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BIOSYNTHESIS OF HERNIARIN: THE ISOMERIZATION STAGE

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Abstract—The isomerization step in the biosynthesis of herniarin in lavender plants has been shown to be non-enzymic. The reaction is light catalysed.

INTRODUCTION

COUMARINS have been found widely distributed in nature, the majority having been isolated from plants.[1,2] The coumarins may be divided into two main groups, those with an oxygenated substituent in the 7-position and those in which this substituent is absent. As a result of tracer experiments, biosynthetic pathways leading to the formation of coumarins have been elaborated.[3] Although the routes involved in the biosynthesis of the 7-oxygenated and non-oxygenated compounds are different in detail the overall pathways have much in common. Figure 1 illustrates the pathways for coumarin (I) and herniarin (II). One stage which is common to the biosynthesis of all coumarins from plant sources is the isomerization of the *trans*-glucoside to the *cis*-glucoside as illustrated by reactions (a) and (b) in Fig. 1.

For coumarin itself it is well established[4–6] that *o*-coumaryl glucoside can be readily converted to coumarinyl glucoside by u.v. light. Recently, indirect evidence by Haskins, Williams and Gorz[7] and more direct evidence by Edwards and Stoker[8] has shown that in sweet clover the isomerization stage is entirely photochemical, no enzyme being involved. The analogous reaction in the biosynthesis of other coumarins has not been reported. This paper presents the results of investigating the isomerization stage of a typical 7-oxygenated coumarin, herniarin.

RESULTS

The chromatographic mobilities of herniarin and some related compounds in the two solvent systems employed in this study are reported in Table 1. Preliminary experiments showed that 2-hydroxy-4-methoxycinnamic acid tends to isomerize to the corresponding

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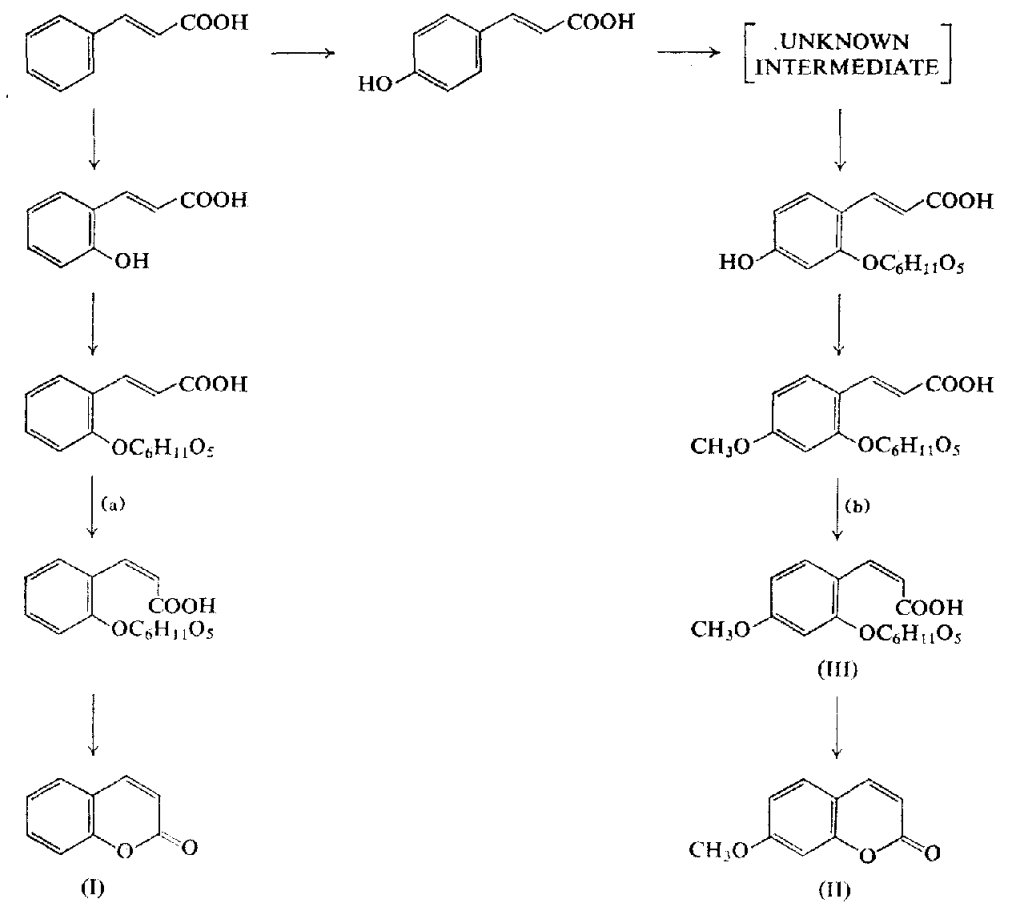

FIG. 1. BIOSYNTHETIC ROUTES TO COUMARIN AND HERNIARIN.

cis-compound in aqueous solution, even in the dark. This makes difficult the interpretation of the results of experiments in which 2-hydroxy-4-methoxycinnamic acid is administered to shoots of *Lavandula* species. Consequently, control experiments were performed using labelled

TABLE 1. CHROMATOGRAPHIC MOBILITIES OF HERNIARIN AND RELATED COMPOUNDS

	R_f value	
	Solvent 1	Solvent 2
Herniarin	0·70	0·55
2-Hydroxy-4-methoxycinnamic acid	0·00	0·00
Coumarin	0·95	0·95
o-Coumaric acid	0·00	0·00

Solvent 1. Chloroform developed plates of Kieselgel G.
Solvent 2. 5% Acetic acid in water developed chromatograms of Whatman 3 MM paper by the descending technique.

