

Letter to the Editor

## Autoxidation of Phorbol Esters under Normal Storage Conditions

The polyfunctional diterpene phorbol and its esters are highly susceptible to autoxidation (2, 3). The widespread use, partially in long-term experiments, of the standard tumor promotor TPA<sup>1</sup> (Chart 1) and other phorbol- or 4 $\alpha$ -phorbol-12,13-diester in cancer and cell research and, in particular, in studies on their metabolism and mechanism of action (4-6) prompted us to investigate in detail the various products arising from TPA and 4 $\alpha$ -phorbol-12,13-diacetate by autoxidation under different conditions of storage. It was found that, in addition to traces of polar products not isolated, the aldehyde **2**, the  $\beta$ -epoxide **3**, the hydroperoxide **4**, the hydroxylated  $\Delta^{5,6}$  isomer **5**, the ketone **6**, and the 6,7-seco hemiacetal **7** are being formed from TPA (Charts 1 and 2).

The structures of the compounds shown in the formulas (except that of the hydroperoxide **4**, which was not isolated) were rationalized by their infrared, mass, nuclear magnetic resonance, and UV spectra and will be discussed elsewhere. Also the results of experiments on the potential irritant and cocarcinogenic activities of **2**, **3**, and **6** to mouse skin will be published separately.

TLC is an efficient means of checking impure samples of TPA for any of the possible compounds formed by autoxidation, as may be seen from Table 1.

### Autoxidation of TPA

**In Solution (Table 2).** Solutions of TPA in acetone, ethyl acetate, or methylene chloride (0.2 mM) were stored at 25° in diffuse daylight and at 4° and -20°, respectively, in the dark.

After 3 months, the solutions kept at 25° showed appreciable amounts of autoxidation products. It is remarkable that autoxidation is most rapid in acetone solution, the  $\beta$ -epoxide **3** being formed as the main product. Autoxidation is slower in ethyl acetate and methylene chloride. If the solutions are kept at 25° in the dark, the extent of autoxidation is reduced.

Only traces of autoxidation products were detected in the solutions stored in the dark at 4° and essentially no degradation occurred in the dark at -20°.

A solution of TPA in DMSO (20 mM), kept at 25° in diffuse daylight, was found to be unchanged for at least 2 weeks; after 3 months under the same conditions, small amounts of the aldehyde **2**, the  $\Delta^{5,6}$ -7-hydroxy compound **5**, and traces of the ketone **6** were found. On keeping DMSO solutions frozen at -20° in the dark, essentially no degradation of TPA was observed for at least 6 months. This finding

<sup>1</sup> The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; TLC, thin-layer chromatography; DMSO, dimethyl sulfoxide.

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is in contrast to the conclusions drawn from a high-pressure liquid chromatography investigation by Rao *et al.* (7). They reported that TPA would be stable in DMSO at 20° for a period of 1 year. We assume that the separation system used by these authors, using diethyl ether:methanol (9:1) as eluent, does not resolve the individual autoxidation products of TPA, as indicated by their chromatogram of phorbol and TPA. Also we found on TLC, with diethyl ether:methanol (9:1) as solvent, that compounds **1** to **7** migrate as 1 band with the front.

**In Solid Form (Table 2).** If stored as a powder at 25° in diffuse daylight for 3 months, TPA is converted preferentially into the hydroperoxide **4** (Chart 2), which appears to be rather stable. Within the same time period, at 4° in the dark this reaction takes place only to a very limited extent. It was not observed at -20° in the dark.

If spread on a large surface as a thin film (on glass plates, beads, or silica gel thin layers) and kept in diffuse daylight at 25°, TPA is oxidized very rapidly. In this case, the 6,7-seco hemiacetal **7** is formed preferentially, in addition to

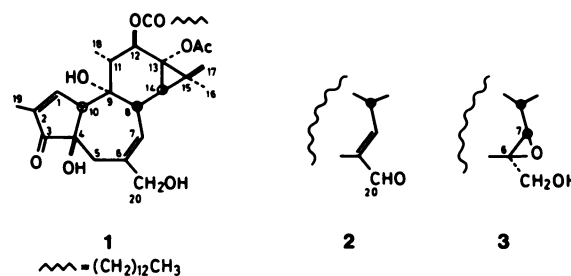


Chart 1. Structures of TPA **1**, aldehyde **2**, and epoxide **3**.

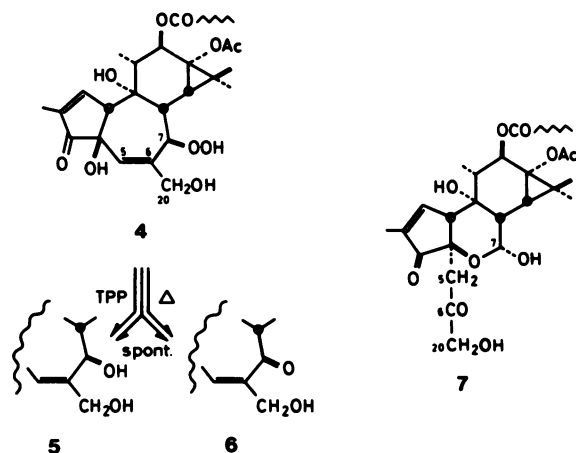


Chart 2. Decomposition of the hydroperoxide **4** to yield hydroxy compound **5** and ketone **6**, respectively, and structure of the 6,7-seco compound **7**.

