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METABOLISM OF LUNULARIC ACID TO A NEW PLANT STILBENE BY *LUNULARIA CRUCIATA*

R. J. PRYCE*

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

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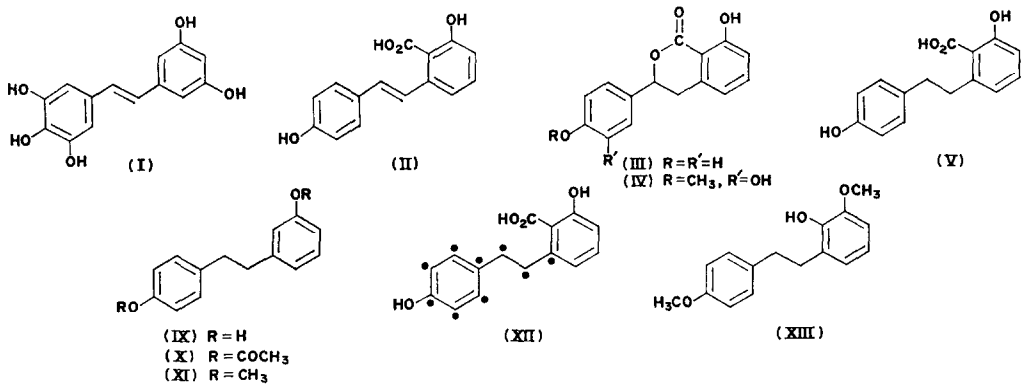
Abstract—The time course of metabolism of the C_{15}-dihydrostilbene carboxylic acid derivative, lunularic acid (V), in the liverwort *Lunularia cruciata* is reported. One of the primary metabolites of ^{14}C-lunularic acid has been identified as a new natural C_{14}-dihydrostilbene derivative named lunularin (IX). Lunularin is the simple decarboxylation product of lunularic acid and is a normal constituent of the liverwort. Incorporations of U-^{14}C-L-phenylalanine, 1-^{14}C-sodium acetate and ^{14}C-hydrangenol (III) into lunularin by *L. cruciata* are also presented. These results establish the previously proposed intermediary role of C_{15}-stilbene carboxylic acids in the phenylpropanoid-polymalonate pathway to the C_{14}-stilbene derivatives of plants. Evidence in support of a hypothesis for the photoperiodic control of liverwort growth by control of lunularic acid synthesis is presented. The antifungal activities of lunularic acid and lunularin are compared in spore germination bioassays.

INTRODUCTION

STILBENE derivatives have been found throughout the plant kingdom from algae and liverworts to conifers. They range in structure[1] from the parent hydrocarbon *trans*-stilbene from *Alnus firma*[2] to polyphenols such as the pentahydroxystilbene (I) from *Vouacapoua macropetala*[3] which can also occur as glucosides and methyl ethers. Hydrangeic acid (II), hydrangenol (III) and phyllodulcin (IV) from *Hydrangea* species[4–6] and lunularic acid (V) from liverworts and algae[7–10] are the only C_{15}-stilbene carboxylic acid derivatives known in



* Present address: Milstead Laboratory of Chemical Enzymology, Shell Research Limited, Sittingbourne, Kent.

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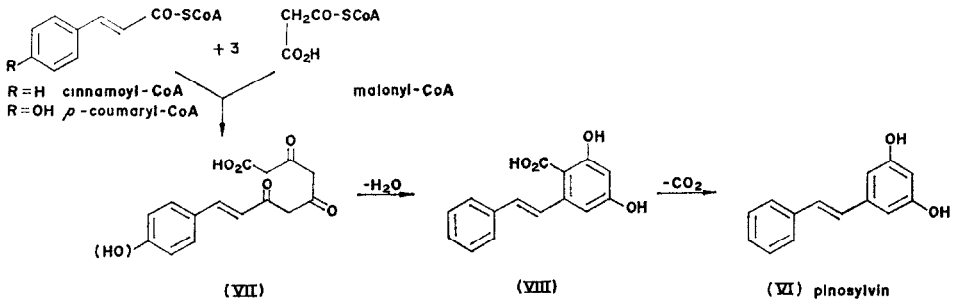
[10] R. J. PRYCE, *Phytochem.* **10**, 2679 (1971).

nature. The dihydrostilbene derivative lunularic acid has an important growth regulatory role in liverworts where it acts as a growth inhibitor apparently replacing the function of abscisic acid of higher plants.[10] Other stilbenes from trees such as pinosylvin (VI) possess fungi-toxic properties and may give protection against wood-rotting fungi.[11]



SCHEME 1. BIOSYNTHESIS OF PINOSYLVIN.

