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BIOSYNTHESIS OF LUNULARIC ACID—A DIHYDROSTILBENE ENDOGENOUS GROWTH INHIBITOR OF LIVERWORTS

R. J. PRYCE

Department of Horticulture, Wye College, University of London, Near Ashford, Kent

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Abstract—Incorporations of U-^{14}C-L-phenylalanine, 1-^{14}C-sodium acetate and ^{14}C-hydrangenol (IV) into lunularic acid (I) by the liverwort *Lunularia cruciata* are reported. The results show, that like other plant stilbene derivatives, lunularic acid can be biosynthesized by the phenylpropanoid–polymalonate pathway. The phenyldihydroisocoumarin, hydrangenol (IV), and/or its isomeric stilbene derivative, hydrangeic acid (VI), are probable intermediates in the biosynthesis of lunularic acid. Although no free or bound hydrangenol or hydrangeic acid could be detected in the liverwort they were found to co-occur with lunularic acid, in an acid labile bound form, in roots of *Hydrangea macrophylla*. A hypothesis for the photoperiodic control of liverwort growth by control of lunularic acid synthesis is presented.

INTRODUCTION

THE DIHYDROSTILBENE derivative lunularic acid (I) was first isolated and characterized as an endogenous growth inhibitor of the liverwort *Lunularia cruciata*.[1,2] Since then it has been found to be apparently universally present in liverworts.[3,4] It has been detected in all algae so far examined but not in mosses, which together with the liverworts comprise the Bryophytes, nor in Pteridophytes.[4] The widespread endogenous growth inhibitor of higher plants, abscisic acid (III),[5] could not be detected in liverworts,[3] nor algae[6] but there is evidence for its occurrence in mosses and Pteridophytes.[6] Lunularic acid is therefore a plant growth inhibitor of some chemotaxonomic and phylogenetic significance and it seems possible that in the lower green plants, liverworts and algae, it may replace the growth regulatory function of abscisic acid of higher plants.

Natural growth regulation in the liverworts *L. cruciata*[7] and *Marchantia polymorpha*[8,9] has been shown to be photoperiodically controlled and evidence for phytochrome involvement in this process has been presented. In the case of *L. cruciata* the amount of extractable growth inhibitory substance, lunularic acid, is reported[2] to be subject to photoperiodic control. Long day growth-inhibitory photoperiods gave rise to more extractable lunularic acid than short day non-inhibitory photoperiods or darkness. The present investigation of the biosynthesis of lunularic acid was undertaken as a first step in a programme designed to investigate further this apparent photoperiodic regulation of lunularic acid metabolism in liverworts.

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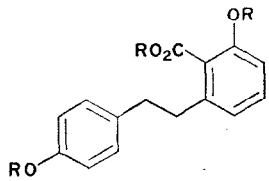

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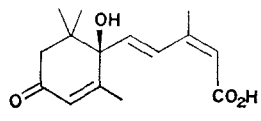
(II) R = CH_3



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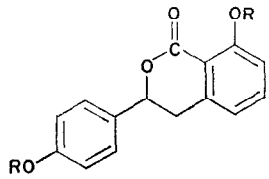

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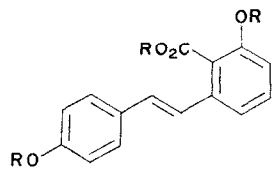
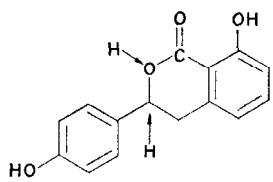
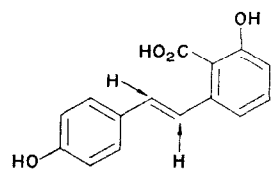


(VI) R = H
(VII) R = CH_3



(VIII)



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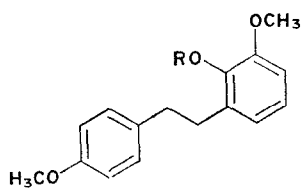

