Triterpene Biosynthesis in the Latex of *Euphorbia lathyris*

EFFECT OF CALMODULIN ANTAGONISTS AND CHLORINATED PHENOXY COMPOUNDS

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ABSTRACT

Recognized calmodulin antagonists and chlorinated phenoxyalkylamines were tested as inhibitors of mevalonate incorporation into triterpenols and their fatty acid esters in a centrifuged pellet from the latex of *Euphorbia lathyris*. The calmodulin antagonists, chlorpromazine (II), fluphenzine, and trifluoperazine were good inhibitors; I_{50} values for II and trifluoperazine were 150 and 55 micromolar, respectively. Inhibition by the phenoxyalkylamines increased with increasing chlorine substitution, and I_{50} for 2-(pentachlorophenoxy)ethyl *N,N*-diethylamine (IX) was 35 micromolar. The calmodulin-stimulated phosphodiesterase catalyzed hydrolysis of cAMP was used as an assay to quantitate the calmodulin antagonism of the tested compounds. Compounds II and IX were calmodulin antagonists over a concentration range similar to their effective range in the biosynthesis of triterpenes. The antagonism of the chlorinated phenoxy compounds increased in parallel to their inhibitory effect upon triterpene biosynthesis.

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[1] and TE (6, 9, 11). Biosynthetic activity was optimum in diluted latex with 0.4 M sorbitol as a component of the diluting buffer, indicating that an osmotically sensitive organelle is involved in triterpene biosynthesis (9). Ponsinet and Ourisson (11) have shown that simple low speed centrifugation of the latex from several *Euphorbia* species disrupts triterpene biosynthesis when acetate is the precursor, presumably by separating portions of the biosynthetic pathway. The pellet from such a simple centrifugation, containing the osmotically sensitive organelle, will efficiently incorporate mevalonate into triterpenes.

Calcium constitutes 7.5% of the dry latex weight and could be important in latex biochemistry (9). Recently the calcium binding protein, calmodulin, has been isolated and purified from *E. lathyris* (10); its amino acid composition is similiar to that of other higher plants. Using gel electrophoresis with *E. lathyris* calmodulin as a standard, it has been shown that the tapped latex contains calmodulin.

Although it is not known if calmodulin plays an important role in the regulation of triterpene biosynthesis in plant latex, there is some evidence that calmodulin participates in mammalian cholesterol biosynthesis. For example, it has been demonstrated that hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase is phosphorylated by a Ca^{2+}, calmodulin dependent kinase *in vitro* (2). Also, antagonists of calmodulin inhibit cholesterol biosynthesis from either acetate or mevalonate in human skin fibroblasts, indicating that calmodulin may be involved in the regulation of an additional enzyme activity further along the pathway than reductase (5).

In the study presented here, we have examined triterpene biosynthesis in a latex pellet in the presence of antagonists of the calcium binding protein, calmodulin. We have used known calmodulin antagonists, the phenothiazines and calmidazolium, as well as chlorinated phenoxyalkylamines which possess a hydrophobic moiety and a positively charged group, two features which make them potential calmodulin inhibitors (13, 17). In addition, we examined the effects of these compounds on calmodulin mediated phosphodiesterase activity.

MATERIALS AND METHODS

Materials. Bovine brain calmodulin was from Bio-Rad.[2] Calmidazolium was purchased from Janssen Pharmaceutica. Fluphenazine was a gift from E. R. Squibb and Sons, Inc., Princeton, NJ. Trifluoperazine was a gift from Smith, Kline and French Laboratories, Philadelphia, PA. R-[5-^{3}H]mevalonic acid, triethylammonium salt (27.6 Ci/mmol) was from Dupont NEN Products. The iodide salt of *S*-adenosylmethionine, chlorpromazine hydrochloride, diethylamine ethyl chloride, 2,6-dichlorophenol, and activator-deficient phosphodiesterase 3′:5′-cyclic nucleotide (crude preparation) were from Sigma. Iodomethane, 3,4-dichlorophenol, 2,4,5-trichlorophenol, and pentachlorophenol were from Aldrich. Elemental analysis was performed by Micro-Analysis, Inc., Wilmington, DE.