The biosynthesis of six phenolic C_{14}-stilbene derivatives[12–16] and the C_{15}-derivatives hydrangenol[17–20] and lunularic acid[10] have been studied in detail using specifically labelled substrates. In all cases results were completely consistent with their being biosynthesized by a phenylpropanoid-polymalonate pathway as originally suggested by Birch and Donovan.[21] This pathway to pinosylvin is shown in Scheme 1 and is supposed to involve the formation of a linear β-triketo acid (VII), which, by an aldol-type cyclising condensation gives rise to a C_{15}-stilbene carboxylic acid (VIII) and then by decarboxylation the C_{14}-stilbenes. To date the proposed intermediate steps of the malonate condensation, cyclisation and decarboxylation have not been established. Poly-β-keto acids such as cinnamoyl triacetic acid (VII) are known as synthetic products[22,23] but have never been detected in plant tissue and although some natural C_{15}-stilbene carboxylic acids are known (see above) they have never been shown to co-occur with C_{14}-stilbenes.[24] Hillis and Ishikura[25] reported a crude enzyme preparation from leaves of *Eucalyptus sideroxylon* which was capable of converting cinnamoyl triacetic acid into pinosylvin but recently Hillis and Yazaki[24] have found this to be completely unrepeatable, neither cinnamoyl triacetic acid nor its *in vitro* cyclisation product, pinosylvic acid (VIII),[22] being converted into pinosylvin.

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[16] G. BILLEK and A. SCHIMPL, in Proceedings of the 2nd Meeting of European Biochemical Societies, Vienna, Vol. 3; Colloquium, *Biosynthesis of Aromatic Compounds* (edited by G. BILLEK), p. 37, Pergamon Press, Oxford (1966).
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[21] A. J. BIRCH and F. W. DONOVAN, *Austral. J. Chem.* **6,** 360 (1953).
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[24] W. E. HILLIS and Y. YAZAKI, *Phytochem.* **10,** 1051 (1971).
[25] W. E. HILLIS and N. ISHIKURA, *Phytochem.* **8,** 1079 (1969).

A recent study of the biosynthesis of lunularic acid (V) in *Lunularia cruciata*[10] confirmed its phenylpropanoid-polymalonate origin and indicated a rapid turnover of this natural growth inhibitory substance in the liverwort. This paper reports an investigation of the metabolism of lunularic acid in *L. cruciata* and shows for the first time that a C_{15}-stilbene carboxylic acid derivative, lunularic acid, can act as a precursor for a new C_{14}-dihydrostilbene derivative, lunularin (IX), which is a normal constituent of the liverwort. Phenylalanine, acetate and hydrangenol (III) which were successfully incorporated into lunularic acid in *L. cruciata*[10] are also incorporated into lunularin. In order to investigate further a hypothesis[10] for the photoperiodic regulation of liverwort growth involving photoperiodic regulation of endogenous amounts of lunularic acid the effect of light and dark on the metabolism of lunularic acid in *L. cruciata* has also been investigated.

RESULTS AND DISCUSSION

^{14}C-Lunularic acid for the metabolic study described below was prepared labelled universally in its phenylpropanoid derived moiety (XII) by feeding thalli of *L. cruciata* with universally labelled phenylalanine.[10] Figures 1–4 show the time course of the metabolism of this ^{14}C-labelled lunularic acid when it was fed from a nutrient solution to cut tips of thalli of *L. cruciata* under tungsten light illumination. This method of administering ^{14}C-lunularic acid was the same as that used for substrates in the earlier biosynthetic study.[10]

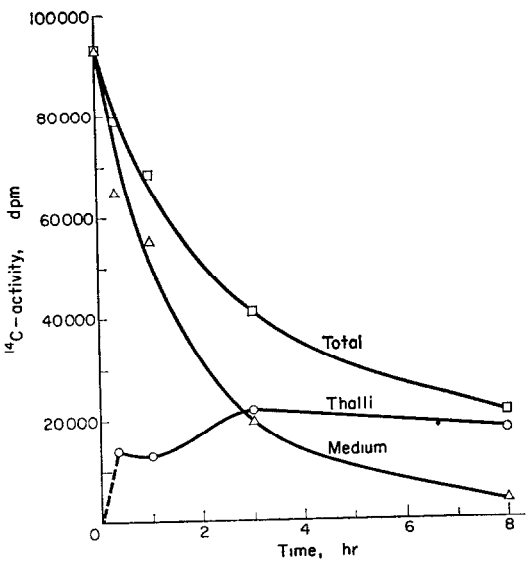

FIG. 1. TIME COURSE OF CHANGE IN TOTAL METHANOL SOLUBLE ^{14}C-ACTIVITY FROM *Lunularia cruciata* THALLI AND INCUBATION MEDIUM DURING INCUBATION WITH ^{14}C-LUNULARIC ACID.
The upper 'total' curve is the sum of the two lower ones.