(X) R = H
(XI) R = CH_3

Structurally lunularic acid (I) is related to the phenyldihydroisocoumarin, hydrangenol (IV), and its isomer hydrangeic acid (VI) which have been isolated from extracts of the common garden hydrangea (*Hydrangea macrophylla*).[10–12] Detailed studies of the biosynthesis of hydrangenol have shown[13–16] that it can, like other stilbene derivatives found in plants,[17] be synthesized *in vivo* by the phenylpropanoid–polymalonate pathway (Scheme 1). It seemed that a similar biosynthetic pathway might give rise to lunularic acid[1] and that hydrangenol and/or its isomer, hydrangeic acid, might be intermediates on this pathway. The results of incorporations of ^{14}C-labelled phenylalanine, acetate and hydrangenol into lunularic acid by the liverwort *Lunularia cruciata*, which are presented here, support this view and confirm that lunularic acid can be synthesized *in vivo* by a phenylpropanoid–polymalonate pathway.

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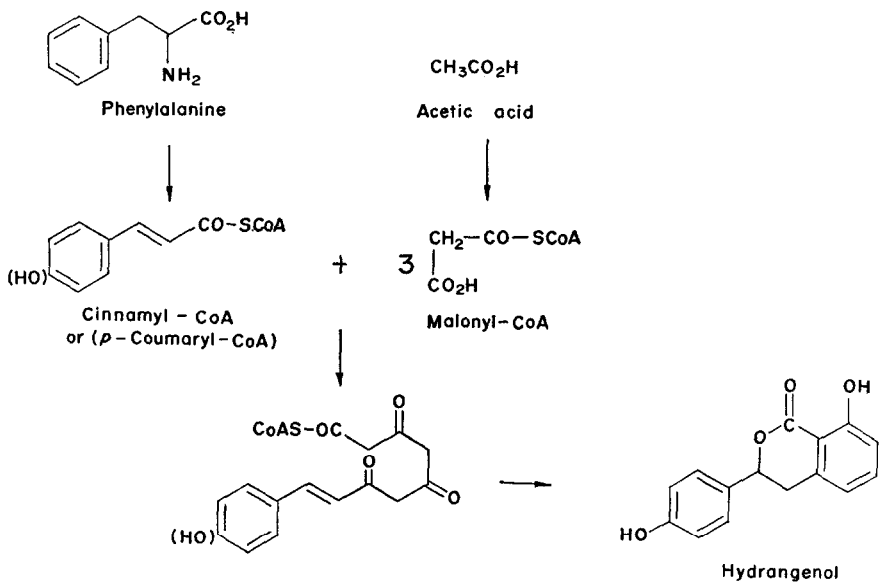

RESULTS AND DISCUSSION

For the present study the liverwort *L. cruciata* was chosen because of its availability and the readily isolable quantities of lunularic acid that it contained. ^{14}C-Labelled phenylalanine, acetate and hydrangenol were fed to the plant and Table 1 shows the results. ^{14}C-Hydrangenol was prepared by feeding root sections of *H. macrophylla* with universally labelled phenylalanine.[14] After each of the feeding experiments, labelled lunularic acid was purified by preparative TLC in three different solvent systems. Finally, it was methylated with diazomethane and, after further preparative TLC, quantitated by GLC as methyl lunularate dimethyl ether (II).[3]

TABLE 1. SYNTHESIS OF LUNULARIC ACID FROM ^{14}C-LABELLED COMPOUNDS BY *Lunularia cruciata**

Compound	Compound fed			Lunularic acid			
	Amount (μM)	Specific activity (μCi/μM)	Incorporation time (hr)	Amount isolated (μM)	Specific activity (μCi/μM)	Incorporation into lunularic acid isolated (%)	Dilution†
U-^{14}C-L-Phenylalanine	$2{\cdot}1 \times 10^{-3}$	$4{\cdot}8 \times 10^{2}$	18	$9{\cdot}1 \times 10^{-2}$	$1{\cdot}7 \times 10^{-1}$	1·6	2800
1-^{14}C-Sodium acetate	$3{\cdot}5 \times 10^{-2}$	$5{\cdot}7 \times 10$	18	$1{\cdot}2 \times 10^{-1}$	$4{\cdot}9 \times 10^{-2}$	0·3	3500‡
^{14}C-Hydrangenol (IV)§	3·1	$1{\cdot}3 \times 10^{-2}$	23	$4{\cdot}7 \times 10^{-2}$	$1{\cdot}1 \times 10^{-2}$	1·3	1·2

* Each feeding experiment was conducted with the same quantity (1 g fr. wt.) of cut ends of *L. cruciata* thalli in the same volume of nutrient solution.

