragment ion at *m/z* 151) indicated a 9,13,14-ortho-(2,4-decadienoate) of 5β-hydroxyresiniferonol-6α,7α-oxide [**1**] which is identical with a toxin isolated from *Ex-*

[2]According to the principles developed for Euphorbiaceae diterpene esters (1,16), as a preliminary assignment of irritants representing single spots in tlc (or peaks in hplc) the term *factor* is used and specified by the systematic botanical name of the species, here for example "*Synaptolepis* factor K or R"; multiplicity of factors in the species concerned is taken care of by indices (see Table 1). Corresponding 20-esters ("cryptic irritants") are assigned by analogy, but with a prime, e.g., "*Synaptolepis* factors K_7 and R_1"(see Table 1). The term *factor* proved useful in the biological context; in the chemical context after successful structure elucidation, the term is abandoned, to be replaced by the semitrivial systematic nomenclature of the structure as introduced previously (2,29) and/or coded as usual by numbers.



1 $R^1=R^2=R^3=R^4=H$ (5β-hydroxyresiniferonol-6α,7α-oxide)
2 $R^1=R^2=R^3=H$, $R^4=OH$ (5β,12β-dihydroxyresiniferonol-6α,7α-oxide)

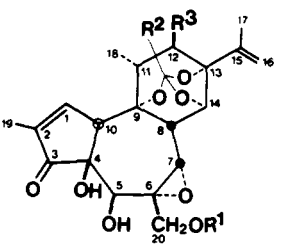
conventional esters of **1**

8 $R^1=R^3=R^4=H$, $R^2=COCH\overset{E}{=}CH(CH_2)_{12}CH_3$

9 $R^1=R^2=R^4=H$, $R^3=COCH\overset{E}{=}CH(CH_2)_{12}CH_3$

10 $R^1=CO(CH_2)_{14}CH_3$, $R^2=COCH\overset{E}{=}CH(CH_2)_{12}CH_3$, $R^3=R^4=H$

FIGURE 1. Daphnane-type diterpene parent alcohols 5β-hydroxyresiniferonol-6α,7α-oxide [**1**], 5β,12β-dihydroxyresiniferonol-6α,7α-oxide [**2**], and esters **8**–**10**.



9,13,14-orthoesters of **1**

3 $R^1=R^3=H$, $R^2=(CH=CH)_2(CH_2)_4CH_3$
4 $R^1=R^3=H$, $R^2=(CH=CH)_2(CH_2)_9CH_3$
5 $R^1=R^3=H$, $R^2=(CH_2)_{14}CH_3$
6 $R^1=R^3=H$, $R^2=CH\overset{E}{=}CH(CH_2)_{12}CH_3$
7 $R^1=CO(CH_2)_{14}CH_3$, $R^2=CH\overset{E}{=}CH(CH_2)_{12}CH_3$, $R^3=H$

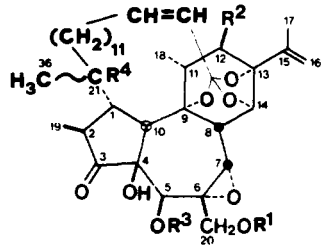
9,13,14-orthoesters of **2**

11 $R^1=H$, $R^2=(CH=CH)_3(CH_2)_2CH_3$, $R^3=OH$
12 $R^1=H$, $R^2=(CH=CH)_2(CH_2)_4CH_3$, $R^3=OH$
13 $R^1=H$, $R^2=(CH=CH)_3(CH_2)_2CH_3$, $R^3=OAc$
14 $R^1=H$, $R^2=(CH=CH)_2(CH_2)_4CH_3$, $R^3=OAc$
15 $R^1=CO(CH_2)_{14}CH_3$, $R^2=(CH=CH)_3(CH_2)_2CH_3$, $R^3=OAc$
16 $R^1=CO(CH_2)_{14}CH_3$, $R^2=(CH=CH)_2(CH_2)_4CH_3$, $R^3=OAc$
17 $R^1=H$, $R^2=(CH=CH)_2(CH_2)_8CH_3$, $R^3=OAc$

FIGURE 2. Structures of 9,13,14-orthoesters of 5β-hydroxyresiniferonol-6α,7α-oxide [**1**] and of 5β,12β-dihydroxyresiniferonol-6α,7α-oxide [**2**].

coecaria agallocha (18). Compound **4** ($[M]^+$ *m/z* 598, intense fragment ion at *m/z* 221) most probably represents the 9,13,14-ortho-(2,4-pentadecadienoate) of **1**.

The ^{1}H-nmr spectroscopic data of **5** were similar to those of simplexin from *Pimelea simplex* (7,19) and of montanin from *Baliospermum montanum* (20). Simplexin is the



18 $R^1-R^4=H$
19 $R^1=CO(CH_2)_{14}CH_3$, $R^2=R^3=R^4=H$
20 $R^1=R^3=R^4=H$, $R^2=OAc$
21 $R^1-R^3=H$, $R^4=OH$
22 $R^1=R^3=Ac$, $R^2=H$, $R^4=OH$

FIGURE 3. Structures of 1α-alkyldaphnane-type intramolecular 9,13,14-ortho-(2-hexadecenoate) of **1**. For numbering of the ansa part of the molecule the interlinking C atom of the tail end of the ansa moieties is assigned C-21. Numbering continues along the straight chain of the 1α-alkyl moiety to the C atom of the orthocarbonyl, C-35; finally side chains of the "ansa" moiety are included in the numbering system, e.g., C-36.

