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THE BIOSYNTHESIS OF STILBENES IN EUCALYPT LEAVES

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Abstract—Compounds which are considered to be intermediates in the biosynthesis of stilbenes have been examined as substrates using acetone powders of *Eucalyptus sideroxylon* leaves under a variety of conditions. No stilbene synthesis could be demonstrated. It is suggested that normally the intermediates do not exist in the free form during biosynthesis.

INTRODUCTION

THE RECENT synthesis of poly-β-keto acids[1-3] has provided comparative compounds to assist the detection of compounds which are theoretically important in the biosynthesis of stilbenes and flavonoids.[4] Cinnamoyl triacetic acid (I) has been tested[5] as a substrate for acetone powders from leaves of a mutant of *Eucalyptus sideroxylon* which contained stilbenes and flavonoids.[6,7] Further studies on this polyketo acid and related compounds with regard to their possible role as intermediates in stilbene formation are reported in this paper.

RESULTS AND DISCUSSION

Theories of biosynthesis of polyphenols propose that the keto acids and similar compounds exist as the activated thiol esters. Whereas these esters may be necessary for flavonoid formation, this activation may not be necessary in the case of stilbene biosynthesis because of the ease with which β-resorcylic acids are formed from the keto acids.[2] Possible stages in the biosynthesis of stilbenes are given in the Scheme 1.

Earlier work[5] reported the conversion at pH 8·2 of cinnamoyl triacetic acid (I) into pinosylvin (V) using acetone powders prepared from leaves of epicormic shoots of a mutant of *Eucalyptus sideroxylon.* Using an improved assay method and a different season's growth of leaves from identical trees we have been unable to confirm this work. Several preparations of acetone powder with substantial phenylalanine ammonia lyase (PAL) activity were prepared at different times of the year but they failed to show pinosylvin synthase activity. Difficulties in obtaining active preparations of another enzyme cyclizing polyketo compounds have also been reported.[8] In the present case, the explanation may lie with the first sample of cinnamoyl triacetic acid which was several years old and possibly contained different enantiomers, in the nature of the epicormic shoots which provided the original powders, or the different method of assay used. Acetone powders and partly purified fractions of these powders have been used under different conditions using a purified sample

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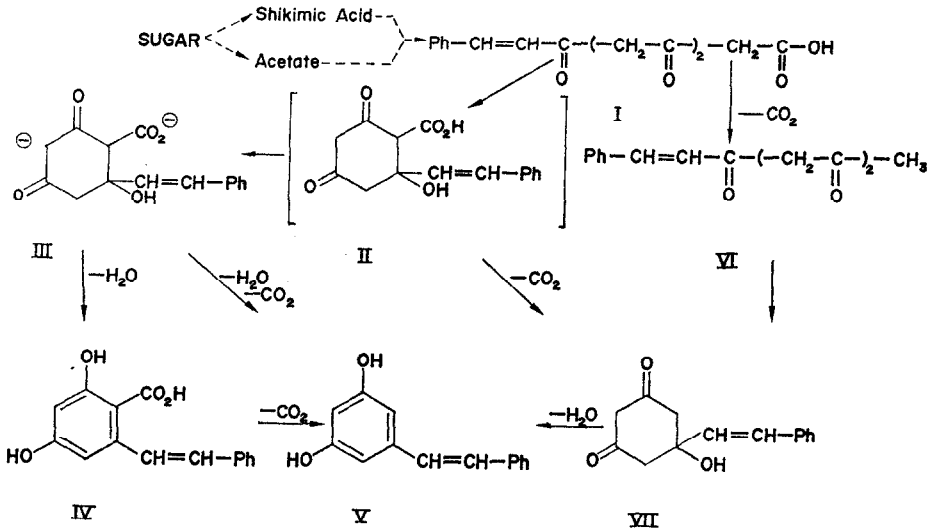

SCHEME 1. BIOSYNTHESIS OF STILBENES.

of cinnamoyl triacetic acid. These conditions include a prolonged incubation period, anaerobic conditions and the addition of several cofactors[9] such as CoA, ATP, $MgSO_4$, glutathione, NADPH and NADP. No evidence of pinosylvin synthesis was found.