† Specific activity of labelled compound fed by specific activity of lunularic acid isolated.

‡ The dilution value of the acetate feeding was calculated on the basis of three molecules of acetate being incorporated into one molecule of lunularic acid.

§ Phenylalanine derived moiety universally labelled with ^{14}C (see text).

From the results shown in Table 1 it is clear that *L. cruciata* can effectively utilize phenylalanine, acetate and hydrangenol for the synthesis of lunularic acid. These incorporations therefore represent a self consistent set of results which confirm that the liverwort *L. cruciata* can synthesize lunularic acid by the anticipated phenylpropanoid–polymalonate pathway (Scheme 1). Furthermore, they suggest that hydrangenol and/or its isomer, hydrangeic acid, may be intermediates on this pathway. The remarkably small dilution of the specific activity of ^{14}C-hydrangenol strongly suggests that hydrangenol is incorporated intact into lunularic acid and, at the same time, indicates a rapid turnover of lunularic acid in the liverwort.

Hydrangenol or its isomer, hydrangeic acid, might act as direct precursors of lunularic acid either by simple benzyl–oxygen reductive cleavage (hydrogenolysis) as in (VIII) or by reduction of the ethylenic double bond (hydrogenation) (IX). Unfortunately, it has not been possible to compare the relative efficiences of hydrangenol and hydrangeic acid as precursors for lunularic acid because in protic solvents hydrangenol gives rise to some hydrangeic acid and vice versa. For example, TLC pure ^{14}C-hydrangenol gave rise to *ca.* 2% hydrangeic acid under the same conditions as those under which the feeding experiments were carried out. It is thus impossible to decide from the feeding experiments which of the two, hydrangenol or hydrangeic acid, are direct precursors of lunularic acid and therefore which type of reductive process, hydrogenolysis (VIII) or hydrogenation (IX), occurs in the liverwort.

Neither hydrangenol nor hydrangeic acid could be detected (limits of detection *ca.* 1 part in 10^8 fresh tissue) in extracts of *L. cruciata* by TLC and GLC analysis before or after acid hydrolysis of the extracts. Therefore, if hydrangenol and/or hydrangeic acid are natural intermediates of lunularic acid biosynthesis in the liverwort they must have relatively small pool sizes. Hydrangenol and hydrangeic acid were analysed after diazomethane methylation by GLC as dimethyl hydrangenol (V) and methyl hydrangeate dimethyl ether (VII) respectively.[11] With roots of *H. macrophylla*, which contain hydrangenol glucoside and no detectable free hydrangenol,[13] examination before and after acid hydrolysis has shown that lunularic acid is present but only in an acid labile bound state together with hydrangenol and hydrangeic acid. The extraction procedure used, however, makes it impossible to say whether it is hydrangenol, hydrangeic acid or both that are present in hydrangea roots.

Recently the isolation of another dihydrostilbene derivative, pellepiphyllin (X), from the liverwort *Pellia epiphylla* has been reported.[18] This stilbene derivative has an unusual oxygenation pattern compared with other plant stilbenes (see Ref. 17) with neither aromatic ring being obviously acetate/malonate derived. In view of the apparently universal occurrence of lunularic acid in liverworts and particularly in *P. epiphylla*,[3] it is tempting to suggest that pellepiphyllin could be derived from lunularic acid in the liverwort by methylation and oxidative decarboxylation processes. Unfortunately, it has not been possible to examine this idea further because no pellepiphyllin nor its further methylated or demethylated derivatives were detectable by TLC and GLC methods in extracts of our samples of *P. epiphylla* or any other liverwort so far examined; *L. cruciata*, *P. endiviifolia* and *Marchantia polymorpha*.