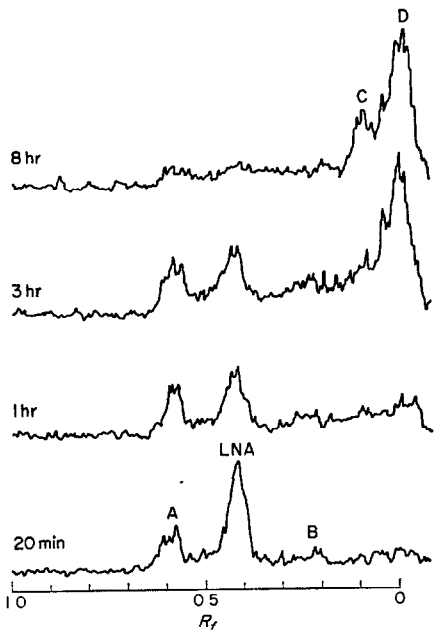

FIG. 2. TLC-RADIOAUTOGRAPHY OF EXTRACTS FROM THALLI OF *Lunularia cruciata* AFTER INCUBATION FOR DIFFERENT TIMES WITH ^{14}C-LUNULARIC ACID (LNA).
All four radioautograms were recorded under the same conditions. Silica gel TLC plates eluted once with EtOAc–$CHCl_3$–HOAc (15:5:1). Standard lunularic acid has R_f 0·43. The total ^{14}C-activity of these extracts before TLC is shown in Fig. 1.

From thalli the total methanol soluble ^{14}C-activity derived from ^{14}C-lunularic acid is seen in Fig. 1 to change little over the 8 hr incubation period whereas *ca.* 90% of the activity from the medium is lost during this period. The third curve in Fig. 1 shows a smooth drop in combined total ^{14}C-activities from thalli and medium during the incubation period and could be due to loss as $^{14}CO_2$ and/or formation of methanol insoluble ^{14}C-compounds but this point was not taken further. Fractions from the thalli taken at different times of incubation with ^{14}C-lunularic acid were analysed individually by TLC-radioautography and the result is shown in Fig. 2. Apart from the peak corresponding to lunularic acid at least four metabolites (*A*, *B*, *C* and *D*) appear and their activity is seen to vary with time. Metabolite *A* is apparently less polar than lunularic acid itself and metabolite *B*, *C* and *D* are more polar. It was evident after extracting and counting each of the TLC bands of activity after the different incubation times (Fig. 2) that as much as 50% of the total activity was being lost, presumably by adsorption onto the silica gel. However, this procedure showed that the activity of lunularic acid and the metabolites *A* and *B* remained almost constant during the 8 hr incubation period and that of *C* and *D* rose sharply during the first 3 hr and then remained constant. In an attempt to derive more information from the TLC-radioautograms in Fig. 2 areas of each of the peaks at each of the time intervals was measured and these are plotted in Fig. 3. Assuming peak areas proportional to total radioactivity it seems that metabolite *A* is the first formed metabolite from lunularic acid with *B*, *C* and *D* appearing later on. The shapes of these peak area or radioactivity curves of lunularic acid, metabolite *A* and metabolite *B* with their sequential maxima of decreasing magnitude may indicate

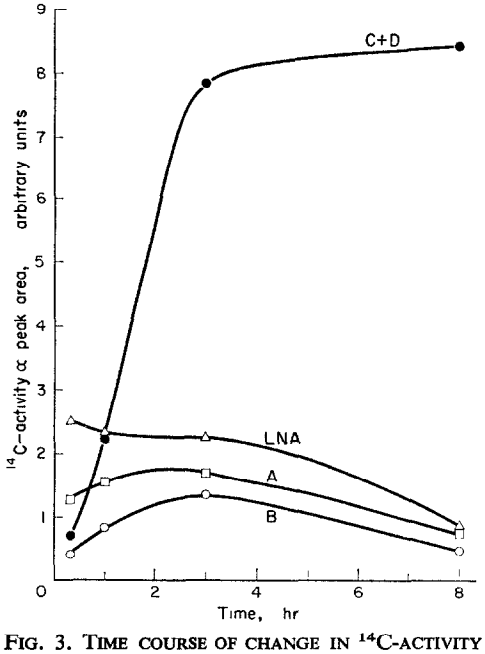

FIG. 3. TIME COURSE OF CHANGE IN ^{14}C-ACTIVITY OF METABOLITES ^{14}C-LUNULARIC ACID (LNA) FROM THALLI OF *Lunularia cruciata*: TLC-RADIOAUTOGRAM (FIG. 2) PEAK AREA MEASUREMENTS. TLC-radioautogram peak areas due to metabolites in Fig. 2 taken as proportional to total radioactivity of metabolite.

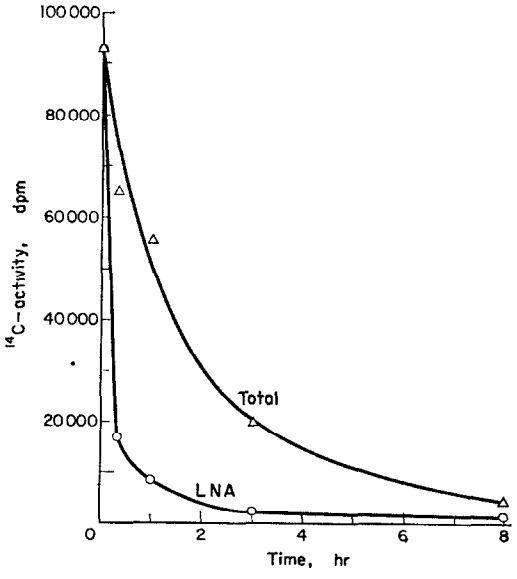

FIG. 4. TIME COURSE OF CHANGE IN ^{14}C-LUNULARIC ACID (LNA) ACTIVITY IN INCUBATION MEDIUM DURING INCUBATION WITH THALLI OF *Lunularia cruciata*. The upper 'total' curve is the ^{14}C-activity of the medium (Fig. 1) before lunularic acid separation by TLC.

that lunularic acid is first transformed into metabolite *A* which gives rise to metabolite *B*. The more polar metabolites *C* and *D* could represent later stages in the metabolism of lunularic acid and may arise through metabolites *A* and *B*.

