Preparation of retinoic acid esters of phorbol derivatives

B. Sorg, G. Fürstenberger, D. L. Berry, E. Hecker, and F. Marks[1]

Institute of Biochemistry, German Cancer Research Center, D-6900 Heidelberg, Federal Republic of Germany, and Western Regional Research Center, Berkeley, CA 94710[2]

Abstract The synthesis of 12-O-retinoylphorbol-13-acetate (RPA), an incomplete tumor promoter (second stage promoter) is described. The preparation starts with phorbol-13-acetate-20-tritylether which is acylated by a carbodiimide method to yield its 12-retinoate. The latter is detritylated by acidic methanol to give RPA. Following an analogous procedure, the 4-methyl-ether of RPA is prepared from 4-O-methylphorbol-13-acetate-20-tritylether.—**Sorg, B., G. Fürstenberger, D. L. Berry, E. Hecker, and F. Marks.** Preparation of retinoic acid esters of phorbol derivatives. *J. Lipid Res.* 1982. **23:** 443–447.

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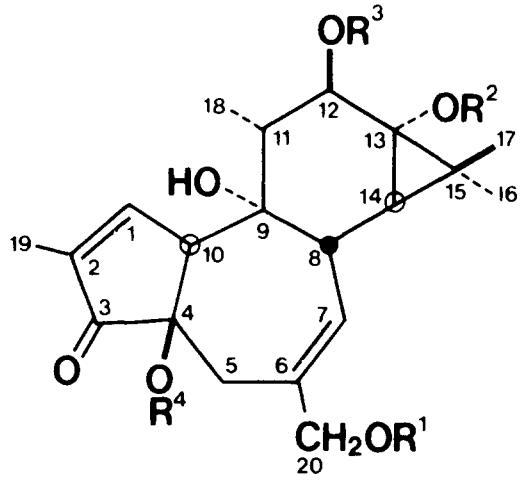
Esters of the diterpene phorbol (*1*) represent a class of compounds which exhibit strong biological effects in a wide variety of cells and tissues in vivo and in vitro (1–3).



1: $R^1 = R^2 = R^3 = R^4 = H$
2: $R^1 = R^4 = H$, $R^2 = CH_3CO$, R^3 = tetradecanoyl ("TPA")
3: $R^1 = R^2 = CH_3CO$, $R^3 = R^4 = H$
4: $R^1 = R^2 = CH_3CO$, R^3 = retinoyl, $R^4 = H$
5: $R^1 = (C_6H_5)_3C$, $R^2 = R^3 = R^4 = H$
6: $R^1 = (C_6H_5)_3C$, $R^2 = CH_3CO$, $R^3 = R^4 = H$
7: $R^1 = (C_6H_5)_3C$, $R^2 = CH_3CO$, R^3 = retinoyl, $R^4 = H$
8: $R^1 = R^4 = H$, $R^2 = CH_3CO$, R^3 = retinoyl ("RPA")
9: $R^1 = R^2 = CH_3CO$, $R^3 = H$, $R^4 = CH_3$
10: $R^1 = R^3 = H$, $R^2 = CH_3CO$, $R^4 = CH_3$
11: $R^1 = (C_6H_5)_3C$, $R^2 = CH_3CO$, $R^3 = H$, $R^4 = CH_3$
12: $R^1 = H$, $R^2 = CH_3CO$, R^3 = retinoyl, $R^4 = CH_3$ ("4-O-Methyl-RPA")

The prototype of these compounds is 12-O-tetradecanoylphorbol-13-acetate (TPA, *2*), the active principle of Croton oil (1, 4). When topically applied to mouse skin, *2* induces inflammation, epidermal cell proliferation, and hyperplasia. Sequential application of *2* onto mouse skin dramatically shortens the latency period of epidermal tumors initiated by chemical carcinogens in subthreshold doses. Since *2* by itself lacks carcinogenic efficacy, it is called a tumor promoter (1).

