

# Triterpene Biosynthesis in the Latex of *Euphorbia lathyris*

## CALMODULIN ANTAGONISTS ARE INEFFECTIVE IN WHOLE LATEX

Received for publication March 24, 1986 and in revised form September 1, 1986

GEORGE J. PIAZZA\*, EDWARD J. SAGGESE, AND KATHLEEN M. SPLETZER

United State Department of Agriculture, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118

### ABSTRACT

The calmodulin antagonists chlorpromazine, fluphenazine, trifluoperazine, and 2-(pentachlorophenoxy)ethyl *N,N*-diethylamine are not inhibitors of acetate incorporation into triterpenols (TOH) and their fatty acid esters (TE) in whole tapped latex from *Euphorbia lathyris*, although prior work demonstrated that these antagonists are good inhibitors of mevalonate incorporation into TOH and TE in a centrifuged pellet from the latex. Antagonist absorption into the endogenous terpene pool is the primary reason for antagonist ineffectiveness in whole latex; changes in the utilized substrate or chemical deactivation of the antagonists were ruled out as factors. A biosynthetically inactive, latex supernatant fraction containing the endogenous terpene pool was prepared. This fraction blocks antagonist action when added to the latex pellet, and proved to be a useful tool for demonstrating that inhibition of triterpene biosynthesis by a calmodulin antagonist is partially reversible.

The tapped latex of *Euphorbia lathyris* can convert acetate to several structurally different tetracyclic triterpenes. In prior studies, two major classes of triterpenes were found to be synthesized *in vitro*: TOH<sup>1</sup> and TE (3, 4). In tapped latex, the terpenoid skeleton, but not the fatty acid moiety of TE, is derived from acetate (3); the fatty acids needed for ester formation are derived from endogenous phospholipids. The pellet from a low speed centrifugation can efficiently utilize mevalonate as a precursor of triterpenes and their esters. In our previous paper (4) we showed that calmodulin antagonists (chlorpromazine, fluphenazine, and trifluoperazine) are very effective inhibitors of triterpene biosynthesis in the latex pellet. Out of several chlorinated phenoxy compounds that were tested, the best calmodulin antagonist, 2-(pentachlorophenoxy) ethyl *N,N*-diethylamine, was the best inhibitor of triterpene biosynthesis. In this paper we present the results of experiments in which calmodulin antagonists and several chlorinated phenoxy compounds were tested as inhibitors of triterpene biosynthesis in whole latex.

### MATERIALS AND METHODS

**Materials.** The sodium salt of [<sup>3</sup>H]acetic acid (100 mCi/mmol), [benzene ring-<sup>3</sup>H]chlorpromazine hydrochloride (22.4 Ci/mmol), and Aquassure were purchased from DuPont NEN Products.<sup>2</sup> High efficiency reverse phase thin layer plates

(HETLC-RPSF) and Silica gel G thin layer plates were from Analtech. Microcrystalline cellulose thin layer plates were from Applied Science. Other materials were obtained as previously described (4).

**Triterpene Biosynthesis in Whole Latex.** Radiolabeled sodium acetate, the compound under test dissolved in methanol, and an equivalent amount of NaOH (if the hydrochloride salt of the compound was used) were placed in a 12 × 75 mm borosilicate culture tube. The solution was dried under a stream of N<sub>2</sub>. To the dried tube was added 20 μl DTT and MgCl<sub>2</sub> dissolved in water, and 200 μl freshly tapped latex. The final concentrations were 8.3 mM acetate (0.2 mCi), 5 mM MgCl<sub>2</sub>, 3 mM DTT, and 0.2 mM of the tested compound. After 3 h at room temperature the latex was placed in a boiling water bath to stop the reaction, and acetate incorporation into TOH and TE was determined as previously described (4).