Fractions from the incubation medium after the different times were subjected to TLC-radioautography and the bands of activity corresponding to lunularic acid were extracted for radioactivity measurement. The result presented in Fig. 4 shows a rapid drop in the lunularic acid content of the incubation medium with *ca.* 80 % being lost in the first 20 min. However, comparison of the lunularic acid curve with the total activity before the TLC separation shows a very large discrepancy. In part at least this could be accounted for by peaks of activity other than lunularic acid appearing in the medium extract but is no doubt also due to inefficient extraction of the silica gel. Other peaks of activity in the medium extract correspond to those of metabolites *A*, *B*, *C* and *D* observed in the thalli extract. After 8 hr these metabolites in the medium were considerably less important than those in the thalli and no further investigation was carried out with the medium. In a blank run with ^{14}C-lunularic acid alone in the incubation medium no trace of any degradation of lunularic acid was detected by TLC-radioautography.

To investigate the effect of light on the metabolism of lunularic acid in *L. cruciata* ^{14}C-lunularic acid was fed simultaneously in tungsten light and darkness to similar samples of thalli in the manner described above. After 18 hr incubation TLC-radioautograms of extracts from light and dark incubated thalli were practically identical qualitatively and quantitatively and both had the appearance of the TLC-radioautogram after 8 hr shown in Fig. 2. Extracts of the corresponding incubation media were also practically identical with each other in all respects.

Combined TLC fractions containing metabolite *A* from the metabolic study described above were subjected to solvent partition. It was found that 93% of the recovered activity from this combined fraction appeared in a weak acid fraction extractable with aqueous sodium hydroxide from an ethyl acetate solution after prior extraction with aqueous sodium bicarbonate. Since, under the same conditions lunularic acid is extractable with aqueous sodium bicarbonate, it was concluded that metabolite *A* must either no longer contain a carboxyl group or that the carboxyl group of lunularic acid was transformed in metabolite *A*. The first possibility suggests that metabolite *A* is simply the previously known *in vitro* decarboxylation product of lunularic acid (IX)[5] and this was indeed shown to be the case.

In TLC-radioautography metabolite *A* and its acetate derivative behaved identically with (IX) and its diacetate (X) respectively. After hydrolysis of the TLC purified acetate derivative of metabolite *A* it was again identical by TLC-radioautography in a different solvent system. This sample of the metabolite was then methylated and shown to be identical with the dimethyl ether (XI) both by TLC-radioautography and GLC. The identity of what appears to be the primary metabolite of lunularic acid in *L. cruciata*, metabolite *A*, with the known *in vitro* decarboxylation product (IX) has therefore been confirmed. By TLC and GLC as the free diphenol (IX), diacetate (X) and dimethyl ether (XI) it has been shown to be a normal and major constituent of the phenolic fraction from *L. cruciata*. Preliminary GLC examination of other liverworts suggests that it may be of equally wide occurrence among the liverworts as its precursor, lunularic acid.[9] The name lunularin is proposed for this new natural C_{14}-dihydrostilbene derivative (IX).

Table 1 shows the results of feeding experiments designed to establish the phenylpropanoid-polymalonate biosynthetic pathway to lunularin through lunularic acid in *L. cruciata*. Isolation of lunularin from fractions available from the recently reported study

of lunularic acid biosynthesis in *L. cruciata*[10] show (Table 1) successful incorprations of phenylalanine, acetate and hydrangenol into lunularin but with a higher dilution of their specific activities than into lunularic acid. Results from a shorter feeding time with lunularic acid are included in Table 1.

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

Compound	Compound fed			Lunularin				Lunularic† acid Dilution‡
	Amount (μM)	Specific activity (μCi/μM)	Incorporation time (hr)	Amount isolated (μM)	Specific activity (μCi/μM)	Incorporation into lunularin isolated (%)	Dilution‡	
U-^{14}C-L-phenylalanine	$2\cdot1 \times 10^{-3}$	$4\cdot8 \times 10^{2}$	18	2·2	$1\cdot6 \times 10^{-3}$	0·36	300 000	2800
1-^{14}C-sodium acetate	$3\cdot5 \times 10^{-2}$	$5\cdot7 \times 10$	18	1·3	$4\cdot7 \times 10^{-4}$	0·03	360 000§	3500§
^{14}C-hydrangenol (III)‖	3·1	$1\cdot3 \times 10^{-2}$	23	1·9	$1\cdot0 \times 10^{-3}$	5·2	13	1·2
^{14}C-lunularic acid (XII)‖	$9\cdot5 \times 10^{-2}$	1·1	1	1·8	$2\cdot3 \times 10^{-3}$	0·41	480	—

* Each feeding experiment was conducted with the same quantity (1g fr. wt) of cut tips of *L. cruciata* thalli in the same volume of nutrient solution (6 ml).