Studies using various phorbol ester derivatives and other skin mitogens, which evoke only a weak or no promoting efficacy, reveal that irritation and epidermal hyperproliferation elicited by phorbol ester tumor promoters are necessary but not sufficient conditions of tumor promotion (5–7). Study of these compounds called "incomplete tumor promoters" has led to the subdivision of the process of tumor promotion into at least two stages as originally reported by Boutwell (8) and Slaga et al. (9). It was shown that neither a few applications of TPA nor long term treatment with those incomplete promoters were able to promote tumor growth in initiated mouse skin. When, however, both treatments were combined, i.e., a limited number of TPA treatments followed by repeated applications of an incomplete promoter, a strong tumor response was observed. The concept of two-stage

Abbreviations: TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography; NMR, nuclear magnetic resonance; v/s, vanillin/sulfuric acid.

[1] To whom reprint requests should be sent.
[2] D. L. Berry.

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tumor promotion may have important implications for a better understanding of the mechanism of tumor promotion.

One way to weaken or to abolish tumor-promoting efficacy of a phorbol ester without impairing its mitogenic and irritant activities is to introduce double bonds into the long chain fatty acid residue at the position 12 of the phorbol moiety (5). Since the polyunsaturated retinoic acid is a strong inhibitor of skin tumor promotion (10) but not of phorbol ester-induced epidermal hyperproliferation, a phorbol ester carrying a retinoic acid at position 12 may be expected to be a non-promoting skin mitogen. The partial synthesis of the new phorbol ester 12-O-retinoylphorbol-13-acetate (RPA, *8*) and some derivatives thereof (*7, 12*) is reported, as *8* has indeed been found to be a potent irritant skin mitogen without tumor-promoting potency. Moreover, it is the most potent "second-stage promoter" known, in that the repeated administration of *8* to mouse skin accomplishes the promoting effect of only a single TPA (*2*) application. Thus, this compound allows testing of the concept of two-stage tumor promotion as well as the design of experiments that are expected to reveal the events induced by *2* critical for tumor promotion in mouse skin (11).

MATERIALS AND METHODS

Mass and NMR spectra were obtained on Varian MAT (Bremen, Federal Republic of Germany), model MAT 711 and Bruker Physik (Karlsruhe, F.R.G.), model HX 90 spectrometers, respectively. For the NMR spectra, tetramethylsilane was used as internal standard. Analytical HPLC was carried out on a Waters (Bad Königstein, F.R.G.) machine using a 4.6 × 250 mm silica gel column (from Knauer, Oberursel, F.R.G.). The eluent both for analytical and preparative work was nitrogen-purged ethyl acetate–heptane 2:1 (including 0.05 vol % of water). In all cases UV detection was used (254 nm). Preparative HPLC was performed on a DuPont (Bad Nauheim, F.R.G.) preparative chromatograph using a 21.2 × 250 mm (DuPont) or a 16 × 250 mm (Knauer) silica gel column. The fraction giving rise to the largest peak of the chromatogram was collected by hand according to visual inspection. Analytical TLC plates were inspected under UV light (254 nm), sprayed with vanillin/sulfuric acid (v/s), and finally heated to about 120°C for full development of the colors. The buffer used in working up procedures was a phosphate buffer, about pH 7. Sodium sulfate was used for the drying of organic extracts.

Phorbol-13,20-diacetate (*3*)

Ten g (24.4 mmol) phorbol (crystallized from ethanol) (*1*) were suspended in 200 ml of tetrahydrofuran and 200 ml of dichloromethane. The flask was immersed in an ice bath and 77 ml of triethylamine (dissolved in 100 ml of dichloromethane) followed by 52 ml of acetic anhydride (dissolved in 100 ml of tetrahydrofurane) were added in one portion. The mixture was purged with nitrogen and stirred for 24 hr, then placed in an ice bath and roughly neutralized by adding 3 M sulfuric acid. Two hundred ml of water was added thereafter and the aqueous layer was extracted three times with 500 ml of dichloromethane. The combined organic extracts were washed with 1 M hydrochloric acid and then thoroughly stirred (mechanical stirrer) with 250 ml of buffer for about 15 hr. The organic layer was then twice extracted with 150 ml of 5% sodium carbonate solution, washed with buffer, dried, and evaporated. After dissolving in boiling ether, crystallization started at once, yielding 9.37 g (20.92 mmol, 86%) of *3*. For physical data see (12).