**Partitioning of Compound II (Chlorpromazine) in Latex.** Separate samples were prepared for each indicated time point containing 0.2 mM compound II (2 × 10<sup>5</sup> dpm) in 100 μl freshly tapped latex. At the indicated time, the sample was diluted with 200 μl of a buffer containing 10 mM Na-phthalate (pH 5.5), 10 mM MgCl<sub>2</sub>, 10 mM KCl, 30 mM CaCl<sub>2</sub>, and 0.4 M sorbitol. The sample was centrifuged for 5 min at 8800g. The supernatant was withdrawn, and the pellet was resuspended in 200 μl of the water. This suspension was transferred to a vial and sonicated for 10 min. The supernatant was boiled and recentrifuged for 15 min. The aqueous fraction of the supernatant was transferred to a vial. The terpene layer was dissolved in 1 ml of isopropanol and transferred to a vial. After adding 18 ml Aquassure, the radioactivity in the pellet, aqueous fraction of the supernatant, and terpene fraction was determined.

**Analysis of Compound II Decomposition Products.** After incubating 0.2 mM compound II in latex for 3 h, the latex was fractionated by a procedure identical to that described above, except that the latex volume was doubled and radiolabeled compound II was increased 100-fold. A portion of each fraction (pellet, aqueous fraction of the supernatant, and terpene layer) was applied to a thin layer plate, and after development the position of radioactivity on the plate was determined by a LB 2832 Berthoid automatic TLC linear analyzer. TLC systems: (a) reverse phase: i) methanol, water, ammonium hydroxide (50:50:0.5), ii) ethanol, ammonium hydroxide (100:0.5); (b) cellulose: methanol, diethyl ether, ammonium hydroxide (15:25:0.5); and (c) Silica Gel G: ethyl acetate, ethanol, butanol, ammonium hydroxide (60:40:15:1). The R<sub>F</sub> values of compound II in TLC systems a, b, and c were 0.41, 0.89, and 0.49, respectively.

**Time Course of Triterpene Biosynthesis in the Presence of Compound II and Latex Supernatant.** A centrifuged latex pellet was prepared from 2 ml of latex as described before (4), and resuspended in 700 μl reaction buffer containing 3.6 μM radio-

<sup>1</sup> Abbreviations: TOH, triterpenols; TE, fatty acid esters of triterpenols.

<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

labeled mevalonate (0.07 mCi), 3 mM DTT, and 0.1 mM *S*-adenosylmethionine. At each time point up to 105 min, a 75  $\mu$ l aliquot was withdrawn and placed in a boiling water bath. After 60 min an aqueous solution (40  $\mu$ l) of compound II (pH 5.5) was added to the latex pellet; final concentration: 0.2 mM. After 105 min the latex supernatant (250  $\mu$ l) and additional compound II (21  $\mu$ l—concentration maintained at 0.2 mM) were added to the latex pellet. After addition of the latex supernatant, a 150  $\mu$ l aliquot was withdrawn and placed in a boiling water bath at the indicated time. All aliquots were analyzed for incorporation of mevalonate into TE and TOH. Incorporation was corrected for dilution by compound II or latex supernatant. The time course experiment was repeated four times. Each data set was normalized to a constant scale by multiplying the data set by an appropriate factor, such that the average incorporation at the 75, 90, and 105 min time points was set equal. This procedure is valid since only the relative rate of incorporation in the presence of inhibitor or supernatant is of interest.

## RESULTS

Table I shows results of experiments with whole latex (first column). In these experiments the extent of incorporation of radiolabeled acetate into TOH and TE in 3 h was measured in the presence of the compound under test and expressed as a percent of a control containing no added compound. The data obtained with the centrifuged latex pellet (4) are shown for comparison in the second column. The most striking aspect of the data in Table I is that in whole latex all of the tested compounds have little effect on triterpene biosynthesis, although some compounds (II, III, IV, VIII, and IX) strongly inhibited biosynthesis in the centrifuged latex pellet system.

Another notable aspect of the data is that there is more experimental variability in the whole latex assays. To achieve errors of approximately 10%, 12 to 18 repetitions were necessary with whole latex, about 4 times more than required in the latex pellet assays. At least in part, the higher variability in the whole

latex assays is due to the relatively low specific activity of precursor acetate as compared to the mevalonate utilized in the latex pellet assays. Each experimental repetition resulting in the data displayed in Table I was performed with a different latex batch. To test whether variability could result from the use of different latex batches, an experiment was performed with a single batch of latex, divided into fractions to give 10 separate assays. The variability in the resulting data was identical to that where separate latex batches were used (data not shown). Thus, latex batch variability is not a factor in the observed data variability.