The present results suggest a possible mechanism for the photoperiodic growth response of liverworts involving control of endogenous levels of lunularic acid. In the proposed pathway to lunularic acid, phenylalanine would, by analogy with higher plant meta-

[18] V. BENESOVA and V. HEROUT, *Collection Czech. Chem. Commun.* **35**, 1926 (1970).

bolism,[17,19] be converted into *trans*-cinnamic acid as in Scheme 1. This process is catalysed in higher plants by the enzyme phenylalanine ammonia-lyase, the activity of which can be photoperiodically controlled by a system involving phytochrome.[19,20] If this is also the case in liverworts, present results suggest that photoperiodic control of their growth could arise from control of lunularic acid synthesis at the stage of the phenylalanine to *trans*-cinnamic acid conversion by control of the activity of phenylalanine ammonia-lyase. This hypothesis and other points concerning the metabolism of lunularic acid are the subject of further investigation.

EXPERIMENTAL

Materials. U-^{14}C-L-Phenylalanine and 1-^{14}C-sodium acetate were obtained from U.K.A.E.A., Amersham, Bucks. *L. cruciata* was grown on compost in short days (8 hr) of low light intensity in a glasshouse. Pure (TLC and GLC) crystalline samples of lunularic and hydrangeic acids were prepared from pure crystalline hydrangenol obtained[13] from roots of *H. macrophylla* by the published procedures.[1,11]

Nutrient solution for feeding experiments. The mineral nutrient solution (pH 6·7) used was similar to one described by SCHWABE[21] and was of the following composition (concentration, mg/l H_2O in parentheses): $NaNO_3$ (456), $Na_2HPO_4.12H_2O$ (150), $Ca(NO_3)_2.4H_2O$ (633), $Ca(H_2PO_4)_2.H_2O$ (52·8), $CaCl_2.6H_2O$ (37), $MgSO_4.7H_2O$ (125), K_2SO_4 (185), $FeCl_3.6H_2O$ (1), H_3BO_3 (2·86), $MnSO_4.4H_2O$ (2·04).

Radioactivity measurements. TLC–radioautography was performed with a Tracerlab radiochromatogram scanner. The radioactivity of samples of lunularic acid and hydrangenol was measured at infinite thinness on steel planchettes (50 cm dia.) with a Beckman Widebeta II counter, ^{14}C efficiency *ca.* 25%, background < 2 counts/min.

Chromatography. The chromatographic properties of compounds referred to are recorded in Table 2. Methylated derivatives were prepared by prolonged (12 hr) treatment of MeOH solutions of the compounds with excess ethereal CH_2N_2 at room temp.

TABLE 2. THIN LAYER AND GAS–LIQUID CHROMATOGRAPHIC PROPERTIES OF COMPOUNDS DESCRIBED

Compound	TLC R_f values (silica gel) Solvent system* 1	2	3	4	GLC Retention indices Retention time (min) in parentheses Column 1·5% XE-60	1% OV-17†
Lunularic acid (I)	0·40	0·32	0·45			
Methyl lunularate dimethyl ether (II)				0·62	3038 (4·8)‡	2736 (6·1)‡
Hydrangeic acid (VI)	0·33	0·26	0·45			
Methyl hydrangeate dimethyl ether (V)				0·35	3407 (13·2)‡	2977 (13·6)‡
Hydrangenol (IV)	0·60	0·39	0·52			
Dimethyl hydrangenol (V)				0·62	3843 (18·6)§	3175 (13·2)§
Pellepiphyllin (X)	0·49				2735 (4·5)‖	2528 (6·9)‖
Methyl pellepiphyllin (XI)					2600 (2·9)‖	2462 (5·4)‖

* (1) EtOAc–$CHCl_3$–HOAc (15:5:1); (2) Benzene–MeOH–HOAc (20:4:1); (3) Diisopropyl ether–HOAc (19:1); (4) Benzene–EtOAc (7:3).

† N_2 flow rate 60 ml/min and injection heater 250° in all cases.

‡ Column temp. 213°.

§ Column temp. 228°.

‖ Column temp. 196°.