Phorbol-20-tritylether (*5*)

To 2.9 g (7.08 mmol) of phorbol (crystallized from ethanol) (*1*) suspended in 10 ml of pyridine, 6.7 g of trityl chloride was added. After 2.5 days of stirring, the mixture was hydrolyzed by adding 50 ml of brine. The aqueous layer was then extracted with 500 ml of ethyl acetate that subsequently was washed with 1 M HCl and buffer, dried, and evaporated. The residue was dissolved in a small volume of trichloromethane and subjected to silica gel column chromatography. Excess trityl alcohol was eluted with trichloromethane. Switching to ethyl acetate, 3.5 g (5.78 mmol; 82%) of TLC-pure *5* was obtained as a resinous material.

Phorbol-13-acetate-20-tritylether (*6*)

To a solution of 2.30 g (3.80 mmol) phorbol-20-tritylether (*5*) (14) in 70 ml of tetrahydrofuran and 70 ml of dichloromethane, 7.54 ml of triethylamine and 5.50 ml of acetic anhydride were added in one portion. After 4 hr stirring, the mixture was diluted with 800 ml of ethyl acetate and extracted wth 1 M hydrochloric acid. The organic layer was thoroughly stirred for 3 hr with 150 ml of buffer, then extracted with 100 ml of 5% sodium carbonate solution, washed with buffer, dried, and evaporated. Crystallization from dichloromethane yielded 2.00 g of *6*. The mother liquor (0.96 g) was separated on a column, affording 0.30 g additional *6*. Total yield: 93%. $F_p(CH_2Cl_2)$: 225–226°C. R_f value: 0.50 (ethyl acetate–petroleum ether 2:1); mass spectrum m/e = 648 (M^+); NMR spectrum ($CDCl_3$-D_2O): δ = 3.99 (d, J = 10 cps, 1 H, 12-H), 3.55 (s, 2 H, 20-H_2), 2.13 ppm (s, 3 H, CH_3CO).

4-O-Methylphorbol-13-acetate (*10*)

Five hundred mg (1.08 mmol) of 4-O-methylphorbol-13,20-diacetate (*9*) (15) was dissolved in 50 ml of a so-

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lution prepared from 1.57 ml of perchloric acid (70%) and 1170 ml of methanol. Twenty hr later, 100 ml of buffer was added and the mixture was extracted twice with ethyl acetate, dried, and evaporated. Separation on a column using ethyl acetate–petroleum ether 2:1 as eluent gave 360 mg (0.86 mmol, 79%) of TLC-pure material.

4-O-Methylphorbol-13-acetate-20-tritylether (*11*)

Three hundred sixty mg (0.86 mmol) of 4-O-methylphorbol-13-acetate (*10*) was dissolved in 2.5 ml of pyridine and 1195 mg of tritylchloride was added. After 3 days of stirring, buffer was added and the mixture was extracted twice with ethyl acetate. The organic layer was washed subsequently with 1 M hydrochloric acid and then with buffer. Separation on a column using dichloromethane–ether 10:1 as eluent yielded 399 mg (0.60 mmol, 70%) *11*; R_f value: 0.30 (ether–petroleum ether 4:1); mass spectrum: m/e = 644 (M^+-H_2O); NMR spectrum ($CDCl_3$): δ = 3.99 (d, J = 10 cps, 1 H, 12-H), 3.57 (s, 2 H, 20-H_2), 3.20 (s, 3 H, CH_3OH), 2.4 ppm (s, 3 H, CH_3CO).