Table 1  
Characteristics of autoxidation products of TPA in TLC

Compound	R <sub>F</sub> values <sup>a</sup> in Systems			Color <sup>b</sup>	M.W. (m/e) <sup>c</sup>
	A	B	C		
TPA ( <u>1</u> )	0.55	0.67	0.21	Rusty brown	616
Aldehyde ( <u>2</u> )	0.93	0.93	0.79	Gray-brown	614
$\beta$ -Epoxide ( <u>3</u> )	0.63	0.74	0.24	Dark brown	632
Hydroperoxide ( <u>4</u> )	0.75	0.81	0.29	Violet	NI <sup>d</sup>
$\Delta^{5,6}$ -7-OI ( <u>5</u> )	0.24	0.46	0.07	Light brown	632
$\Delta^{5,6}$ -7-On ( <u>6</u> )	0.83	0.91	0.36	Violet	630
Seco-compound ( <u>7</u> )	0.30	0.52	0.16	Green-brown	630 (M <sup>+</sup> - 18)

<sup>a</sup> TLC on precoated plates, silica gel 60 HF 254 (E. Merck, Darmstadt, West Germany). A, dry diethyl ether; B, diethyl ether, water saturated; C, chloroform:ethyl acetate (3:2). Each system without chamber saturation (solvents are of analytical grade; E. Merck).

<sup>b</sup> On spraying the plates with vanillin:sulfuric acid and exposure to 110°.

<sup>c</sup> Determined by mass spectrometry.

<sup>d</sup> NI, not isolated.

Table 2  
Estimated relative amounts of autoxidation products of TPA stored under various conditions

Compound	Relative amounts (%) <sup>a</sup>							
	Stored for 3 mos. at 25° in diffuse daylight					After 4 days in diffuse daylight as thin solid film on		
	In solution				As solid (powder)	Glass plate	Glass beads	Silica gel
	Acetone <sup>b</sup>	Ethyl acetate <sup>b</sup>	Methylene chloride <sup>b</sup>	DMSO <sup>c</sup>				
<u>2</u>	10	5	5	<5	5	5	5	5
<u>3</u>	25	<5	<5	—	<5	5	5	5
<u>4</u>	— <sup>d</sup>	—	—	—	15	5	<5	<5
<u>5</u>	—	—	—	5	Traces	Traces	<5	<5
<u>6</u>	<5	5	5	Traces	<5	5	5	5
<u>7</u>	—	—	—	—	Traces	30	10	20

<sup>a</sup> Estimated from TLC (Table 1, System B) by relative intensities of the spots in UV light (254 nm), referring to TPA spot as reference.

<sup>b</sup> At a concentration of 0.2 mM (applied to TLC plates after concentrating 2-ml equivalents to dryness in a vacuum and dissolving the residue in 50  $\mu$ l of ethyl acetate).

<sup>c</sup> At a concentration of 20 mM (applied directly to TLC plates).

<sup>d</sup> —, no detectable spot on TLC.

the different products obtained from dissolved and powdered TPA (see Table 2). This rapid conversion is dependent on light and the reaction is slowed down in the dark.

### Autoxidation of 4 $\alpha$ -Phorbol

In contrast to the variety of autoxidation products obtained from TPA, a sample of 4 $\alpha$ -phorbol-12,13-diacetate yielded the corresponding 20-aldehyde as the only conversion product after storage for 1 year at room temperature in the dark (yield, about 30%). Storage in the dark is mandatory to avoid the known "lumi reaction" of 4 $\alpha$ -phorbol, which is independent of the presence of oxygen (1, 3).

### Partial Synthesis of Autoxidation Products

**Hydroxy Compound 5 and Ketone 6.** Dye-sensitized photooxygenation of TPA, using a hematoporphyrin:pyri-

dine solution and the light of a fluorescent lamp (with maxima at about 480 and 580 nm), gives rise to a mixture of the hydroperoxide 4 and the ketone 6. After reflux of the irradiated solution for 15 min, ketone 6 is obtained as the only product; treatment of the isolated reaction mixture with triphenylphosphine leads to reduction of 4 giving rise to 5, whereas 6 remains unchanged (Chart 2).

**Aldehyde 2 and  $\beta$ -Epoxide 3.** The aldehyde 2 is obtained by reaction of TPA either with manganese dioxide or with the chromium trioxide:pyridine reagent (3). Treatment of TPA with perbenzoic acid yields the corresponding  $\beta$ -epoxide.

The compounds obtained by partial synthesis are identical (TLC, mass, infrared, nuclear magnetic resonance) to those isolated from autoxidized TPA.

As a result of this investigation, it is strongly recommended to store TPA (and related phorbol-12,13-diester, as well as phorbol itself) either as a solid or in solution at -20° in the dark. For most purposes stock solutions in

acetone, methylene chloride, ethyl acetate, or DMSO may be used up to about 3 months, if stored at 4° in the dark. At room temperature, solutions of TPA in methylene chloride and ethyl acetate (at concentrations of 0.2 mM) or DMSO (20 mM) do not show any detectable autoxidation if stored in diffuse daylight for about 14 days; acetone solutions should not be stored under these conditions.

For a rapid check of purity of TPA, TLC on silica gel using water-saturated diethyl ether as solvent is recommended. The purification method of choice is preparative TLC on silica gel using 2 successive runs in hexane: peroxide-free diethyl ether (1:5) as solvent.

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