TABLE 2. CONVERSION OF 2-HYDROXY-4-METHOXYCINNAMIC ACID-α-^{14}C TO HERNIARIN IN *Lavandula* SPECIES

Pretreatment	Extraction method*	Quantity of herniarin isolated (mg)	Specific activity of herniarin (dpm/m mole × 10^{-5})	% of original radio-activity in the herniarin isolated	Herniarin impurity in precursor solution (%)
IN DARKNESS					
None	Method 1†	1·15	2·92	0·32	0·97
None	Method 1†	1·20	2·83	0·30	0·97
Heated	Control† method 1	1·49	5·46	0·78	0·97
Heated	Control† method 1	1·07	6·85	0·70	0·97
None	Method 2	0·97	3·38	1·29	3·01
None	Method 2	0·73	3·46	0·94	3·01
Heated	Control method 2	0·82	7·85	2·29	3·01
Heated	Control method 2	0·77	8·00	2·19	3·01
IN LIGHT					
None	Method 1	1·23	21·7	5·86	0·97
None	Method 1	1·26	21·4	5·92	0·97

* Four experiments were performed in each series, only two typical results are quoted. For details of method 1 and method 2, see experimental. Amounts of cinnamic acid fed were as follows: 3·0 ml of 0·0048 M solution of 2-hydroxy-4-methoxycinnamate, $4{\cdot}054 \times 10^7$ dpm/m mole (method 1); 3·0 ml of 0·0013 M solution of 2-hydroxy-4-methoxycinnamate, $4{\cdot}054 \times 10^7$ dpm/m mole (method 2); 3·0 ml of 0·0021 M solution of 2-hydroxy-4-methoxycinnamate, $4{\cdot}054 \times 10^7$ dpm/m mole (method 1—Light).

† *Lavandula spica* used. In all other experiments *L. officinalis* plants were used.

TABLE 3. THE METABOLISM OF HERNIARIN-3-^{14}C BY *Lavandula spica* PLANTS

Compound fed	Quantity of herniarin isolated (mg)	Specific activity of herniarin isolated (dpm/m mole × 10^{-6})	% of original herniarin not metabolized
Herniarin-3-^{14}C (4·0 ml; 0·00038 M; $3{\cdot}87 \times 10^7$ dpm/m mole)	2·21	1·61	23·4
	1·99	1·34	25·8

precursor mixed with ground heat-killed plant material to give some indication of the amount of non-enzymic isomerization occurring at the pH of the cell sap. The results of the feeding and control experiments are shown in Table 2. Also recorded in this table are the results of feeding experiments carried out in artificial light and the determination of the amount of herniarin in precursor solutions. A second method for the isolation of herniarin with β-glucosidase was used, in case the normal glucosidases of the plant were unable to hydrolyse *cis*-2-glucosyloxy-4-methoxycinnamic acid (III) to herniarin.

Since precursor solutions of 2-hydroxy-4-methoxycinnamic acid contained some herniarin, plants were also fed ^{14}C-labelled herniarin to give an indication of its rate of metabolism. These results are given in Table 3.

DISCUSSION

The isomerization of 2-hydroxy-4-methoxycinnamic acid in aqueous solution in the dark[11] tends to complicate the interpretation of the results of feeding experiments. This isomerization makes it virtually impossible to prepare solutions of 2-hydroxy-4-methoxycinnamic acid devoid of herniarin.

In all control experiments in which killed plant material was incubated with precursor solution, the amount of radioactivity in the herniarin isolated was only slightly less than was originally present in the herniarin impurity of the precursor solution. This would suggest that since no appreciable synthesis or breakdown of herniarin has occurred under these conditions, the lower value for radioactivity associated with the isolated herniarin represents the efficiency of the extraction method.

The results of the test experiments in the dark all show much lower values for the amounts of radioactivity in the herniarin. In the presence of an isomerase the amount of radioactivity in the herniarin would be expected to increase unless herniarin is rapidly metabolized. However, in the absence of an isomerase, the radioactivity of the herniarin originally present as impurity in the precursor solution would not change. The observed reduction in the amount of radioactivity in the herniarin could be explained by the absence of an isomerase, i.e. no new herniarin is formed from the labelled 2-hydroxy-4-methoxycinnamic acid while some of the original herniarin is further metabolized. That this interpretation is correct is borne out by the observation that, when herniarin itself is administered to *Lavandula spica* plants, about 75 per cent is metabolized in 24 hr (Table 3). This value is of the same order as that for the loss of radioactivity associated with herniarin in the test experiments.