Acylation of *6* affording retinoate *7*

The diacetate *3* and the 4-O-methyl derivative *11* were acylated in an analogous fashion. One hundred sixty-two mg (0.25 mmol) of phorbol-13-acetate-20-tritylether (*6*) was dissolved in 0.25 ml of dichloromethane including 8 mg (0.066 mmol) of 4-(N,N-dimethylamino) pyridine. In a separate flask, 368 mg (1.23 mmol) of retinoic acid was dissolved in 0.5 ml of dry N,N-dimethylformamide and 2 ml of dichloromethane. The solution was chilled to 0°C and 276 mg (1.34 mmol) of N,N′-dicyclohexylcarbodiimide was added. After 10 min, 1.25 ml of this solution was transferred to the phorbol derivative. The flask was briefly purged with nitrogen, protected from light, and stirred for 23 hrs. After this time interval, water and ethyl acetate were added and the organic layer was washed subsequently with 1 M hydrochloric acid and buffer, dried, and finally evaporated to dryness. The crystalline residue, due to the presence of dicyclohexylurea, was suspended in about 10 ml of ether–petroleum ether 4:1 and filtered by syringe through a 0.5 μm filter. The solvent was distilled off, leaving a resinous material that was dissolved in 1–2 ml of ether–petroleum ether 1:3, and introduced onto a 1.5 × 20 cm silica gel column. Elution with ether–petroleum ether 1:3 gave 153 mg of a fraction containing mainly *7*(*7* gives rise to the lowest of the most intense TLC spots, R_f value, and coloration: see below). Subsequent purification on two plates (40 cm × 2 mm), developed in ether–petroleum ether 1:3 gave 112 mg (0.12 mmol, 48% yield) TLC-pure *7*; R_f value: 0.40 ether–petroleum ether 1:1, coloration: yellow; after spraying with v/s intensively red and brownish-red after heating); mass spectrum: m/e = 930 (M^+).

12-O-(all-*trans*)-Retinoylphorbol-13-acetate (RPA, *8*)

One hundred eight mg (0.12 mmol) of *7* were dissolved in 10 ml of a solution prepared from 0.34 ml of perchloric acid (70%) brought to 100 ml with methanol. The mixture was purged with nitrogen, protected from light, and stirred for 45 min. Work-up of the reaction was the same as for *10*. The mixture (124 mg) was separated by TLC or preparative HPLC using ethyl acetate–heptane 2:1 (containing 0.05 vol % of water) as eluent. In the case of HPLC, the mixture was separated in a single run and 48 mg of *8* (0.07 mmol, 58% yield) was obtained; R_f value: 0.35 (ethyl acetate–petroleum ether 2:1, coloration: as *7*); mass spectrum: m/e = 688 (M^+), 670 (M^+-H_2O), 628 (M^+-acetic acid), 389 (M^+-retinoic acid anion); NMR spectrum ($CDCl_3$, see Fig. 1): δ = 7.56 (m, 1-H), 5.65 (m, 7-H), 5.44 (d, J = 10 cps, 12-H), about 4 (broad s, beginning AB character, 20-H_2), 3.25 (m, 8-H, 10-H), 2.75 (m, 19-H_3), 1.10 and 1.15 (s, s, 16/17-H_3), 0.88 ppm (m, 14-H). After shaking with D_2O, changes occur at different positions, the most notable being the disappearance of the singlet at 5.60 ppm (OH-9). Retinoic acid moiety (see (16)): 7.00 (m, 11′-H), 6.43–5.88 (m, 12′-H, 10′-H, 8′-H, 7′-H), 5.72 (m, 14′-H), 2.36 (m, 20′-H_3), 2.00 (m, 19′-H_3), 1.71 (m, 18′-H_3), 1.01 ppm (s, 16′/17′-H_3).

4-O-Methylphorbol-13-acetate-12-(all-*trans*)-retinoate (*12*)

Acylation of *11* was similar to that of *7*. The resulting 12-retinoate was detritylated using conditions corresponding to the preparation of *8* from *7*. R_f value: 0.35 (ethyl acetate–petroleum ether 2:1; coloration: as *7*); mass spectrum: m/e = 702 (M^+); NMR spectrum ($CDCl_3$): δ = 5.44 (d, J = 10 cps, 1 H, 12-H), 4.02 (s, 2 H, 20-H_2), 3.27 (s, 3 H, CH_3O), 2.13 ppm (s, 3 H, CH_3CO).

RESULTS AND DISCUSSION

The partial synthesis of 12-O-retinoylphorbol-13-acetate (RPA; *8*) was carried out as a two-step procedure, starting with phorbol-13,20-diacetate (*3*) (12) or with phorbol-13-acetate-20-tritylether (*6*). *3* was synthesized from phorbol (*1*) by a new selective acetylation procedure for the hydroxyl functions 13 and 20 using limited amounts of acetic anhydride and triethylamine in dichloromethane–tetrahydrofuran. Acylation of *3* to yield its 12-retinoic acid ester *4* was performed with the in situ-formed anhydride of retinoic acid (prepared by the N,N′-dicyclohexylcarbodiimide method), in the presence of the powerful acylation catalyst 4-(N,N-dimethylamino)pyridine (13). Attempts to esterify the 12-OH function by means of the mixed anhydride of tosic and retinoic acid or with retinoic acid imidazolide failed.