Birch[10] has speculated that non-aromatic aldol cyclization products may be intermediates in the formation of β-resorcylic acids and related compounds from triketo acid precursors. Above pH 6·1, cinnamoyl triacetic acid (I) changes to the aldol dianion (III) (the aldol (II) being unstable).[11] Spectral examination showed that the rate of formation of the aldol dianion is faster at 6·1 than at 8·2 which in turn is faster than that at pH 9·7. It takes at least 1 hr to completely change cinnamoyl triacetic acid to the aldol dianion at pH 8·2 and room temp. A solution of the dianion at pH 8·2 is unchanged after at least 10 months at 4°, however, it is quickly changed to pinosylvic acid (IV) and small amounts of pinosylvin (V) under acidic conditions. At pH 5, the pinosylvin formed amounted to about 3% of the pinosylvic acid. The rate of formation of pinosylvic acid and pinosylvin from the aldol dianion increases rapidly with the decrease of the pH below 5. Incubation of the aldol dianion III with acetone powder at pH 8·2 in the presence of magnesium ion did not result in decarboxylation and the production of pinosylvin.

Stilbene acids (e.g. IV) have been postulated[4,12] as intermediates but only those in the 3-deoxy form have been found in nature.[12] The fluorescent properties of pinosylvic acid (IV) enables detection of 0·0004 mg. However, examinations of chromatograms of extracts of tissues containing stilbenes have failed to find compounds with the chromatographic properties of pinosylvic acid (Table 1) or of those expected for relevant substituted stilbene acids. Furthermore, although facile decarboxylation of β-resorcylic acids in basic conditions has been reported,[2,13] tests with different preparations of acetone powder from *E. sideroxylon* leaves failed to decarboxylate IV under incubation conditions at pH 8·2. Pinosylvic

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TABLE 1. PROPERTIES OF PINOSYLVIN AND POSSIBLE INTERMEDIATES IN BIOSYNTHESIS

Properties	Pinosylvin	Pinosylvic acid	Cinnamoyl triacetic acid	Cinnamoyl diketone	Aldol dianion
R_f value (paper chromatography) in					
6% acetic acid	0·09	0·11	u	0	u
Butanol–acetic acid–water (6:1:2)	0·93	0·94	u	0·95	u
Butanol–ethanol–1·5 N NH_4OH (4:1:3)	0·83	0·52	u	0·90	0·70 0·52 0·16* 0·09*
R_f value (TLC) in					
Benzene–dioxane–acetic acid (90:25:4)	0·65	0·68	u	0·90	u
Chloroform–ethyl acetate–formic acid (5:4:1)	0·63	0·70	u	0·75	u
Benzene–methanol (9:1)	0·30	0·02	0* 0·55†	0·60	0
Toluene–ethyl formate–formic acid (5:4:1)	0·50	0·56	u	0·68	u
Methanol–chloroform–petrol (1:2:7)	0·14	0·04	0* 0·25†	0·35	0
Fluorescence in u.v.	purple	green-blue	green-blue on paper; yellow on TLC	yellow	green to dark blue
λ_{max} (nm)	300 309	253 300	229 350	228 362	255 284

* Main spot.
† Values variable.
u, Unstable in solvent.

acid was heated at 30° for 3 hr in aq. buffers at pH 1·0, 3·0, 5·0, 6·1, 7·1, 8·2 and 9·7 but there was no decarboxylation. The pinosylvin which formed on acidification of alkaline solutions of the aldol dianion apparently does not arise directly from pinosylvic acid. During acidification the unstable aldol II may have been formed and this could give rise to pinosylvin by decarboxylation to VII before dehydration.