† Result of incorporations into lunularic acid in the same feeding experiments.[10]

‡ Specific activity of labelled compound fed by specific activity of lunularin or lunularic acid isolated.

§ Calculated on the basis of three molecules of acetate being incorporated into one molecule of lunularin or lunularic acid.

‖ Phenylalanine derived moiety universally labelled with ^{14}C—see text.

At the present time nothing more is known about the nature of the other metabolites of lunularic acid in thalli of *L. cruciata*. Examination of extracts of *L. cruciata* before and after acid hydrolysis gives no indication of the presence of bound, acid labile forms such as glycosides of lunularic acid and lunularin. There was evidence for the existence of such an acid labile bound form of lunularic acid in extracts of *Hydrangea macrophylla*.[10] The recently reported dihydrostilbene derivative, pellepiphyllin (XIII), from the liverwort *Pellia epiphylla*[26] seemed a potential metabolite[10] of the liverworts' ubiquitous lunularic acid. However, by TLC it did not correspond to any of the metabolites observed in this investigation. This is consistent with our recently reported inability to find pellepiphyllin or any of its methylated or demethylated derivatives in our samples of *P. epiphylla*, *L. cruciata* or other liverworts.[10]

Lunularic acid has previously been shown to possess some antifungal activity in spore germination bioassays.[27] In Table 2 its activity in this test against four test organisms is compared with that of its metabolite, lunularin. Lunularin appears to be less effective than lunularic acid and both compounds have somewhat lower antifungal activity than some

TABLE 2. FUNGAL SPORE GERMINATION BIOASSAY OF LUNULARIN AND LUNULARIC ACID*

Compound tested	Minimum concentration (μg/ml) for complete inhibition of germination with spores of the test fungi†.			
	Alternaria brassicicola	*Botrytis cinerea*	*Septoria nodorum*	*Uromyces fabae*
Lunularin	250	250	100	50
Lunularic acid	50	100	50	25

* Germination *in vitro* of spores in 0·2% sucrose solution. Incubation in dark at 25° for 18 hr. Simultaneous testing of lunularin and lunularic acid with the same batches of spores.

† Controls gave 90–100% germination.

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

[27] R. S. BURDEN, W. W. SCHWABE and I. F. M. VALIO, unpublished observations.

other natural stilbenes.[11] However, it is perhaps interesting to note that liverworts do not apparently suffer any fungal diseases and that two major constituents of at least one liverwort do have antifungal properties.

The results presented above confirm the previously indicated[10] rapid turnover of lunularic acid (V) by the liverwort *L. cruciata*. The identity of one of its primary metabolites has been established as a new natural dihydrostilbene derivative, lunularin (IX), which is a normal constituent of the liverwort. To the author's knowedge this is the first observation of the co-occurrence of a C_{15}-stilbene carboxylic acid and its decarboxylation product, a C_{14}-stilbene derivative, in the plant kingdom. Of more significance is the demonstration that this C_{15}-stilbene carboxylic acid, lunularic acid, is converted into the C_{14}-stilbene derivative, lunularin, by the plant thus establishing the proposed decarboxylation step (Scheme 1) in the proposed[21] phenylpropanoid-polymalonate pathway to the naturally occurring plant C_{14}-stilbenes. Hillis and co-workers have suggested[24,25] that an hydroxyl *para* to the carboxyl group, as in pinosylvic acid (VIII), may be necessary for the decarboxylation to occur. This suggestion was based on the observation that the only known naturally occurring C_{15}-stilbene derivatives containing the carboxyl group in question, (II), (III), (IV) and (V), have no such *para*-hydroxyl function. However, the present results which clearly show the *in vivo* decarboxylation of lunularic acid, having no *para*-hydroxyl to its carboxyl group, suggest that the *para*-hydroxyl is not obligatory for the decarboxylation step in C_{14}-stilbene biosynthesis. The possibility that lunularic acid was being decarboxylated *in vitro* during the work up of the metabolites has been ruled out by blank experiments and indeed lunularic acid is at least as stable to *in vitro* decarboxylation below its melting point as pinosylvic acid.[24]