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Transesterification of *4* by weakly acidic methanol (12) only yielded impure 12-O-retinoylphorbol-13-acetate (*8*). Since detritylation with acidic methanol occurs much faster than deacetylation, phorbol-13-acetate-20-trityl-ether (*6*) turned out to be a much better starting material. *6* was obtained from the known phorbol-20-tritylether (*5*) (14) using a similar acetylation procedure as that for the synthesis of the diacetate *3* from *1*. *6* was acylated to its 12-retinoate *7* in an analogous fashion to the acylation of the diacetate *3*. *7* was carefully purified by column chromatography and subsequent TLC. The detritylation was performed by mild acidic conditions, yielding 12-O-retinoylphorbol-13-acetate (RPA; *8*). The crude *8* was purified by TLC or by HPLC.

Analogous to RPA (*8*), the 4-O-methyl-RPA (*12*) was prepared by acylation of 4-O-methylphorbol-13-acetate-20-tritylether (*11*) and subsequent detritylation. Synthesis of *11* commenced with the known 4-O-methylphorbol-13,20-diacetate (*9*) (15). The 20-acetate from *9* was cleaved off selectively by means of methanol–perchloric acid (12) to yield 4-O-methylphorbol-13-acetate (*10*). *10* was tritylated by the standard method to give *11*.

In the mass spectrum, RPA (*8*) showed the expected molecular ion m/e = 688. The NMR spectrum (**Fig. 1**) was in full agreement with the expected structure of 12-O-(all-*trans*)-retinoylphorbol-13-acetate (RPA; *8*). Concerning the all-*trans*-retinoate, conclusions can be drawn from the fact that all-*trans*-retinoic acid and its methyl ester produce very similar NMR spectra to the non-diterpene part of the NMR-spectrum of *8* (Fig. 1). This is clearly noticeable in the region above 5.5 ppm, where, as has previously been shown (16), isomeric methyl retinoates differ in a clearcut manner. 4-O-methyl-RPA (*12*) displayed a molecular ion of m/e = 702 in the mass spectrum and the NMR spectrum proved the structure to be the 4-methyl ether of RPA (*8*).

As shown in **Table 1,** RPA did not produce tumors when repeatedly applied in a two-stage carcinogenesis