According to the accepted metabolic pathway for triterpene formation acetate precedes mevalonate as a precursor. Therefore, use of precursor acetate in the whole latex experiments (mevalonate was used as precursor in the latex pellet assays) should not be responsible for abolition of the effectiveness of compounds II, III, IV, VIII, or IX. To test this theory, compound IV was added to whole latex with mevalonate as the precursor. The results of five repetitions were TOH:  $103 \pm 11\%$  and TE  $98 \pm 12\%$ , results that are identical within experimental error to those shown in Table I with acetate as precursor.

The partitioning of radiolabeled compound II (chlorpromazine) in whole latex was determined according to the protocol given in "Materials and Methods." Figure 1 shows the results of one typical experiment. The majority of compound II partitioned into the terpene fraction. The pellet fraction (the site of triterpene biosynthesis from mevalonate) contained the least amount of compound II. Over the time period of 15 to 108 min this pattern did not change significantly.

The oxidation product of compound II, chlorpromazine sulfide, is a relatively poor antagonist of calmodulin (5). The bio-oxidation of compound II has been shown to occur both *in vivo* (2) and *in vitro* (1). Thus it could be hypothesized that compound II is inactive as an inhibitor in whole latex because it is rapidly oxidized. Accordingly, compound II was incubated in latex for 3 h (the usual time of the triterpene biosynthetic assays). The

Table I. Effect of Compounds on Triterpene Biosynthesis

Compound		Triterpene Biosynthesis				
		Whole latex <sup>a</sup>		Latex pellet		
		%	N <sup>b</sup>	%	N <sup>b</sup>	
I	Calmidazolium	TOH	114 $\pm$ 11	14	120 $\pm$ 11	5
		TE	112 $\pm$ 10		52 $\pm$ 3	
II	Chlorpromazine	TOH	91 $\pm$ 13	13	9 $\pm$ 2	3
		TE	84 $\pm$ 11		7 $\pm$ 1	
III	Fluphenazine	TOH	95 $\pm$ 14	13	31 $\pm$ 3	4
		TE	91 $\pm$ 5		26 $\pm$ 8	
IV	Trifluoperazine	TOH	97 $\pm$ 14	14	3 $\pm$ 1	3
		TE	104 $\pm$ 7		1 $\pm$ 1	
V	2,6-dichloroanisole	TOH	106 $\pm$ 10	12	99 $\pm$ 3	4
		TE	97 $\pm$ 9		87 $\pm$ 7	
VI	2-(2,6-dichlorophenoxy)-ethyl <i>N,N</i> -diethylamine	TOH	97 $\pm$ 10	18	87 $\pm$ 6	4
		TE	121 $\pm$ 9		72 $\pm$ 8	
VII	2-(3,4-dichlorophenoxy)-ethyl <i>N,N</i> -diethylamine	TOH	79 $\pm$ 10	14	61 $\pm$ 7	4
		TE	111 $\pm$ 6		79 $\pm$ 8	
VIII	2-(2,4,5-trichlorophenoxy)-ethyl <i>N,N</i> -diethylamine	TOH	102 $\pm$ 11	13	17 $\pm$ 6	4
		TE	108 $\pm$ 9		20 $\pm$ 4	
IX	2-(pentachlorophenoxy)-ethyl <i>N,N</i> -diethylamine	TOH	84 $\pm$ 8	15	1 $\pm$ 1	3
		TE	88 $\pm$ 7		2 $\pm$ 1	

<sup>a</sup> Control incorporation rates: TOH, 0.015–0.045; TE, 0.06–0.12 nmol h<sup>-1</sup> ml<sup>-1</sup> latex. <sup>b</sup> N is the number of experimental repetitions.