The results of the incorporation of potential precursors into lunularin in *L. cruciata* (Table 1) and the higher dilution of their specific activities compared with their simultaneous incorporations into lunularic acid supports the intermediary role of lunularic acid in lunularin biosynthesis. Together with the results and conclusion presented previously[10] concerning the biosynthesis of lunularic acid and those conclusions of other workers referred to in the introduction, the known stilbene metabolism of the liverwort *L. cruciata* might be via hydrangeic acid (II) and lunularic acid (V) in an analogous manner to Scheme 1. The natural intermediary role of hydrangenol (III) and hydrangeic acid (II) has been discussed previously[10] and remains in doubt, but there is no doubt that one or both of these C_{15}-stilbene derivatives can serve as precursors for lunularic acid and lunularin. Practically nothing of the other details of biosynthesis are known either in *L. cruciata* or any other stilbene producing plant.[24] The existence and intermediary role of hypothetical β-triketo acids such as (VII) has never been satisfactorily demonstrated in any plant and this fact alone has prompted Hillis[24] to suggest that they might only exist covalently bound to enzymes. The inability to find pinosylvic acid (VIII) co-occurring with pinosylvin (VI) in *Eucalyptus sideroxylon* together with the inability of crude enzyme systems to convert pinosylvic acid into pinosylvin also gave rise to the suggestion that the whole process from the β-triketo acid (VII) to C_{14}-stilbene may involve covalently bound substrates to multienzyme complexes.[24] This latter suggestion clearly does not apply to *L. cruciata* where lunularic acid as well as lunularin are freely extractable from the liverwort and the liverwort can successfully utilise exogenously supplied lunularic acid for lunularin biosynthesis.

Of the enzymology of stilbene biosynthesis little is known at the present time.[24] In preliminary experiments with crude cell-free enzyme systems from *L. cruciata*[28] no lunu-

[28] R. J. PRYCE, unpublished observation.

laric acid nor lunularin was detectably produced from phenylalanine. Cinnamic acid was the major product, indicating the presence of phenylalanine ammonia-lyase (PAL) (see Ref. 29 and references therein) activity in the preparation. The same crude enzyme preparation was also incapable of converting hydrangenol into lunularic acid or lunularin. Much thereafter remains to be discovered concerning stilbene metabolism in plants. Investigations are in progress into the enzymology of this metabolic pathway in *L. cruciata* which passes through the endogenous growth regulator of liverworts, lunularic acid.

In an earlier publication[10] a hypothesis was proposed for the photoperiodic regulation of liverwort growth involving photoperiodic regulation of lunularic acid biosynthesis. It was proposed that the observed photoperiodic variation in extractable amounts of the growth inhibitory substance, lunularic acid, could be accounted for by the known photoactivation of PAL (Ref. 30 and references therein) and hence photoactivation of lunularic acid biosynthesis. A necessary requirement for the hypothesis is that lunularic acid should be transformed at equal rates in light and dark. Therefore, the results presented above, which indicate similar rates and pathways for lunularic acid metabolism in light and dark, support the hypothesis. Recent reports showing that cinnamic acid 4-hydroxylase[31] and *p*-coumarate: CoA ligase[32] activities are light stimulated suggests two further points for photoperiodic regulation of lunularic acid synthesis in liverworts.

EXPERIMENTAL

M.ps were measured on a Kofler block and are uncorrected. *L. cruciata* and ^{14}C-labelled compounds were obtained as previously described.[10] TLC-radioautography and other radioactivity measurements were carried out as previously described.[10] MS was performed with an AEI MS9 instrument operating at 70 eV using the direct insertion probe.

TABLE 3. THIN LAYER AND GAS-LIQUID CHROMATOGRAPHIC PROPERTIES OF LUNULARIN AND ITS DERIVATIVES*

Compound	TLC R_f values (silica gel) Solvent system†				GLC Retention indices Retention time (min) in parentheses Column	
	1	2	3	4	1·5%XE-60‡	1%OV-17‡
Lunularin	0·64	0·38	0·56			
Lunularin dimethyl ether				0·56	2433 (2·7)‡ (5·6)§	2316 (4·9)
Lunularin diacetate	0·68			0·47	2917 (12·4)	2625 (16·1)
Lunularic acid	0·44					
Methyl lunularate dimethyl ether				0·51	3038 (12·0)	2736 (22·2)
Hydrangenol		0·43	0·51			
Hydrangenol dimethyl ether				0·47		
Hydrangenol diacetate				0·33		

* Some properties of lunularic acid and hydrangenol and their derivatives determined at the same times, under the same conditions, are included to enable correlation with data published previously.[10]

† (1) EtOAc–$CHCl_3$–HOAc (15:5:1); (2) Benzene–MeOH–HOAc (20:4:1); (3) Diisopropylether–HOAc (19:1); (4) Benzene–EtOAc (8:2).

‡ Column temp. 187°, injection heater 250°, N_2 flow rate 60 ml/min unless otherwise indicated.

§ Column temp. 171°.

[29] T. SWAIN and C. A. WILLIAMS, *Phytochem.* **9**, 2115 (1970).
[30] P. SCHOPFER and B. HOCK, *Planta* **96**, 248 (1971).
[31] D. W. RUSSELL, *J. Biol. Chem.* **246**, 3870 (1971).
[32] K. HAHLBROCK, E. KUHLEN and T. LINDL, *Planta* **99**, 311 (1971).

Chromatography. The chromatographic properties of lunularin and its relevant derivatives are recorded in Table 3 and supplement those previously presented.[10] Methylated derivatives were prepared as previously.[10] TLC and GLC was carried out as previously described.[10] Lunularin and its derivatives were located on TLC plates by their blue fluorescence under UV light.