Synthesis of Inhibitors. *2,6-Dichloroanisole (V).* The 2,6-dichlorophenol (3.2 g) was dissolved in 50 ml of acetone. Potassium carbonate (4.1 g) was added to the solution. The stirred solution was heated to reflux temperature, and 4.2 g iodomethane was added dropwise over a 0.5 h period. After 6 h at reflux temperature, the solution was filtered and evaporated. The crude residue (2.6 g) was purified by vacuum distillation, b.p. 70 to 71°C at 0.75 mm. Anal. Calcd for $C_7H_6Cl_2O$: C, 47.48; H, 3.66. Found: C, 47.65; H, 3.58.

β-(2,4,5-Trichlorophenoxy)ethyl N,N-Diethylamine Hydrochloride (VIII) (15). The 2,4,5-trichlorophenol (4.93 g) was dissolved in 13.3 ml of 0.5 N NaOH. The solution was stirred and heated to reflux temperature while blanketed with a stream of N_2. Diethylaminoethyl chloride (3.44 g) in 20 ml of water was added dropwise over 0.5 h. The solution was maintained at the reflux temperature for an additional 3 h. The cooled solution was extracted three times with 50 ml diethylether. The combined

[1] Abbreviations: TOH, triterpenols; TE, fatty acid esters of triterpenols; m.p., melting point; b.p., boiling point.

[2] 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.

extracts were washed with 50 ml of 5% NaOH and two 50 ml portions of water. The ether layer was removed, dried over $NaSO_4$, and filtered. A current of anhydrous HCl gas was passed through the stirred solution at 5°C. The hydrochloride salt, a white precipitate, was recovered by filtering. The hydrochloride salt was recrystallized from isopropyl alcohol to give a white crystalline material (2.29 g) m.p. 177 to 178.5°C. Anal. Calcd for $C_{12}H_{17}Cl_4NO$: C, 43.26; H, 5.14; N, 4.20. Found: C, 43.42; H, 5.14; N, 4.18.

Other Compounds. The other phenoxy compounds were synthesized by a procedure similar to that used for compound VIII. β-(2,6-Dichlorophenoxy)ethyl *N,N*-diethylamine hydrochloride (VI), m.p. 116 to 118°C. Anal. Calcd for $C_{12}H_{18}Cl_3NO$: C, 48.26; H, 6.07; N, 4.68. Found: C, 48.96; H, 6.29; N, 4.68. β-(3,4-Dichlorophenoxy)ethyl *N,N*-diethylamine hydrochloride (VII), m.p. 109 to 111°C. Anal. Calcd for $C_{12}H_{18}Cl_3NO$: C, 48.26; H, 6.07; N, 4.68. Found C, 48.07; H, 6.04; N, 4.58. β-(Pentachlorophenoxy)ethyl *N,N*-diethylamine hydrochloride (IX), m.p. 184 to 186°C. Anal. Calcd for $C_{12}H_{15}Cl_6NO$: C, 35.85; H, 3.76; N, 3.48. Found: C, 36.10; H, 3.72; N, 3.51.

Plant Materials. *Euphorbia lathyris* was grown from seeds collected from wild plants in coastal Northern California. Seedlings were grown in 8-inch clay pots in a glasshouse. Sunlight was supplemented with 12 h of artificial light per day using 1000 W metal halide lamps. Tapping was begun when the plants were about 3 months old and 35 cm tall. The latex was taken from the upper 10 cm of the plant as previously described (9).