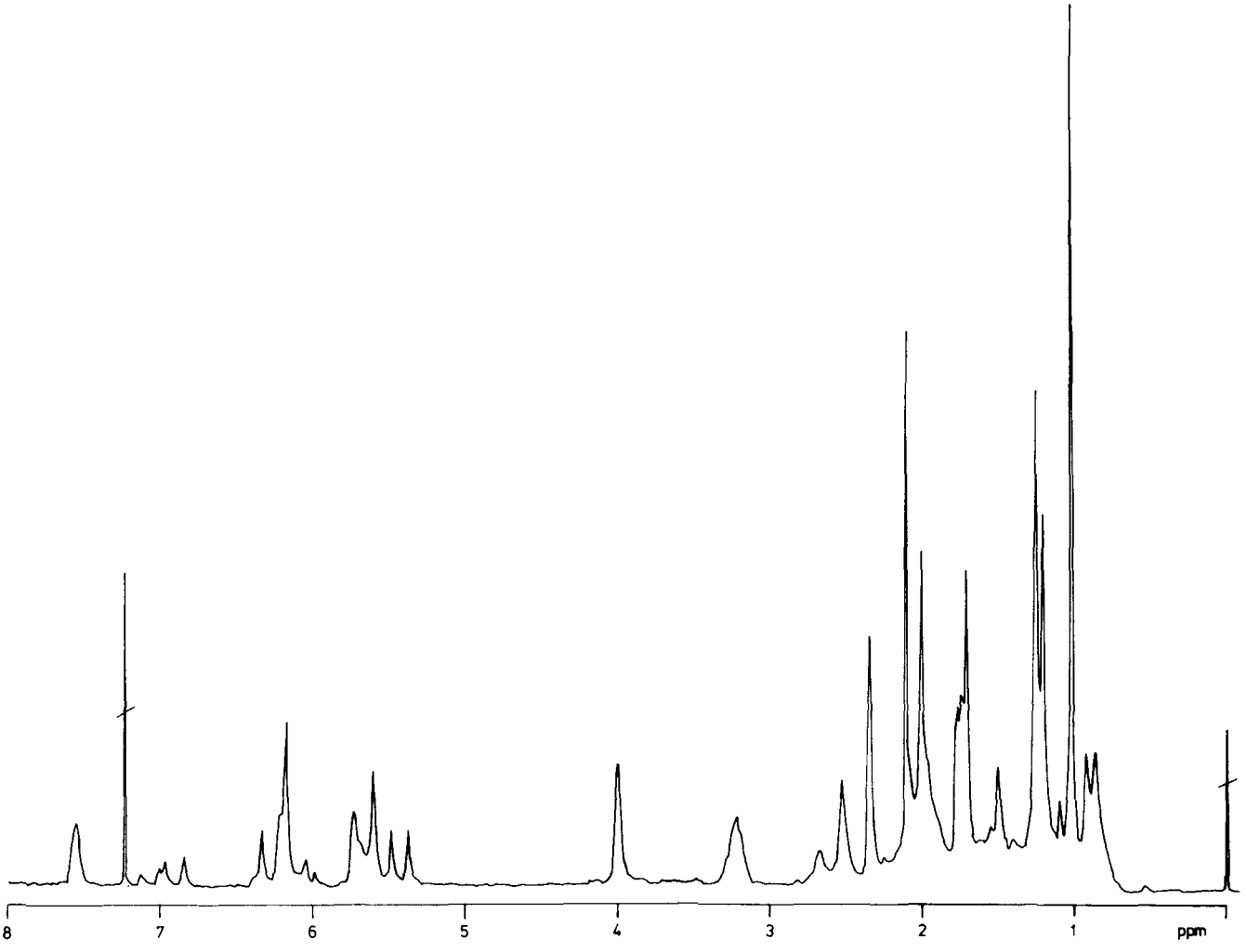

Fig. 1. ^{1}H-NMR spectrum ($CDCl_3$) of 12-O-(all-*trans*)-retinoylphorbol-13-acetate (RPA, *8*).

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TABLE 1. Two-stage tumor promotion in mouse skin

Treatment		Tumor Development					
		After 12 Weeks		After 15 Weeks		After 18 Weeks	
First	Second	Rate	Yield	Rate	Yield	Rate	Yield
TPA (1×; 10 nmol)	TPA (35×; 10 nmol)	93	8.1	93	8.4	93	8.5
TPA (1×; 20 nmol)	Acetone (35×)	0	0	0	0	0	0
TPA (2×; 20 nmol)	Acetone (35×)	0	0	0	0	0	0
Acetone (2×)	RPA (34×; 10 nmol)	0	0	0	0	0	0
TPA (1×; 20 nmol)	RPA (35×; 10 nmol)	38	2.8	50	3.4	60	4.1
TPA (2×; 20 nmol)	RPA (34×; 10 nmol)	50	2.7	75	5.0	94	5.7

For each group, 16 female NMRI mice (7 weeks old) were initiated by topical application of 100 nmol dimethylbenz(a)anthracene (dissolved in 0.1 ml of acetone) on the shaved back skin. One week later, the treatments with acetone, TPA, and RPA were started. At the end of the experiment, 94% of the animals were alive. The tumor-promoting activity was measured as tumor rate (tumor bearing animals/survivors) and as tumor yield (number of tumors/survivors). For other details of the two-stage carcinogenesis experiment see Ref. 11.

experiment. When, however, one or two applications of the strong promoter TPA, which by themselves did not promote tumor growth, were followed by repeated administrations of RPA, a strong tumor response was observed which was comparable to that obtained by respective TPA applications. The characterization of RPA as an "incomplete tumor promoter" thus strongly supports the concept of two-stage tumor promotion (9, 11).

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