^{14}C-lunularic acid metabolism in L. cruciata. ^{14}C-Lunularic acid (1·1 μCi/μM) labelled universally in its phenylpropanoid moiety (XII) was prepared by feeding thalli of *L. cruciata* with U-^{14}C-L-phenylalanine (4·8 × 10^2 μCi/μM) as previously described.[10] 4 equal samples (0·5 g fr. wt) of cut tips of thalli of *L. cruciata* were floated separately on nutrient solution (3 ml each) containing ^{14}C-lunularic acid (4·2 × 10^{-2} μCi, 1·1 μCi/μM) in petri dishes (5 cm dia.). They were then incubated at room temp. in continuous tungsten light illumination (*ca.* 1000 lx) for different lengths of time. After 20 min, 1 hr, 3 hr and 8 hr one of the incubations was stopped by removing the thalli, quickly washing (H_2O), then extracting with EtOH in a soxhlet for 12 hr. The washings of each batch of thalli were combined with the corresponding batch of incubation medium and evaporated to dryness *in vacuo* at 35°. The EtOH extracts from each batch of thalli were evaporated to dryness *in vacuo* and the residues taken up individually in hot MeOH (4 × 5 ml) filtered and evaporated to dryness *in vacuo*. Similarly each batch of corresponding used incubation medium combined with respective washings of thalli provided MeOH soluble fractions. The ^{14}C-activity of these samples of MeOH soluble material from thalli and medium (medium plus washings) after incubations for the times indicated are shown in Fig. 1. A blank incubation of ^{14}C-lunularic acid with thalli absent was carried out for 8 hr and worked up as above to give a MeOH soluble fraction.

The four MeOH soluble extracts of thalli above were each subjected to TLC-radioautography (solvent 1, Table 3) and the results are shown in Fig. 2. Reference samples of lunularic acid (R_f 0·43) and pellepiphyllin (R_f 0·62) were run on the side of each of the four TLC plates used. Bands corresponding to lunularic acid and the metabolites *A*, *B*, and *C* and *D* combined, from each of the four extracts were extracted and aliquots of their radioactivity measured. Similarly the four samples of MeOH solubles from incubation media were subjected to TLC-radioautography (solvent 1) and each of the four lunularic acid bands were extracted and measured for radioactivity (Fig. 4). TLC-radioautography of the blank incubation extract showed only lunularic acid and nothing corresponding to any of the metabolites *A*, *B*, *C* or *D*. After heating a cold sample of lunularic acid with 1 N HCl on a steam bath for 1 hr no lunularin (metabolite *A*) formation could be detected by TLC or GLC.

To investigate the effect of light and dark on the metabolism of lunularic acid in thalli of *L. cruciata* ^{14}C-lunularic acid (6·1 × 10^{-2} μCi, 1·1 μCi/μM) was fed to each of two equal samples of thalli (1 g fr. wt). One sample was kept in the dark for 18 hr and the other was incubated in light. MeOH solubles from the light and dark incubated thalli and their corresponding incubation media were examined by TLC-radioautography (solvent 1).

Identification of metabolite A. TLC fractions containing metabolite *A* from above were combined (6·2 × 10^{-3} μCi) and dissolved in EtOAc (12 ml). The EtOAc solution was extracted first with 5% $NaHCO_3$ (4 × 3 ml) and then with 1 N NaOH (4 × 3 ml). Both of these combined extracts were acidified with conc. HCl and extracted with EtOAc (3 × 4 ml) to give, after drying (Na_2SO_4) and evaporation to dryness *in vacuo* strong and weak acid fractions respectively. The remaining EtOAc after the NaOH extraction was dried (Na_2SO_4) and evaporated to dryness *in vacuo* to give the 'neutral' fraction. Radioactivity measurement of the 'neutral', strong and weak acid fractions showed that 93% of the recovered activity was associated with the weak acid fraction (2·2 × 10^{-3} μCi); strong acid fraction (5·0 × 10^{-5} μCi) and 'neutral' fraction (1·3 × 10^{-4} μCi).

The weak acid fraction containing metabolite *A* was mixed with pure, cold lunularin (IX) (1 mg) and subjected to TLC-radioautography (solvent 1). After extracting the active band corresponding to pure lunularin it was dissolved in dry pyridine (150 μl) and acetic anhydride (300 μl), left to stand for 1 hr at room temp. and then evaporated to dryness *in vacuo* at room temp. The acetate derivative thus formed was subjected to TLC-radioautography (solvent 4) and the active band corresponding to lunularin diacetate (X) (see below) was extracted and hydrolysed by refluxing under N_2 with 6% KOH in 90% aq. MeOH (2 ml) for 4 hr. After cooling, diluting (H_2O, 15 ml) and acidification (conc. HCl) the hydrolysis product was extracted into EtOAc (3 × 5 ml), dried (Na_2SO_4) and evaporated to dryness *in vacuo*. TLC-radioautography of the hydrolysis product (solvent 3) showed one active band corresponding to lunularin which was extracted, methylated with CH_2N_2 and then on TLC-radioautography (solvent 4) showed one active band corresponding to lunularin dimethyl ether (XI) (see below). This TLC purified sample of active methylated metabolite A plus cold lunularin dimethyl ether (6·9 × 10^{-4} μCi) showed only one peak on GLC (XE-60) column corresponding to lunularin dimethyl ether.