Triterpene Biosynthesis Assay. Freshly tapped latex (100–200 μl per assay) was diluted 3-fold with ice-cold buffer containing 10 mM Na-phthalate (pH 5.5), 10 mM $MgCl_2$, 10 mM KCl, 30 mM $CaCl_2$, and 0.4 M sorbitol. The diluted latex was centrifuged for 5 min (including starting and stopping time) in an Eppendorf model 5413 desk top centrifuge (8800*g*). The supernatant was removed, boiled, and extracted with hexane to provide carrier triterpenes. The pellet was resuspended in 200 μl buffer per assay. In addition to the resuspended pellet each assay contained 0.01 mCi (1.0 nmol) R-[5-^{3}H]mevalonic acid, 0.66 μmol DTT, 22 nmol *S*-adenosylmethionine, the indicated amount of inhibitor, and enough NaOH to neutralize the inhibitor, if the HCl salt was used. The final volume was 0.22 ml. After 3 h at room temperature, the reactions were quenched by placing them in a boiling water bath. The carrier triterpenes in 20 μl acetone were added, and the reactions were washed twice with 2-ml aliquots of water. The triterpenes were extracted with acetone and analyzed using TLC, as previously described (9).

Phosphodiesterase Assay. The assay was performed as described (3) except that assays were conducted at room temperature, and $CaCl_2$ (18 μM) and calmodulin (0.1 or 1 μg/0.9 ml total reaction volume) were present. In addition, each assay tube was recentrifuged immediately before determining *A* at 650 nm.

Data Analysis. All assays containing the compound under test were compared to a control containing no added compound. The control rate was assigned the value of 100%. All experimental errors are reported as standard errors.

RESULTS

Table I shows the effect of all compounds at a concentration of 200 μM on triterpene biosynthesis. Compounds I to IV are recognized calmodulin inhibitors (1, 17). All are good inhibitors of triterpene biosynthesis, except compound I which does not inhibit TOH biosynthesis. Compound IV is a particularly effective inhibitor and totally eliminates biosynthesis. The clearest trend noted with the phenoxy compounds (V–IX) is that increasing chlorine substitution causes increasing inhibition. None of the di-chlorinated phenoxy compounds were good inhibitors. There are, however, small differences in their effects upon biosynthesis: addition of the diethylamino side chain increases inhibition (compare V and VI); rearrangement of the chlorines from the 2,6 to the 3,4 positions shifts inhibition toward TOH (compare VI and VII).

The right column of Table I shows the effect of all compounds on the phosphodiesterase catalyzed hydrolysis of cAMP. All assays were performed in the presence of 0.1 μg bovine brain calmodulin and 200 μM inhibitor. Phosphodiesterase hydrolyzes cAMP even in the absence of calmodulin (16). The rate of hydrolysis with no added calmodulin is reduced to 48 ± 3% (data not shown). Therefore, compounds I to IV at 200 μM may be unspecifically inhibiting the basal phosphodiesterase catalyzed hydrolysis as well as being antagonistic toward calmodulin. This has been observed before (7). It should be noted, however, that the commercial phosphodiesterase used in these studies could contain some endogenous calmodulin; thus, it is not possible to accurately quantitate nonspecific inhibition using this data.

Considering the phenoxy compounds (V–IX) only, it can be seen that there is a correlation, albeit imperfect, between antagonism toward calmodulin enhancement of phosphodiesterase activity and inhibition of triterpene biosynthesis.

Figures 1 and 2 show the concentration dependent inhibition of triterpene biosynthesis by compounds II and IX. Both TOH and TE biosynthesis are equally inhibited by increasing concentrations of either of these two compounds. The concentrations of compounds II and IX required for 50% inhibition of biosynthesis are about 150 and 35 μM, respectively. The value for 50% inhibition for compound IV is 55 μM (data not shown). Figure 1 shows compound II to be a less effective inhibitor than is reported in Table I. This difference is probably due to variation in the plant material as the two experiments were performed several months apart.

Figures 3 and 4 report complete concentration curves for compounds II and IX in the phosphodiesterase assay. Each concentration curve was repeated twice with both 0.1 and 1.0 μg added calmodulin. At the higher calmodulin concentration more compound is required to achieve an equivalent amount of inhibition. This causes the curve at higher calmodulin to be shifted to the right. This is the behavior expected for a true calmodulin antagonist, rather than a direct inhibitor of the phosphodiesterase enzyme.