9,13,14-orthodecanoate and montanin the 9,13,14-orthododecanoate of **1**, whereas **5** ($[M]^+$ at *m/z* 616) represented the 9,13,14-orthohexadecanoate of **1**.

Compound **6** is a 9,13,14-orthoester of **1** with 2*E*-hexadecenoic acid ($[M]^+$ *m/z* 614). The ^{1}H-nmr spectrum was similar to that of **5**, and the olefinic protons at 6.3 (dt) and 5.65 ppm (d, $J=16$ Hz) could be decoupled indicating both the position and *E*-configuration of the double bond. An additional substance was isolated ($[M]^+$ *m/z* 852), and its ^{1}H-nmr data proved the structure of **7** to be the 20-hexadecanoate of **6**.

Compounds **8** and **9** were convertible into each other, either by diluted acids or on Si gel (2D tlc). Hence, purification was carried out by reversed phase hplc. A fraction containing a mixture of **8** and **9** yielded, upon treatment with 0.5% $HClO_4$/MeOH, a single product, the orthoester **6**. In the mass spectra of both **8** and **9**, the $[M-H_2O]^+$ peak (*m/z* 614) was registered corresponding to the $[M]^+$ peak of **6**. In the ^{1}H-nmr spectrum of **8**, as compared to that of **6** (Table 2), the signal of 14-H was paramagnetically shifted to 5.67 and that of 8-H to 3.66 ppm, indicating the ester position at OH-14 of the diterpene parent alcohol **1**. The relative paramagnetic shift of 8-H might be explained by the proximity of the OH-9 and the C-14 ester function, respectively. The ^{1}H-nmr spectrum of **9** (Table 2) indicated an esterified hydroxyl group at 13, because the signals for 16-H_2 were paramagnetically shifted to 5.25 ppm as compared to **8** and that for 8-H to 3.36 ppm (9,14-diol in **9** embedding 8-H) as compared to **6**. A sharp singlet at 5.0 ppm might correspond, as in tigliane derivatives, to the proton of the hydroxyl group at C-9. Thus, structure **9** might be tentatively assigned the 13-ester of the parent **1**; as an alternative consideration the 9-ester could not entirely be excluded.

Compound **10** ($[M]^+$ *m/z* 870), whose signal for 20-H_2 was paramagnetically shifted to 4.40 ppm (Table 2), was the 20-hexadecanoate of **8**.

Esters of 5β,12β-dihydroxyresiniferonol-6α,7α-oxide [**2**].—Compounds **13** ($[M]^+$ at *m/z* 584) and **14** ($[M]^+$ at *m/z* 586) represented diesters of the daphnane type carrying an acetate and an unsaturated acid moiety. In their ^{1}H-nmr spectra they exhibited, compared to the 9,13,14-orthoesters of 5β-hydroxyresiniferonol-6α,7α-oxide [**1**] (see above), an additional singlet of one proton at ca. 5.0 ppm (12-H) and a singlet of an acetyl group at ca. 2.0 ppm. The chemical shifts of the protons on the β-sides of the

TABLE 2. ^{1}H nmr (90 MHz) Data of Diterpene Esters **8–10** as Compared to Those of Compound **6**.

Proton	δ value (ppm, $CDCl_3$) in compound			
	8	**9**	**10**	**6**
1-H	7.65	7.65	7.73	7.62
14-H	5.67	4.41	5.58	4.42
16-H_2	5.10	5.25	5.10	4.98
5-H	4.26	4.27	4.02	4.27
10-H	3.89	3.99	3.64	3.85
20-H_2	3.79 (AB)	3.86	4.40 (AB)	3.84 (AB)
8-H	3.66	3.36	3.60	2.96
7-H	3.17	3.36	3.25	3.46
17-H_3	1.90	1.83	1.85	1.83
19-H_3	1.80	1.83	1.85	1.83
9-OH	n.d.[a]	5.0[b]	n.d.[a]	—
Olefinic protons of the 2*E*-hexadecenoic acid moiety				
α (d)	5.94	5.90	5.90	5.67
β (dt)	7.10	6.95	7.05	6.32

[a]Not determined.
[b]Tentatively assigned.

molecules, i.e., 14-H at 4.76 and 8-H at 3.51 ppm, were typical for the presence of an additional 12β-acyloxy substituent in the resiniferonol derivative **1**. Base catalyzed transesterification of both compounds furnished products with the ^{1}H-nmr signals for 12-H at ca. 3.9 ppm, as required for structures **11** and **12** with a free hydroxyl at C-12. Thus, the structures of **13** and **14** might be deduced as derivatives of 5β,12β-dihydroxyresiniferonol-6α,7α-oxide [**2**], both carrying a 12-acetyl group but differing in the acid moiety of their orthoesters. In compound **13** the 9,13,14-ortho-(2,4,6-decatrienoate) and in **14** the 9,13,14-ortho-(2,4-decadienoate) moiety is present, which is consistent with the typical uv-absorption bands of the triene and diene systems. Additionally, a mixture of two compounds (**15** and **16**, $[M]^+$ *m/z* 822 and 824) was isolated, representing most likely the 20-hexadecanoates of **13** and **14**, respectively.

Compound **17** furnished a ^{1}H-nmr spectrum similar to that of **14**. By its mass spectrum ($[M]^+$ *m/z* 642) it was identified as 12-acetoxyhuratoxin, previously isolated as "subtoxin A" from *P. simplex* (19).