Biosynthesis of lunularin. The EtOAc fractions remaining after extraction of the strong acids with 5% $NaHCO_3$ from feeding experiments previously reported with thalli of *L. cruciata*[10] had been dried (Na_2SO_4), evaporated and stored and were used for this investigation. These EtOAc fractions from feedings of U-^{14}C-L-phenylalanine, 1-^{14}C-sodium acetate and ^{14}C-hydrangenol were each redissolved in EtOAc (12 ml) and extracted with 1 N NaOH (4 × 4 ml). Each NaOH extract was acidified with conc. HCl and extracted with EtOAc (3 × 5 ml) which was dried (Na_2SO_4) and evaporated to dryness *in vacuo* to give the corresponding weak acid fractions. In a new experiment ^{14}C-lunularic acid (1·0 × 10^{-1} μCi, 1·1 μCi/μM) was fed to thalli.

of *L. cruciata* (1g fr. wt.) as before[10] in nutrient solution (6 ml) and tungsten light (*ca.* 1000 lx) for 1 hr After this time thalli were extracted in the usual way[10] to give a weak acid fraction similar to those described above. The weak acid fractions from each of the four feedings experiments were then treated identically. They were each subjected to TLC in solvent 1 and, with the help of TLC-radioautography and UV visualization, the lunularin bands were extracted and then subjected to further preparative TLC in solvent 3 and 2 in that order. The essentially pure lunularin samples obtained after TLC in solvent 2 were then methylated and subjected to further preparative TLC in solvent 4 prior to quantitation by GLC and planchette radioactivity counting of an aliquot.

Lunularin and its derivatives. Lunularin was prepared essentially as previously described.[5] Lunularic acid (V) (25 mg), prepared in quantitative yield by hydrogenolysis of hydrangenol (III) in EtOH over 5% Pd/C, was melted and kept above its m.p. for 1 min using the heat of a micro bunsen burner. The total product was then fractionally sublimed at 160°/0·1mm Hg to give pure crystalline lunularin (IX) m.p. 105–107° (Lit.[5] m.p. 108–109°) (12 mg) M^+-214 (base peak *m/e* 107—dibenzylic cleavage).

Lunularin diacetate was prepared by dissolving lunularin (350 μg) in dry pyridine (100 μl) and Ac_2O (200 μl) and leaving the mixture to stand at room temperature for 1 hr. After this time the reaction mixture was evaporated to dryness *in vacuo* at room temp. to give lunularin diacetate (X) M^+-298 (*m/e* 149—dibenzylic cleavage, was 4% of base peak *m/e* 107—dibenzylic cleavage after loss of 2 × CH_2CO from M^+ [*m/e* 256 and *m/e* 214]) which was >95% pure by TLC and GLC analysis.

Lunularin dimethyl ether was prepared by treating a MeOH solution of lunularin with excess ethereal diazomethane for 12 hr at room temp. then evaporating the solution. This gave lunularin dimethyl ether (XI) M^+-242 (base peak *m/e* 121—dibenzylic cleavage) which was >95% pure by TLC and GLC analysis.

Detection of lunularin in L. cruciata. Thalli of *L. cruciata* were extracted with EtOH in a Soxhlet as previously described.[10] This extract was separated into strong and weak acid fractions as above. TLC of an aliquot of the weak acid fraction in solvent 1 showed the presence of a major component of the same R_f as lunularin. After acetylation and methylation of aliquots of the weak acid fractions TLC in solvent 4 showed major components of the same R_f values as lunularin diacetate and lunularin dimethyl ether respectively. In these three TLCs just described lunularin and its derivatives were detected by their UV fluorescence and also after spraying the plates with a solution of 4% $Ce(SO_4)_2$ in 10% H_2SO_4 and heating. The presence of lunularin as a major component of the weak acid fraction from *L. cruciata* was confirmed by GLC (XE-60 and OV-17 columns) of both acetylated and methylated weak acid fractions. No increase in the amount of lunularin present in weak acid fractions from *L. cruciata* could be detected after prior acid hydrolysis (1 N HCl on a steam bath for 1 hr) of an aliquot of evaporated EtOH extracts. Similarly no increase in extractable amounts of lunularic acid could be detected in strong acid fractions. These quantitative measurements were carried out by GLC with methylated extracts.

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Note added in proof. A personal communication from Professor V. Herout states that, due to a wrong identification, pellepiphyllin was isolated from *Pellia neesiana* and not from *P. epiphylla* as originally noted.[26] This correction corroborates the author's inability to detect pellepiphyllin in *P. epiphylla*.[10]

Key Word Index—Lunularia cruciata; Hepaticae; Bryophyta; biosynthesis; stilbenes; lunularic acid; lunularin; growth inhibitor metabolism; antifungal.