DISCUSSION

Our interest in the chlorinated phenoxyalkylamines stems from work showing that treatment of young guayule plants with compound VII resulted in a 2- to 6-fold increase in terpenoid (rubber) synthesis (12, 18). We found that compound VII is a weak inhibitor of triterpene biosynthesis in *E. lathyris*. However, the treatment period in the guayule study was 3 weeks, and the entire plant was treated with compound VII. Because of the difference in experimental protocol and the difference in the plant species used, our results do not necessarily refute this earlier work.

Potential calmodulin inhibitors contain a hydrophobic group and a positively charged moiety. In addition, effective calmodulin inhibitors satisfy at least two other criteria: (a) the distance between the hydrophobic and positively charged groups is optimal, and (b) the hydrophobic group has a partial negative charge. Criterion b has received little attention in discussions of calmodulin antagonists. The partial negative charge requirement may be related to the need for antagonists to form a charge transfer complex with calmodulin as discussed by Reid (13). With most calmodulin inhibitors criterion b is satisfied by placing an electron withdrawing halogen atom on an aromatic group and inserting an electron donating moiety nearby. Relatively inactive analogs of calmodulin inhibitors such as chlorpromazine sulfoxide or W-5 are inactive because the transfer of charge to the hydrophobic group is reduced by adding an electronegative oxy-

Table I. *Effect of Compounds on Triterpene Biosynthesis and Phosphodiesterase Activity*

	Compound		Triterpene Biosynthesis[a]		Phosphodiesterase[b]
			%	N^c	%
I	Calmidazolium	TOH	120 ± 11	5	40 ± 4
		TE	52 ± 3		
II	Chlorpromazine	TOH	9 ± 2	3	43 ± 3
		TE	7 ± 1		
III	Fluphenazine	TOH	31 ± 3	4	25 ± 4
		TE	26 ± 8		
IV	Trifluoperazine	TOH	3 ± 1	3	35 ± 5
		TE	1 ± 1		
V	2,6-dichloroanisole	TOH	99 ± 3	4	101 ± 3
		TE	87 ± 7		
VI	2-(2,6-dichlorophenoxy)ethyl *N,N*-diethylamine	TOH	87 ± 6	4	98 ± 3
		TE	72 ± 8		
VII	2-(3,4-dichlorophenoxy)ethyl *N,N*-diethylamine	TOH	61 ± 7	4	75 ± 2
		TE	79 ± 8		
VIII	2-(2,4,5-trichlorophenoxy)-ethyl *N,N*-diethylamine	TOH	17 ± 6	4	60 ± 5
		TE	20 ± 4		
IX	2-(pentachlorophenoxy)ethyl *N,N*-diethylamine	TOH	1 ± 1	3	54 ± 5
		TE	2 ± 1		

[a] Control incorporation rates: TOH, 5.7–8.4 × 10^{-4}; TE, 1.8–5.1 × 10^{-3} nmol h^{-1} mg^{-1} protein. [b] Phosphodiesterase was assayed in triplicate. [c] N is the number of experimental repetitions.

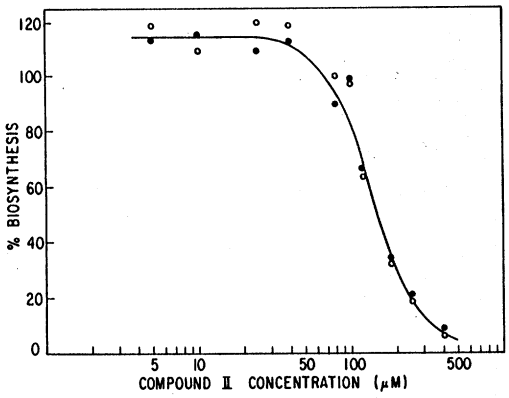

FIG. 1. The effect of compound II on TOH (●) and TE (○) biosynthesis.