[19] T. SWAIN and C. A. WILLIAMS, *Phytochem.* **9**, 2115 (1970).

[20] H. MOHR, C. HUAULT, H. LANGE, L. LOHMANN, I. RISSLAND and M. WEIDENER, *Planta* **83**, 267 (1968).

[21] W. W. SCHWABE, *Ann. Bot.* **15**, 417 (1951).

TLC was carried out on silica gel layers (250 μ thick). The compounds described were located on TLC plates by their blue or blue–green fluoresence under UV light. For preparative purposes bands on TLC plates of the same R_f values as pure substances under the same conditions were removed, saturated with water, and eluted with MeOH.

GLC was performed with a Pye series 104 dual column chromatograph with dual flame ionization detectors and injection heaters. The instrument was fitted with silanized glass columns (1·5 m × 4 mm i.d.) packed with stationary phases (XE-60 or OV-17) adsorbed on Gas-Chrom Q (80–100 mesh). Quantitative measurements were made by GLC peak area measurements using linear detector response calibrations obtained with the appropriate pure substances.

Feeding experiments with L. cruciata. U-^{14}C-L-Phenylalanine, 1-^{14}C-sodium acetate and ^{14}C-hydrangenol (see below) were all fed in exactly the same manner to freshly cut tips of *L. cruciata* thalli (0·5–1 cm long). The radioactive compounds were added in small volumes of 2% aqueous EtOH, water or EtOH respectively, to the mineral nutrient solution (6 ml) which was divided equally into two petri dishes (5 cm dia.). The maximum concentration of EtOH present in the nutrient solution in the case of hydrangenol was 1%. Thalli of *L. cruciata* were floated on the nutrient solution (0·5 g fr. wt. of thalli per petri dish) and incubation was carried out at room temp. under tungsten light illumination. (*ca.* 1000 lux) for the times shown in Table 1. After incubation the thalli were collected and washed with water before extraction with EtOAc in a Soxhlet for 12 hr. The EtOH extract was evaporated to dryness *in vacuo* and the residue taken up into EtOAc (3 × 4 ml) which was then extracted with 5% $NaHCO_3$ (4 × 4 ml). After acidification of the $NaHCO_3$ extract with conc. HCl extraction with EtOAc (3 × 5 ml) gave the strong acid fraction which was dried (Na_2SO_4) and evaporated to dryness *in vacuo*. These lunularic acid containing strong acid fractions from each of the three feedings were then treated in the same following manner.

The total strong acid fractions were first subjected to TLC in solvent system 1 and, with the help of TLC-radioautography and UV visualization, the lunularic acid bands were extracted then subjected to further preparative TLC in solvent systems 2 and 3. The essentially pure lunularic acid obtained after chromatography in solvent system 3 was methylated and subjected to further preparative TLC in solvent system 4 prior to quantitation by GLC and planchette radioactivity counting of an aliquot. With the phenylalanine and acetate feedings labelled lunularic acid accounted for >90% of the total radioactivity of the strong acid fractions as shown by radioautography during the TLC purification procedures. The hydrangenol feeding gave rise to at least two other more strongly labelled acids than lunularic acid but these were readily separated during the TLC purification.

In a control feeding experiment with ^{14}C-hydrangenol, which was carried out exactly as described above except that no *L. cruciata* thalli were used, TLC-radioautography indicated that hydrangeic acid (*ca.* 2%) had been formed—no lunularic acid was detectable.