1α-ALKYLDAPHNANE-TYPE DERIVATIVES.—Compound **18** was obtained in the highest yield from both species. It exhibited the same molecular ion as compound **6** but was not visible on tlc under uv light (254 nm). In the ^{1}H-nmr spectrum, the typical signals for two olefinic protons corresponding to the unsaturated acid moiety, as present in **6**, were apparent. Yet, in **18** the signals for an olefinic 1-H and an allylic 19-H_3 of the diterpene moiety, as seen in **6**, were missing. A carbonyl vibration band in the ir spectrum at 1745 cm^{-1} supported the presence of a cyclopentanone system and hence, saturation of the 1,2 double bond in **18**, as compared to **6**. A doublet at 3.2 ppm ($J = 10$ Hz) was assigned 10-H, coupling only with one vicinal proton 1-H. Therefore, as compared to **6**, a substitution at C-1 of the diterpene moiety, i.e., with C-21, was assumed leading to the new 1α-alkyldaphnane structure **18** (Figure 3). Analysis of the 500 MHz ^{1}H-nmr spectrum of **18** showed the presence of a secondary methyl group (C-36; Figure 3) and indicated a branching point within the ansa-type macrocyclic ring system. In agreement with the spectroscopic data the branching point is assumed to be

indentical with the interlinking C atom of the ansa moiety (Figure 3).[3] In the 500 MHz ^{1}H-nmr spectrum of **18**, furthermore, signals of very low intensity were registered, which could not be assigned. Further purification by repeated hplc on reversed-phase columns indicated the presence of a C-21 epimeric compound as shown in other pairs of the 1α-alkyldaphnane type, i.e., for the tumor promoters, such as *Pimelea* factors P_2, P_3 (5) and S_6, S_7 (7). The pairs P_2/P_3 and S_6/S_7 proved to be epimers with respect to C-21.[4] The prototype of this skeleton was first derived for the tumor-inhibiting compound gnidimacrin (22) by X-ray diffraction analysis.

In addition to **7**, compound **19** could be detected in both species as another nonpolar DTE yielding **18** upon base-catalyzed transesterification. Presumably **19** is the 20-hexadecanoate of **18**.

Compound **20** exhibited ^{1}H-nmr data similar to those of **18**. However, an additional singlet (12-H) in the ^{1}H-nmr spectrum of **20** superimposed with the signal for 16-H_2 at 5.0 ppm, and the signals of 14-H and 8-H appearing at lower field than that of **18** (see also the differences of esters of **1** and **2**), were indicative of 12β-acyloxy substitution of **18**. The molecular ion (*m/z* 672) and the additional acetyl signal in the ^{1}H-nmr spectrum (ca. 2.0 ppm) supported the structure of **20** as the 12β-acetoxy derivative of **18**.

The molecular ion at *m/z* 630 of compound **21** was 16 mass units higher than that of **18**. Most of the signals in the ^{1}H-nmr spectra were very similar. However, a paramagnetic shift of 10-H from 3.2 to 3.62 ppm was observed, and the doublet at 0.9 ppm assigned to 36-H_3 in **18** was missing in the spectrum of **21**. Thus, most probably, the additional hydroxyl group was located at C-21 furnishing a tertiary methyl group Me-36 whose signal may be superimposed on the signal of the methylene protons. Acetylation of **21** gave the diacetate **22** exhibiting in the ^{1}H-nmr spectrum the signals of two acetates, the doublets of two methyl groups, and a singlet of a further methyl group at 1.47 ppm. The signal for 10-H was paramagnetically shifted because of a sterical proximity of the 21-hydroxyl group. Thus, the structure of **21** was tentatively assigned as the 21-hydroxy derivative of **18**.

Some interesting new DTE structures have been obtained from both *Synaptolepis* species in this study. Of these the 1α-alkyldaphnane structures with their ansa-type macrocyclic ring system, e.g., **18**, deserve particular attention. Formally (and perhaps also biogenetically), structures such as **18** may be derived from long chain structures such as **6** by interlinkage of the tail end, e.g., of the 9,13,14-ortho-(2-hexadecenoate) with C-1 of the daphnane skeleton and simultaneous saturation of the 1,2-double bond.

In view of a renewed interest in utilizing DTE, especially daphnane- and 1α-alkyldaphnane types, as antineoplastic agents (23–25,31), the putative irritant and tumor-promoting activities of the new DTE structures should be analyzed and considered critically (2,13,14).

Irritant activity—structure/activity relations.—For convenience of comparison of irritant activities of all esters isolated, instead of the ID_{50}^{24} their irritancies $I^{24} = 1/ID_{50}^{24}$ may be used. In the case of daphnane or 1α-alkyldaphnane types, they are compared to those of simplexin as standard, i.e., to the 9,13,14-orthodecanoate of **1** (Tables 3,4).

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[3]Previously (15), the signal for 10-H in the ^{1}H-nmr spectrum of **18** was reported erroneously to be at 3.0 ppm, whereas correctly, it was at 3.20 ppm. Furthermore, the assignment for 1α-alkyldaphnane derivatives of the secondary methyl groups provided previously (20) was revised in a recent publication (21): 400 MHz ^{1}H-nmr decoupling experiments with *Pimelea* factor P_2 proved the chemical shifts of 18-H_3 at 1.44 ppm and 30-H_3 at 0.82 ppm. Accordingly our 500 MHz ^{1}H-nmr decoupling experiments with **18** allowed the assignments of 36-H_3 at 0.88, 19-H_3 at 1.12, 18-H_3 at 1.27, 1-H at 2.07, 2-H at 2.26, and 21-H at 2.42 ppm.

[4]M. Fellhauer *et al.*, in preparation.