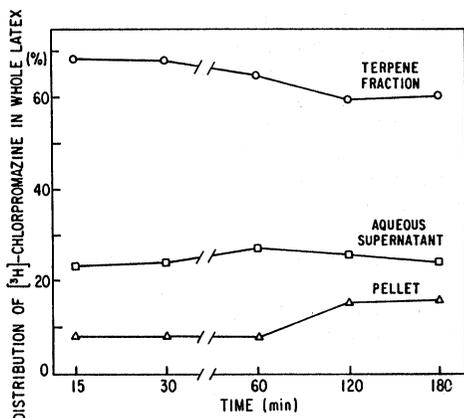


FIG. 1. The partitioning of [<sup>3</sup>H]chlorpromazine in whole latex. The data are derived from one representative experiment.

Table II. Effect of Latex Supernatant on Compound II-inhibited Triterpene Biosynthesis by Latex Pellet

Experiments were performed in the presence of 0.2 mM compound II, and the results compared to control containing no added compound II as described in the text.

	Pellet <sup>a</sup>	Pellet + Supernatant	Pellet + Supernatant (corrected) <sup>b</sup>
	%		
TOH	19 ± 4	182 ± 28	161 ± 24
TE	14 ± 3	176 ± 23	165 ± 21

<sup>a</sup>Each value represents an average ± SE of eight determinations. <sup>b</sup>Correction was for terpenoid biosynthesis occurring in the added supernatant.

latex was fractionated and a portion of each fraction was analyzed by TLC. Samples that were analyzed immediately after the 3 h incubation showed only one peak corresponding to unchanged chlorpromazine in the three different TLC systems described in "Materials and Methods" (data not shown). It should be noted, however, that if samples were stored frozen and repeatedly analyzed, then a metabolite of compound II was detected by TLC. Thus, compound II may be metabolized by latex, but this metabolism is slow, slow enough to be of no consequence in the triterpene biosynthetic assays.

The partitioning experiments (Fig. 1) showed that the majority of compound II partitioned into the terpene fraction of whole latex. It was reasoned that if the terpene fraction was mixed with the centrifuged pellet, the inhibitory effects of compound II on triterpene biosynthesis should be reduced or eliminated. After the supernatant is boiled, the isolated terpene fraction does not redisperse in an aqueous buffer. Even if the supernatant is boiled, but not centrifuged, the supernatant is no longer colloidal, and the terpenes flocculate into nonsoluble, large, visible particles. Neither the isolated terpenes nor the boiled supernatant were able to significantly overcome compound II inhibition of triterpene biosynthesis.

Experiments were conducted in which the supernatant (not boiled) was added to compound II followed by the addition of the pellet. Triterpene biosynthesis, with mevalonate as precursor, was restored by this treatment (Table II). Experiments were conducted containing the supernatant and compound II (no added pellet) to confirm that triterpene biosynthesis did not arise from the added supernatant and did, in fact, occur in the pellet. Biosynthesis from the supernatant alone was very poor, and the right hand column of Table II displays data corrected for the small supernatant contribution. Corrected biosynthetic rates of the pellet plus supernatant containing compound II, are greater

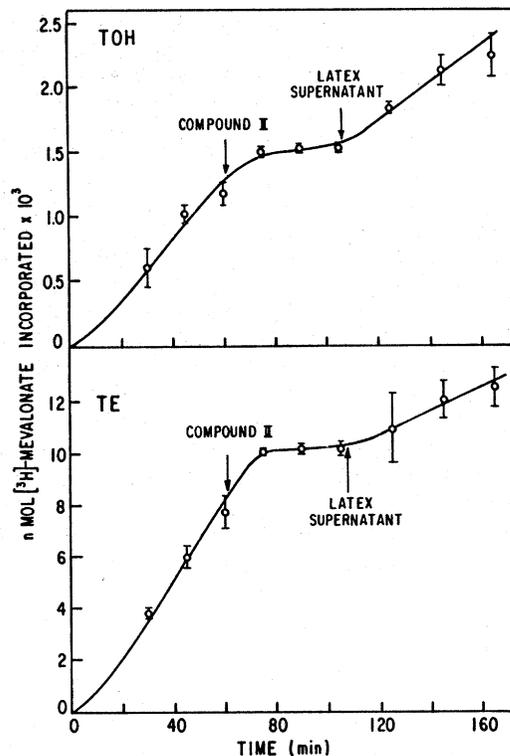


FIG. 2. The influence of compound II and latex supernatant on the time course of terpenoid biosynthesis. Each value is the mean of four normalized experiments. Bars represent the SE of the mean.

than control rates (pellet plus no added compound II).