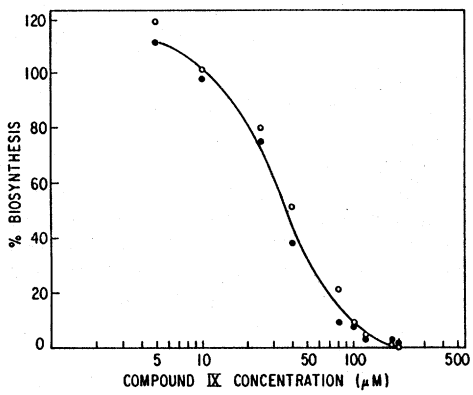

FIG. 2. The effect of compound IX on TOH (●) and TE (○) biosynthesis.

gen in the former case or by eliminating the halogen atom in the latter case. The phenoxyalkylamine compounds studied here contain an oxygen atom instead of the sulfur found in many calmodulin antagonists. Oxygen is a relatively poor electron donor compared to sulfur. Recognition of this fact makes it clear why the hydrophobic phenol ring needs to be substituted with five halogen atoms before a very effective calmodulin antagonist is produced. Although compound IX is a good calmodulin antagonist, it is not the best antagonist of bovine brain calmodulin (17). In part this may be due to insufficient partial charge on the hydrophobic phenol ring, or perhaps because the best calmodulin antagonists found to date contain two or more aromatic regions (13).

In addition to their impact upon calmodulin action, calmodulin antagonists may interact with a variety of nonpolar cellular components through hydrophobic interactions (4, 8). However, since the phenoxyalkylamine series have widely differing abilities to inhibit triterpene biosynthesis, it is clear that hydrophobicity alone is insufficient to yield a good inhibitor of triterpene biosynthesis. It is therefore likely that a hydrophobic interaction between the antagonists and the nonpolar intermediates of the triterpene pathway is not a mediating factor in inhibition. The pattern of inhibition given by the phenylalkylamine series and low I_{50} values given by the accepted calmodulin antagonists demonstrate that specific interactions are occurring. The phosphodiesterase assays show that calmodulin interacts with the inhibitors with the required specificity, and therefore the data indicate the involvement of calmodulin in triterpene biosynthesis. However, the possibility that the antagonists are acting on a protein with a binding site similar to that of calmodulin cannot be eliminated (1, 14).

The presence of a limiting membrane around the site of biosynthesis makes direct tests of calmodulin involvement impractical, as exogenously added calmodulin cannot penetrate to

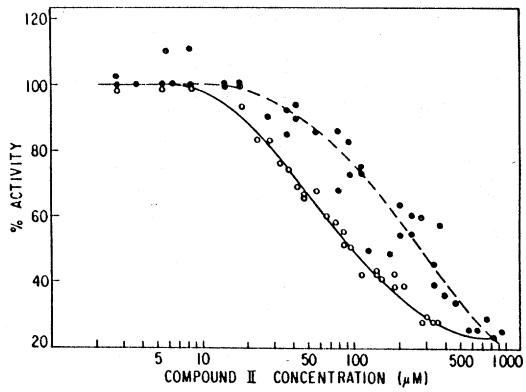

FIG. 3. The effect of compound II on phosphodiesterase activity with 0.1 μg (○) and 1.0 μg (●) added calmodulin.

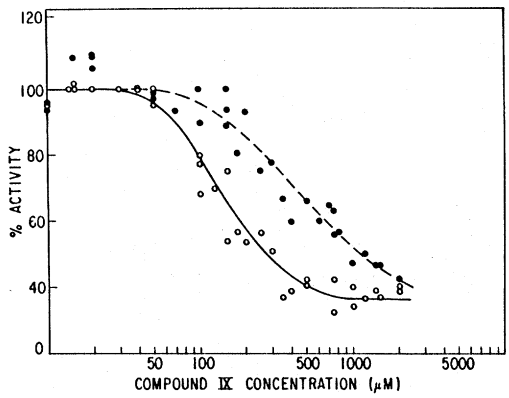

FIG. 4. The effect of compound IX on phosphodiesterase activity with 0.1 μg (○) and 1.0 μg (●) added calmodulin.

the interior of the organelles, and triterpene biosynthesis does not occur when the organelles are broken. Nevertheless it should be possible to determine, by analysis of intermediate levels and enzymic assay, the point of antagonist action and to subsequently test for calmodulin involvement in a direct manner. Future investigations will be directed toward this goal.

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