TABLE 3. Irritant Dose 50 (ID_{50}^{24}) and Irritancy (I^{24}) on the Ear of NMRI Mice of Some of the Daphnane-type DTE Isolated from *Synaptolepis kirkii* and *Synaptolepis retusa*. Diterpene esters (DTE) Are Arranged According to Parent Alcohol (**1** or **2**), Degrees of Esterification, and Unsaturation of Ester Moieties.

Parent alcohol	DTE structure	Ester moieties	ID_{50}^{24} (nmol/ear)[a]	I^{24} $(nmol)^{-1}$
1	**3**	9,13,14-ortho-(2,4-decadienoate)	0.15	6.7
	4	9,13,14-ortho-(2,4-pentadeca-dienoate)	0.020	50
	5	9,13,14-orthohexadecanoate	0.040	25
	6	9,13,14-ortho-(2*E*-hexadecenoate)	0.020	50
	7	9,13,14-ortho-(2*E*-hexadecenoate)-20-hexadecanoate	0.16	6.3
	8	14-(2*E*-hexadecenoate)	0.035	29
	9	13-(2*E*-hexadecenoate)	0.080	13
	10	14-(2*E*-hexadecenoate)-20-hexadecanoate	20	0.15
2	**12**[b]	9,13,14-ortho-(2,4-decadienoate)	0.92	1.1
	14	9,13,14-ortho-(2,4-decadienoate)-12-acetate	0.27	3.7
	11[c]	9,13,14-ortho-(2,4,6-decatrienoate)	0.81	1.2
	13	9,13,14-ortho-(2,4,6-decatrie-noate)-12-acetate	1.1	0.91
	16/15[d]	9,13,14-ortho-(2,4-decadienoate)-12-acetate-20-hexadecanoate 9,13,14-ortho-(2,4,6-decatrienoate)-12-acetate-20-hexadecanoate	2.7	0.37
	17	9,13,14-ortho-(2,4,-tetradeca-dienoate)-12-acetate	0.030	33

[a]For simplexin as standard: $ID_{50}^{24} = 0.013$ nmol/ear, $I^{24} = 77$ $nmol^{-1}$ (7).
[b]Obtained by partial transesterification of **14**.
[c]Obtained by partial transesterification of **13**.
[d]Obtained as mixture (see also Table 1).

As compared to simplexin, derived from parent alcohol **1**, the orthoester **3** carries in the aliphatic chain two conjugated double bonds and was about 1/10 as active as simplexin. In comparable tigliane types, as a rule, the contrary would have been expected (2,3,26). Extension of the aliphatic chain in the orthoester group of **3** to yield **4** raises the irritancy to about the level seen in simplexin. The orthoester **5**, with the saturated hexadecanoic acid, was less active than simplexin. This may be taken as an indication that in **5** the lipophilic/hydrophilic balance necessary for optimal irritancy (2,26) was shifted beyond the optimum, as perhaps is present in simplexin. Introduction of one

TABLE 4. Irritant Dose 50 (ID_{50}^{24}) and Irritancy (I_{24}) on the Ear of NMRI Mice of Some of the 1α-Alkyldaphnane-type Diterpene Esters (DTE) Isolated from *Synaptolepis kirkii* and *Synaptolepis retusa*.

DTE structure	Ester moieties	ID_{50}^{24} (nmol/ear)[a]	I^{24} $(nmol)^{-1}$
18	intramolecular 9,13,14-ortho-(2-hexadecenoate) of **1** (ansa-type isomer of **6**)	0.0015	667
19	20-hexadecanoate of **18**	0.019	53
21	21-hydroxy derivative of **18**	0.016	6
20	12β-acetoxy derivative of **18**	0.010	100

[a]For simplexin as standard: $ID_{50}^{24} = 0.013$ nmol/ear, $I^{24} = 77$ $nmol^{-1}$ (7).

double bond as in **6** increases the irritancy. Esterification of **6** in position 20 with hexadecanoic acid depresses the irritancy considerably. This effect was shown to be common to, but more or less pronounced in, all DTE of the tigliane, ingenane, and daphnane types (2,3,26). It represents the structural principle of "cryptic" irritants (and promoters), because they may be hydrolyzed selectively at the 20-ester group by chemical or enzymatic means yielding highly active irritants and promoters (2,26). If, instead of a 9,13,14-orthoester group, the parent **1** carries the 2*E*-hexadecenoic acid in a secondary or tertiary ester group in the 14 or 13 positions, respectively, as in **8** or **9**, the irritancy was slightly decreased as compared to the corresponding orthoester structure **6**. If the primary hydroxyl group at C-20 in the 14-ester **8** is esterified, as in compound **10**, irritancy was considerably diminished as expected for cryptic irritant types.

The 9,13,14-ortho-(2,4-decadienoate) **12** derived from parent alcohol **2** carries, as compared to **3**, an additional hydroxyl group in position 12 and exhibits decreased irritancy. In this case, by introduction of a hydrophilic group the lipophilic/hydrophilic balance was shifted, resulting obviously in an irritancy. Accordingly, if this hydroxyl group is acetylated as in **14**, the irritancy is increased as compared to **12**. An additional double bond in the ester moiety as in structure **11** essentially does not increase the irritancy compared to **12**. A similar response of irritancy as observed in the pair **12/14** could have been expected also in the pair **11/13**. Apparently the contrary appears to be the case; however, this may be due to the variability of the biological assay. The decrease in irritancy to be expected for the cryptic irritant types **16** and **15**, as compared to **14** and **13**, respectively, is beyond the variability of the assay. Structure **17**, with an increased lipophilicity as compared to structure **14**, exhibited an irritancy increased by a factor of about 10, reminiscent of the increase in the pair **3/4**.