Since latex supernatant can block compound II inhibition, an experiment was designed to test whether latex supernatant could reverse compound II inhibition: compound II was added to a latex pellet actively synthesizing triterpenes, and after full inhibition was achieved, latex supernatant was added. Figure 2 shows the average result of four experiments normalized to the same scale as described in "Material and Methods." About 15 min was required for full inhibition of biosynthesis by compound II (final concentration: 0.2 mM). Addition of latex supernatant partially restored TOH biosynthesis to 63% of the initial rate (Fig. 2, top). The rate of TE biosynthesis in the presence of latex supernatant recovered only partially to 30% of the rate before compound II was added (Fig. 2, bottom).

## DISCUSSION

The data displayed in Table I show that effective inhibitors of triterpene biosynthesis in latex pellet assays (compounds II, III, IV, VIII, and IX) are rendered ineffective in whole latex assays. With mevalonate as the precursor (rather than acetate) compound IV was an ineffective inhibitor in tapped latex, thus ruling out the possibility that the use of acetate as a precursor is responsible for the abolition of the effectiveness of the inhibitors. No breakdown or metabolism of compound II could be detected during the assays. Thus, chemical degradation or inactivation of the inhibitors does not explain their ineffectiveness in whole latex.

Compound II was found to be absorbed by the terpene fraction of latex. The word "terpene" is used loosely here to denote the water insolubles (after boiling) of latex supernatant. This fraction contains over 90% of the endogenous latex triterpenes, phospholipids, and may contain other compounds as well. The partitioning data shown in Figure 1 should not be taken as an absolute measurement of the distribution of compound II in whole latex

since no data is available to show that the procedures used to partition the various fractions do not change the distribution of compound II. Nevertheless, considering all of the data, absorption of the inhibitors and a resultant lowering of their free concentration is the best explanation for their ineffectiveness in whole latex.

An experimental result that corroborates this explanation was that the addition of latex supernatant (still in the colloidal state, not boiled) completely overcame all compound II inhibition (Table II). Insufficient surface area for absorption is a reasonable explanation for the inability of the isolated, noncolloidal terpenes to effectively block compound II inhibition.

The data in Figure 2 show that compound II inhibition of TOH and TE biosynthesis is partially reversible. The inability of the latex supernatant to completely reverse inhibition may be due to very tight binding of compound II to the active site of biosynthesis. The latex supernatant could therefore not remove enough inhibitor to completely reactivate biosynthesis. The reversible portion of TOH and TE biosynthesis is consistent with the simple binding of compound II to its site of action. As was

discussed before (4), this site of action is probably calmodulin.

Calmodulin antagonists are molecules with hydrophobic properties and as such can nonspecifically interact with other hydrophobic components of a cell. As far as we are aware this is the only reported case in which these interactions are of such severity that antagonist action is completely blocked. It is possible that calmodulin antagonists may not be effective probes in other lipid-rich plant systems.

#### LITERATURE CITED

1. CAVANAUGH DJ 1957 Oxidation of chlorpromazine by peroxidase and catalase. *Science* 125: 1040-1041
2. CHAN TL, G SAKALIS, S GERSHON 1974 Quantitation of chlorpromazine and its metabolites in human plasma and urine by direct spectrodensitometry of thin-layer chromatograms. *Adv Biochem Psychopharmacol* 9: 323-333
3. NEMETHY EK, C SKRUKRUD, GJ PIAZZA, M CALVIN 1983 Terpenoid biosynthesis in *Euphorbia* latex. *Biochim Biophys Acta* 760: 343-349
4. PIAZZA GJ, EJ SAGGESE, KM SPLETZER 1987 Triterpine biosynthesis in the latex of *Euphorbia lathyris*: effect of calmodulin antagonists and chlorinated phenoxy compounds. *Plant Physiol* 83: 177-180
5. WEISS B, W PROZIALECK, M CIMINO, MS BARNETTE, TL WALLACE 1980 Pharmacological regulation of calmodulin. *Proc NY Acad Sci* 356: 319-345