^{14}C-*hydrangenol.* Sections (1 to 2 cm long; 10 g fr. wt.) of washed roots from 2·5-month-old rooted cuttings of *H. macrophylla* were placed in the mineral nutrient solution (20 ml) containing U-^{14}C-L-phenylalanine (12·5 μCi, 477 μCi/μM). Incubation was carried out for 42 hr in the dark at room temp. The root sections were then collected, washed (H_2O) and extracted with EtOH in a soxhlet for 24 hr. The dry residue, obtained by evaporation of the EtOH extract *in vacuo*, was refluxed with 10% HCl (50 ml) for 1 hr then the reaction mixture evaporated to dryness *in vacuo*. The dry hydrolysis residue was taken up in EtOAc (4 × 15 ml) and extracted first with 5% $NaHCO_3$ (6 × 15 ml) and then with 1 N NaOH (5 × 15 ml). The NaOH extract was acidified with conc. HCl and extracted with EtOAc (3 × 20 ml) which was then dried (Na_2SO_4) before evaporating to dryness *in vacuo*. The hydrangenol containing weak acid fraction thus obtained (43 mg) was subjected to the same TLC purification sequence as described above for the *L. cruciata* feeding experiments to give pure hydrangenol m.p. 179–181° (reported 180–181°[13]) (8·3 mg, specific activity 1·26 × 10^{-2} μCi/μM, 3·3% incorporation). The amount of hydrangenol was determined by CH_2N_2 methylation of an aliquot followed by quantitative GLC analysis. Methylation of hydrangenol gave rise to some methyl hydrangeate dimethyl ether (VII) as shown by TLC-radioautography and GLC. The amount of methyl hydrangeate dimethyl ether formed was taken into account during the quantitative GLC analysis of the hydrangenol. The radioactivity of the hydrangenol was determined by planchette counting of an aliquot.

Investigation of the occurrence of hydrangenol, hydrangeic acid and lunularic acid in L. cruciata *and roots of* H. macrophylla. (a) *L. cruciata.* A washed sample of *L. cruciata* (20 g fr. wt.) was macerated in 80% aq. EtOH (200 ml) and allowed to soak for 18 hr. After filtration the aq. EtOH extract was divided into two equal parts prior to evaporation of them both to dryness *in vacuo*. One half of the dry extract was hydrolysed on a steam bath with 1 N HCl (50 ml) for 1 hr then evaporated to dryness *in vacuo*. The acid hydrolysed and unhydrolysed extracts were then treated identically. The dry extract was taken up in EtOAc (4 × 15 ml) which was then extracted with 5% $NaHCO_3$ (3 × 20 ml) and 1 N NaOH (3 × 20 ml). Acidification with conc. HCl of these $NaHCO_3$ and NaOH extracts and extraction into EtOAc (3 × 25 ml), which was dried (Na_2SO_4) and evaporated to dryness *in vacuo*, gave the strong acid and weak acid extracts, respectively. TLC of these fractions from both unhydrolysed and acid hydrolysed extracts in solvent system 1 showed the presence of lunularic acid in both strong acid fractions but gave no indication of the presence of hydrangeic acid or hydrangenol in any strong or weak acid extracts. GLC analyses of the methylated (CH_2N_2) extracts on both columns gave the same result. Extraction of *L. cruciata* with EtOH in a Soxhlet gave similar results.

(b) *Roots of H. macrophylla.* Various samples of roots of *H. macrophylla* have been extracted similarly to that described in (a), the only difference being that EtOH Soxhlet extraction was used. In all cases no hydrangenol, hydrangeic acid nor lunularic acid could be detected in weak or strong acid fractions except after acid hydrolysis of the EtOH extractives. In one such case after extraction of roots (572 g fr. wt.) and acid hydrolysis of the extract the combined acid fractions contained hydrangenol (*ca.* 200 mg), hydrangeic acid (*ca.* 8 mg) and lunularic acid (*ca.* 3 mg) as indicated by TLC and GLC analysis.

Investigation of the occurrence of pellepiphyllin in liverwort extracts. Weak acid and neutral fractions from the liverworts *L. cruciata*, *P. epiphylla*, *P. endiviifolia* and *M. polymorpha* were obtained from each after extraction with EtOH in a Soxhlet then separating the EtOAc solubles of these extracts in a similar manner to the extractions described above. The neutral fractions were those which remained in the EtOAc layer after NaOH extraction and washing with 2 N HCl, 5% $NaHCO_3$ and water.

TLC analysis of the weak acid fractions in solvent system 1 gave no indication of any pellepiphyllin in these extracts. Nothing corresponding to pellepiphyllin or methyl pellepiphyllin could be detected by GLC in the weak acid or neutral fractions before or after methylation with CH_2N_2.

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