Of all polyfunctional diterpenes described in this paper, the 1α-alkyldaphnane-type DTE derived from **1** exhibited the highest irritancies (Table 4). The ansa-type isomer of **6**, compound **18**, is more than 10 times as active as either **6** or the daphnane standard simplexin. Again, esterification of the primary hydroxyl group at C-20 (as in compound **19**) diminished irritancy, as compared to the pair **6/7**. Also, introduction of a 12β-acetoxy group (as in compound **20**) lowered the irritancy of **18**. Interestingly, **21**, carrying presumably a hydroxyl group at C-21, i.e., in the more hydrophobic ansa part of the molecule, was by two orders of magnitude less irritant than the nonsubstituted **18**. It seems that irritancy is especially sensitive toward hydrophilic alteration in the ansa part of the molecule.

Tumor promoting activity (Table 5)—*structure/activity relations*.—Sufficient amount of compound for the assay of tumor promoting activity was available for the daphnane types **6**, **13**, and **14** and for the 1α-alkyldaphnane types **18** and **20**. The survival rates document that in all experimental groups almost no animals were lost during the entire promotional period (24 weeks).

As expected from its high irritancy, the daphnane type 9,13,14-ortho-(2*E*-hexadecenoate) **6** exhibited high promoting activity; it is dose dependent. As expected, too, the low irritancy C-12 acetoxy derivatives **14** and **13** showed marginal or no tumor-promoting activity, respectively, at doses of $p = 20$ nmol/application. The 1α-alkyldaphnane types, i.e., **18** and its 12-acetoxy derivative **20**, were very potent tumor promoters even at a dose of $p = 5$ nmol/application. At a dose of $p = 2.5$ nmol, **18** still reached, after a longer latency period, high tumor rates and tumor yields. At the same dose **20** was significantly less active.

Specific cellular receptor(s) of DTE postulated previously (2) were detected recently. They are related to the inositolphosphate/diacylglycerol second messenger system (26,27). Therefore, the interest in antineoplastic compounds of this type may be revived, provided they are of high antineoplastic and little or no irritant and promoting

TABLE 5. Tumor-promoting Activity of Some *Synaptolepis* Diterpene Esters on the Back Skin of NMRI Mice in the Standard Experiment,[a] Expressed as Tumor Rate and Average Tumor Yield, Respectively, at 12 and 24 Weeks of Standard Exposure to the Promoter.

Structural type	Structure	Number of mice	Dose (nmol)	Tumor rate[b] at week		Average tumor yield[c] at week		Survival rate (%) at week 24	Synoptic rating
				12	24	12	24		
Daphnane	Simplexin[d]	28	20	20	71	0.6	3.0	100	+++
			10	4	54	0.2	1.7	93	
	6	16	10	94	100	4.7	9.7	94	++++
		16	5	6	69	0.13	2.9	100	
	13	16	20	0	0	0	0	100	0
	14	15	20	0	14	0	0.36	88	(+)
1α-Alkyldaphnane . .	**18**	15	5	60	86	1.3	2.7	94	+++
		15	2.5	6.7	87	0.33	2.9	100	
	20	15	5	33	100	0.33	4.4	99	++
		16	2.5	0	27	0	0.6	94	

[a]Initiation: i = 100 nmole of 7,12-dimethylbenz[*a*]anthracene (DMBA); promotion: twice weekly dose of the promoter (29).
[b]Tumor-bearing animals/survivors in percent.
[c]Total number of tumors/survivors.
[d]Synoptic Rating Relative to the Activity of Simplexin (see Experimental: Biological assays).
[e]See Hafez *et al.* (7).

activities (12,23–25). Promising antineoplastic agents of this kind, the presently described daphnane- and 1α-alkyldaphnane types, may carry highly unsaturated orthoester moieties and C-12 acyloxy groups (e.g., the structural type **13** or **14**). Similarly, in the case of tumor-promoting structures of DTE (28), computer-assisted molecular modelling may turn out to be a useful tool in developing further this basically new type of antineoplastic principle (see also footnote 5).

EXPERIMENTAL

BIOLOGICAL ASSAYS.—The irritant activity was determined according to the standard procedure (29) as irritant dose 50 (ID_{50}^{24}) on the ear of NMRI mice, read 24 h after administration. It is expressed also as irritancy $I^{24} = 1/ID_{50}^{24}$, for convenience of comparison with the daphnane type standard simplexin (Tables 3,4). For reference, the data of TPA, the tigliane type standard, are $ID_{50}^{24} = 0.016$ nmol/ear (29) or $I^{24} = 63\ nmol^{-1}$. Tumor-promoting activity was assayed on the back skin of groups of 16 NMRI mice according to the standard procedure (29,30). To express skin-tumor-promoting activity semi-quantitatively (30) relative to that of simplexin as a standard, certain essential parameters of the protocol of each group (i.e., the promoting dose employed, the latency period for the first tumor to appear in the group, and the time course of tumor rates and yields) are assessed synoptically over a set period of exposure (24 weeks). The results of such synopses are expressed in five categories by the symbols 0, (+), ++, +++, and ++++ and related to the activity of simplexin at 20 nmol assigned +++ (Table 5). TPA at $p = 10$ nmol was assigned ++++ (30); see also footnote 5.