There is a possibility the route to pinosylvin might lie through the cinnamoyl diketone (VI) which would not necessitate the formation of pinosylvic acid. However, the ketone is a stable compound, it is insoluble in aq. media and incubations with it as substrate did not produce pinosylvin. Methanol solutions showed a characteristic u.v. absorption spectrum, and 80 min after acidification with 5 M HCl at room temp., the change in its spectrum was very slight. No evidence of cinnamoyl diketone in chromatograms of extracts of leaves was seen.

Although intermediates other than those considered here may be involved in stilbene formation it has been noted[14,15] that the intermediates in the postulated biosynthetic pathways from other polyketo compounds have not been found free in the tissues concerned. It has been suggested[14,15] that these polyketo chains exist covalently coupled to

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multienzyme complexes on which subsequent variations in the molecules take place before the products are released in the final form. Presumably also in normal circumstances the intermediates in stilbene biosynthesis in eucalypt leaves do not exist in the free form.

EXPERIMENTAL

Plant Material

The leaf samples, examined at intervals for pinosylvin synthase, pinosylvic acid decarboxylase and PAL activity, were obtained from branches of trees of *Eucalyptus sideroxylon* stilbenoid chemovar. Natural branches were collected on 9, 17, 23 and 30 June 1969, epicormic branchlets were collected on 7 July 1969, both types of branches on 21 July 1969, natural branches on 20 October 1969, 10 and 24 November 1969, 30 December 1969, 10 March 1970, 3 April 1970 and both types of branches on 20 April 1970.

Preparation of Acetone Powder

The methods for the preparation of acetone powder and its purification by dialysis and fractionation on Sephadex G-50 were essentially the same as those used previously.[5]

Enzyme Assays

Pinosylvin synthase activity. The reaction mixture contained 200 mg of acetone powder or 20 mg of purified powder and 1·25 μmoles of cinnamoyl triacetic acid in 4 ml of 0·05 M tris-HCl buffer (pH 8·2). When cofactors were used 5 μ moles of ATP, $MgSO_4$, glutathione and cinnamoyl triacetic acid as well as 0·3 μmole of CoA were included in a total volume of 2 ml of tris-buffer.[9] Incubations with additions of these cofactors along with NADP and NADPH (1 μmole) were also examined separately. The reaction mixtures were incubated without shaking for 1, 3 and 6 hr in test tubes at 30°. Anaerobic conditions were maintained by N_2 stream. At the end of the incubation period, the reaction mixture was immediately extracted with Et_2O (4 × 7 ml), the extract was evaporated to dryness in vacuum and the residue resolved on a chromatoplate (250 μ thickness) of silica gel GF254 using the solvent MeOH–$CHCl_3$–petrol (90–110°) (2:4:7, v/v). Pinosylvin when present was detected by u.v. light, the band extracted rapidly with Et_2O (4 × 7 ml) which was then evaporated to dryness in vacuum. The residue was dissolved in 5 ml MeOH, and from the u.v. spectrum the absorbance at 312 nm was determined and the amount of pinosylvin calculated from a standard curve.

For incubation of the aldol dianion, the reaction mixture consisted of 200 mg of acetone powder or 20 mg of purified powder, 2 ml of 0·04% aldol dianion solution and 2 ml of 0·05 M tris-HCl buffer (pH 8·2). The reaction products were examined as above. Incubations with cinnamoyl diketone (0·1 mg) as the substrate were examined in the same way.

Pinosylvic acid decarboxylase activity. The reaction mixture contained 200 mg of acetone powder or 20 mg of purified powder, 0·1 mg of $MgCl_2$ 6 H_2O and 0·2 mg of pinosylvic acid in 4 ml of 0·05 M tris-HCl buffer (pH 8·2) or 0·05 M citrate buffer (pH 6·1). The reaction mixtures were incubated for 1, 3 and 6 hr without shaking in test tubes at 30°. After the incubation, the reaction mixtures were extracted with Et_2O (4 × 7 ml), and the extracts examined for pinosylvin as above.

Phenylalanine ammonia lyase activity. The method used was essentially the method of Koukol and Conn[16] followed by separation of the cinnamic acid on TLC plates.[17] All acetone powders had appreciable PAL activity.

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