Although the results obtained by extraction methods (1) and (2) are not directly comparable because different precursor solutions and plant materials were used, the same interpretation can be applied, indicating that the plant enzymes are able to fully hydrolyse *cis*-2-glucosyloxy-4-methoxy-cinnamic acid to herniarin.

That photochemical isomerization does take place is well shown since approximately twenty times more radioactivity was found in the herniarin in plants exposed to light than in the corresponding plants kept in the dark (Table 2).

The results quoted in this paper indicate that the isomerization stage in the biosynthesis of herniarin is a photochemical step, no isomerase enzyme being involved. Consequently, this isomerization stage has now been shown to be photochemical both for coumarin itself[8] and for a typical 7-oxygenated coumarin. It is probable therefore that the isomerization step in the biosynthesis of all plant coumarins is entirely photochemical. At the present time the only microbial coumarin to be examined biosynthetically is novobiocin and this has been shown to arise by an atypical oxidative cyclization route.[12]

EXPERIMENTAL

Plant Material

Lavandula spica plants (6 months old) were grown from seed in the greenhouse. *Lavandula officinalis* plants (1 yr old) were grown in soil outdoors.

Radioactive Compounds

2-Hydroxy-4-methoxycinnamic acid-α-^{14}C and herniarin-3-^{14}C were synthesized according to the methods of Brown.[9,10]

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Administration of Labelled Compounds

The 2-hydroxy-4-methoxycinnamic acid-α-^{14}C was dissolved in the calculated quantity of 5% $NaHCO_3$ solution just prior to use and the pH adjusted to 7·5–8·0 when necessary. Shoots (about 4 g) were cut and the stems trimmed under water. The cut ends were immersed in 3 ml of the precursor solution (for concentrations see Table 2). As solutions were taken up by the shoots water was added to keep the cut ends immersed. Metabolic periods of 24 hr were used, plants being either kept in darkness throughout this time or continuously exposed to artificial light using a Siemens Sieray 250-watt lamp, after uptake of the precursor solution.

Isolation of Herniarin (Method 1)

After the metabolic period the plant material was ground with acid-washed sand (about 2 g) and transferred with 10 ml of distilled water to a vial sealed with a polythene cap. After allowing to stand in the dark at 21° for 1 hr, acetone (5 ml) was added. The vial was mechanically shaken for 1 hr. The extract was then strained through muslin, the filtrate acidified with 2 N HCl (2 ml) and extracted with ether (2 × 20 ml). The ether layer was evaporated, dried and chromatographed on Kieselgel G plates with chloroform. The fluorescent area corresponding in position to herniarin was scraped off the plate and the herniarin extracted by shaking for 1 hr with acetone. The silica gel was removed by centrifugation and the acetone supernatant chromatographed on Whatman No. 3MM paper using 5 per cent acetic acid as developing solvent. The area corresponding to herniarin was cut out and eluted with absolute ethanol. Aliquots of this eluate were taken for measurement of radioactivity and for u.v. spectrophotometric assay.

Isolation of Herniarin (Method 2)

After the incubation period, the plant material was ground with 0·1 M phosphate buffer (pH 5·0), transferred to a vial and allowed to stand for 1 hr. The vial was then immersed in boiling water for 5 min, cooled and 2 ml of a β-glucosidase solution added (3 mg/ml). After incubation for 48 hr at 21° the herniarin was extracted as described in method 1. All isolation techniques were carried out in the dark-room using a 25-watt lamp with a Kodak Wratten OB safelight filter.

Control Experiments (Method 1)

Plant material was ground as described above and allowed to stand 1 hr. The ground plant material was then heated in a boiling-water bath for 5 min to destroy enzymes, allowed to cool and precursor solution (3 ml) added. After 24 hr incubation in conditions similar to those used for the intact plants the herniarin was isolated as before.

Control Experiments (Method 2)

This method was similar to that above except that after incubation with precursor solution, phosphate buffer (10 ml, pH 5·0) and β-glucosidase solution (6 mg in 2 ml) were added. The herniarin was then extracted according to method 2 above.

Quantitative Determination of Herniarin Impurity in the Precursor Solution

Precursor solution (3 ml) was adjusted to pH 7·5–8·0 and allowed to stand 24 hours in the dark. The solution was acidified with 5 ml 2 N HCl and extracted with ether (2 × 20 ml). The ether extract was chromatographed on Kieselgel G plates developed with chloroform. The herniarin area was scraped off and eluted with acetone. Aliquots of the eluate were counted for radioactivity.

Determination of Radioactivity

Ethanol or acetone solutions of compounds were counted in toluene containing 0·6% PPO and 0·05% POPOP using an I.D.L. 6012 Liquid Scintillation Counter. *n*-Hexadecane-1-^{14}C was used as an internal standard in all measurements.

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