PLANT MATERIAL.—Roots of *S. kirkii* (5 kg) were collected by S.F.D. assisted by F.N. Gachathi, Nairobi University Herbarium, Kenya, near Sokoke forest, Kenya, in 1981, and stored under MeOH. Voucher specimens were deposited at the East African Herbarium, Nairobi, Kenya. Roots of *S. retusa* (10 kg) were collected by P. Hougnon, Responsable de l'Herbier National du Bénin, Université Nationale du Bénin, Faculté de Science et Technique, Cotonou, Benin, in 1982, and stored under MeOH. Voucher specimens are deposited at the Herbier National du Bénin, UNB. B.P. 526, Cotonou, R.P. Bénin, Afrique.

METHODS AND EQUIPMENT.—Methods and machinery of multiplicative distribution, analytical, and preparative tlc (usually on SiO_2 if not stated otherwise), have been described previously (6,23). Separations using hplc were carried out on a DuPont 830 chromatograph using Lichrosorb (Merck) RP-18 columns, 10 μm, and MeOH-H_2O systems. Mass spectra were measured with a Varian MAT 711 spectrometer, uv spectra with a Beckman DK 2a uv spectrometer, ir spectra with a Perkin-Elmer spectral photometer 521, and ^{1}H-nmr spectra with a Bruker HX 90 and AM 500 spectrometer. Chemical shifts refer to TMS ($\delta = 0.00$ ppm) as internal standard. All ^{1}H-nmr spectra were measured in $CDCl_3$ and in $CDCl_3/D_2O$, all uv spectra in MeOH.

EXTRACTION AND FRACTIONATION PROCEDURES.—The roots of both *Synaptolepis* spp. (ca. 2.5 kg each) were homogenized in MeOH by an ultra-turrax homogenizor (Jahnke und Kunkel) and filtered; this procedure was repeated 5 times with the filter residue, and the filtrates were combined. After evaporation of the solvents, the MeOH extracts were obtained: *S. kirkii* 132 g, ID_{50}^{24} 5.6 μg/ear; *S. retusa* 147 g, ID_{50}^{24} 2.5 μg/ear. Each of the extracts was partitioned between EtOAc and H_2O, and the aqueous phases extracted 5 times with EtOAc. After drying of the organic phase ($MgSO_4$) and evaporation of the solvent, the EtOAc extracts were obtained: *S. kirkii* 55.5 g, ID_{50}^{24} 0.6 μg/ear; *S. retusa* 53.8 g, ID_{50}^{24} 0.16 μg/ear. The EtOAc extracts were filtered over Si gel columns using EtOAc as solvent. The filtrates (*S. kirkii* 9.5 g and *S. retusa* 15.5 g) were subjected to Craig distributions in petroleum ether-MeOH-H_2O (15:10:0.5) over $n = 2000$ transfers (single withdrawal procedure, $z = 1020$ elements, $V = 10$ ml/10 ml, $T = 20°$ d). The fractions generated were combined in sections according to tlc testing (Table 1). From sections exhibiting irritant activity, irritant *Synaptolepis* factors were isolated by tlc and/or hplc.

ISOLATION AND CHARACTERIZATION OF THE IRRITANT STRUCTURES.—*Daphnane types—Esters of 5β-hydroxyresiniferonol-6α,7α-oxide* [**1**].—Compound **3**.—Combined fractions 71–105 (78 mg) of *S. kirkii* were separated by tlc in CH_2Cl_2-MeOH (95:5) and subsequently by hplc in MeOH-H_2O (80:20) to yield 4.5 mg of **3**; ms *m/z* $[M]^+$ 528, 510, 497, 479, 469, 360, 342, 329, 317, 283, 151 (base peak *m/z*

[5]Comparison of biological activities of DTE with different carbon skeletons, for example, of daphnane and tigliane types, involves certain reservations regarding possible differences in agonists interaction with receptor(s) caused by the different structures of the diterpene moieties. Objections of this kind may be clarified by computer-assisted molecular modeling (28).

> 100); uv λ max (MeOH) nm (ε) 231 (29600), sh 279 (1000), 192 (13400); ^{1}H nmr signals similar to those of huratoxin (15).

Compound **4**.—Combined fractions 251–320 (57 mg) of *S. kirkii* were separated by tlc in CH_2Cl_2-MeOH (95:5) to yield 11 mg of **4**; ms *m/z* $[M]^+$ 598, 567, 549, 360, 342, 329, 317, 283, 269, 221 (base peak *m/z* > 200); uv λ max (MeOH)nm (ε) 231 (17400), 193 (10300); ^{1}H nmr signals similar to those of huratoxin (15).

Compound **5**.—Combined fractions 501–600 (57 mg) of *S. kirkii* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:2:1) to yield 1.8 mg of **5**. Combined fractions 551–650 (197 mg) of *S. retusa* were separated in the same system and subsequently purified by hplc in MeOH-H_2O (97.5:2.5) to yield 5.5 mg of **5**; ms *m/z* $[M]^+$ 616, 598, 585, 567, 383, 340, 323; ^{1}H nmr data similar to those published for simplexin (5,19) and montanin (20) (impurities at δ 5.35 and 3.65).

Compound **6**.—Combined fractions 371–470 (82 mg) of *S. kirkii* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:1:1) to yield 30 mg of **6**. Tlc separation of combined fractions 371–470 of *S. retusa* in Et_2O-petroleum ether-Me_2CO (1:1:1) afforded 105 mg of **6**; ms *m/z* $[M]^+$ 614, 596, 583, 565, 446, 360, 342, 329, 317, 283, 237 (base peak *m/z* > 200); uv λ max (MeOH) nm (ε) 242 (7060), 332 (120), 190 (11300); ir ν max (KBr) 3460 (OH), 1705 (CO), 1635 (C=C) cm^{-1}; ^{1}H nmr acid moiety δ 6.3 (dt, J_1 = 16 Hz, J_2 = 7 Hz, 1 olefinic H), 5.65 (d, J = 16 Hz, 1 olefinic H), 1.28 $(CH_2)_{10}$; all other signals are similar to those described for simplexin (5,19).

Compound **7**.—Combined fractions 1751–2000 (1.4 g) of *S. kirkii* were separated by tlc in Et_2O-petroleum ether (3:1). After rechromatography in the same system, 84 mg of a fraction was obtained which was further separated by tlc in petroleum ether-EtOAc (4:1) and subsequently in cyclohexane-Et_2O (1:1). Besides 10 mg of compound **19** (data below), 8.3 mg of **7** were obtained; ms *m/z* $[M]^+$ 852, prominent fragment ion at *m/z* 256; ^{1}H-nmr data at variance with those in the spectrum of structure **6**; δ 4.30±0.45 (AB, J_{AB} = 12 Hz, 20-H_2), 2.34 (t, CH_2-CO).

Combined fractions 1751–1900 (0.5 g) of *S. retusa* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:4:1). A fraction (100 mg) was obtained containing a compound with the same R_f value as **7**. This fraction was treated with 0.1 M NaOMe/MeOH (50 ml) and worked up by adding buffer, pH 6.8, and extracting with EtOAc. The reaction mixture was separated by tlc in Et_2O-petroleum ether-Me_2CO (1:2:1, twice developed) to yield 3 mg of **18** (see below) and 7 mg of **6**.

Compounds **8** and **9**.—Combined fractions 71–130 (420 mg) of *S. retusa* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:1:1) to yield material that formed structure **6** upon treatment with 0.5% $HClO_4$/MeOH. This material was resolved by reversed-phase hplc in MeOH into compounds **8** (16.5 mg) and **9** (8.7 mg). Both are partially converted to each other when stored in solution or on Si gel. Ms *m/z* $[M - H_2O]^+$ 614 for both structures; ^{1}H nmr see Table 2.

Compound **10**.—Combined fractions 651–750 (248 mg) of *S. retusa* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:4:1) and subsequently in EtOAc-petroleum ether (1:1, twice developed) to yield 19.5 mg of compound **10**; ms *m/z* $[M]^+$ 870; ^{1}H nmr see Table 2.

Esters of 5β,12β-dihydroxyresiniferonol-6α,7α-oxide [**2**].—Compound **13**.—Combined fractions 21–55 (230 mg) of *S. kirkii* were separated by tlc in $CHCl_3$-EtOH (25:1) and subsequently in EtOAc-petroleum ether (4:1). The fraction obtained was finally resolved into compounds **13** (2.1 mg) and **14** (7 mg, see below) by reversed-phase hplc in MeOH-H_2O (80:20). Combined fractions 56–70 (108 mg) of *S. kirkii* were separated by tlc in CH_2Cl_2-MeOH (95:5) and subsequently by reversed-phase hplc in MeOH-H_2O (80:20) to yield compounds **13** (2.5 mg) and **14** (24 mg, see below); ms *m/z* $[M]^+$ 584, 566, 553, 535, 358, 314, 284, 255, 241; uv λ max (MeOH) nm (ε) 278 (29800), 268 (38200), 258 (sh, 30500), 249 (sh, 21900); ^{1}H nmr δ 7.55 (m, 1-H), ca. 5.0 (s-br, s, superimposed, 16-H_2, 12-H), 4.76 (d, J = 2.5 Hz, 14-H), 4.25 (s, 5-H), 3.85 (AB, m, superimposed, 20-H_2, 10-H), 3.53 (s, 7-H), 3.51 (d, J = 2.5 Hz, 8-H), 1.82 (m, superimposed, 17-H_3, 19-H_3), acid moieties 6.72 (dd, J_1 = 15 Hz, J_2 = 10 Hz, 1 olefinic H), 5.6–6.4 (m, 5 olefinic H), 2.0 ppm (s, acetate).

Compound **13** (5 mg) was treated with 0.1 M NaOMe/MeOH (1 ml) for 3 h. After purification by tlc in CH_2Cl_2-MeOH (95:5), compound **11** was obtained (2.5 mg): ms *m/z* $[M]^+$ 542, 149 (base peak *m/z* > 100); uv λ max (MeOH) nm (ε) 279 (18400), 269 (23400), 259 (20200), 249 (16900), 236 (15750), 194 (11300); ^{1}H nmr differences from the spectrum of **13**: δ 3.76 (d, J = 2.5 Hz, 8-H), 3.82 ± 0.07 (AB, J_{AB} = 12 Hz, 20-H_2), 3.91 (s, 12-H), no signal of an acetyl group.

Compound **14**.—For isolation, see compound **13**. Ms *m/z* $[M]^+$ 586, 568, 555, 537, 151; uv λ max (MeOH) nm (ε) 231 (23000), 193 (10100); ^{1}H nmr differences from the spectrum of **13** δ 6.68 (dd, J_1 = 9 Hz, J_2 = 15 Hz, 1 olefinic H), 5.5–6.3 (m, 3 olefinic H).

Compound **14** (16 mg) was treated with 0.1 M NaOMe/MeOH (5 ml) for 3 h. After purification by tlc in CH_2Cl_2-MeOH (95:5) 9.5 mg of reaction product **12** was obtained: ms *m/z* $[M]^+$ 544, 526, 513, 495, 151; ^{1}H nmr differences from the spectrum of structure **11**, only 4 olefinic protons corresponding to the acid moiety.

Mixture of compounds **15** and **16**.—Combined fractions 900–1020 (78 mg) of *S. kirkii* were separated by tlc in EtOAc-petroleum ether (1:1) to yield 2 mg of a mixture of compounds **15** and **16**: ms *m/z* $[M]^+$ 822, 824; ^1H nmr differences from the spectra of structures **13** and **14**: δ 4.36 ± 0.47 (AB, J_{AB} = 12 Hz, 20-H_2).

Compound **17**.—Combined fractions 161–190 (223 mg) of *S. retusa* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:2:1) and subsequently in $CHCl_3$-EtOH (95:5, twice developed) to yield 9 mg of **17**: ms *m/z* $[M]^+$ 642; ^1H nmr similar to that of compound **14**.

1α-Alkyldaphnane types.—Compound **18**.—Combined fractions 161–250 (223 mg) of *S. kirkii* were separated by tlc in $CHCl_3$-MeOH (95:5) to yield 77 mg of **18**.

Combined fractions 191–300 (1.055 g) of *S. retusa* were separated in $CHCl_3$-MeOH (95:5, twice developed) to yield 310 mg of **18**: ms *m/z* $[M]^+$ 614, 596, 583, 571, 555; ir ν max (KBr) 3440 (OH), 1745 (CO), 1685, 1650 (C=C), cm^{-1}; uv λ max (MeOH) nm (ε) 290 (140), 190 nm (11300); ^1H nmr for 90 MHz data of structure **18**, see Zayed *et al.* (15). Erroneously, the chemical shift of 10-H was reported at 3.0 ppm. The correct value is δ 3.20 ($J_{1,10}$ = 12 Hz, 10-H). Two-dimensional ^1H-^1H-correlation (500 MHz) allows assignment of the following protons δ 2.54 (11-H), 2.42 (21-H), 2.26 (2-H), 2.21 (12-H_a), 2.07 (1-H), 1.67 (12-H_b), 1.27 (18-H_3), 1.12 (19-H_3), and 0.88 (36-H_3).

Compound **19**.—For the isolation, see compound **7**. Ms *m/z* $[M]^+$ 852, prominent fragment ion at *m/z* 256, ^1H nmr signals at variance with those in the spectrum of compound **18** δ 4.32 ± 0.40 (AB, J_{AB} = 12 Hz, 20-H_2), 2.34 (t, CH_2-CO).

Compound **20**.—Combined fractions 106–130 (81 mg) of *S. kirkii* were separated by tlc in $CHCl_3$-EtOH (25:1, thrice developed) and subsequently in Et_2O-petroleum ether-Me_2CO (1:2:1, twice developed) to yield 8 mg of compound **20**: ms *m/z* $[M]^+$ 672, 654, 641, 613, 612, 559; ^1H nmr 5.0 (m, s, superimposed, 16-H_2, 12-H), 4.66 (d, J = 2.5 Hz, 14-H), 4.04 (s-br, 5-H), 3.84 ± 0.03 (AB, J_{AB} = 12 Hz, 20-H_2), 3.55 (d, J = 2.5 Hz, partially superimposed with s, 8-H, 7-H), 3.22 (d, J = 12 Hz, 10-H), 1.86 (s-br, 17-H_3), 1.25 [s, d, superimposed, $(CH_2)_{8\text{-}9}$, H_3-18], 1.14 (d, J = 6 Hz, 19-H_3), 0.91 (d, J = 7 Hz, 36-H_3), 6.2 (dt, 1 olefinic H), 5.54 (d-br, J = 16 Hz, 1 olefinic H), 2.04 (s, acetate).

Compound **21**.—Combined fractions 71–130 (420 g) of *S. retusa* were separated by tlc in Et_2O-petroleum ether-Me_2CO (1:1:1) and subsequently in EtOAc-petroleum ether (4:1) followed by CH_2Cl_2-MeOH (95:5, twice developed) to yield 5.5 mg of compound **21**: ms *m/z* $[M]^+$ 630; ^1H nmr data at variance with those of compound **18** 4.18 (s, 5-H), 3.83 ± 0.13 (AB, J_{AB} = 12 Hz, 20-H_2), 3.62 (d, J = 13 Hz, 10-H), 2.99 (d, J = 2.5 Hz, 8-H), 1.10 (d, J = 7 Hz, 19-H_3), no signal for 36-H_3 at 0.9.

Compound **21** (5 mg) was reacted with 0.8 ml of pyridine and 0.4 ml of Ac_2O overnight. After usual work-up and purification by tlc, 2.5 mg of the acetylation product **22** was obtained: ms *m/z* $[M]^+$ 714; ^1H nmr data at variance with those described for compound **21** δ 5.55 (s, 5-H), 4.15 ± 0.56 (AB, J_{AB} = 12 Hz, 20-H_2), 2.12 (s, 2 acetates) and (s, 2.07), 1.47 (s, 36-H_3), 1.25 [d, J = 7 Hz, superimposed to $(CH_2)_{7\text{-}9}$, 18-H_3], 1.15 (d, J = 7 Hz, 19-H_3).

ACKNOWLEDGMENTS

The excellent technical assistance of Miss Petra Hofstetter is gratefully acknowledged. We thank Mrs. A. Schrödersecker, Mrs. D. Kucher, Mrs. G. Perthun, and Mrs. B. Schutzius for careful performace in the animal experiments. We appreciate synoptic evaluation of tumor-promoting activities by R. Schmidt and critical reading of the manuscript by R. Schmidt, Dr. M. Fellhauer, and Dr. B. Sorg.

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Received 29 January 1985