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BIOSYNTHESIS OF SHIKONIN DERIVATIVES FROM L-PHENYLALANINE VIA DEOXYSHIKONIN IN *LITHOSPERMUM* CELL CULTURES AND CELL-FREE EXTRACTS

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; L-phenylalanine; biosynthesis; shikonin derivatives; deoxyshikonin; intermediate; esterification.

Abstract—^{14}C-labelled L-phenylalanine administered to shikonin-producing cell cultures of *Lithospermum erythrorhizon* was incorporated rapidly into deoxyshikonin and then into fatty acid esters of shikonin. A gradual increase in the radioactivity of shikonin derivatives was accompanied by a decrease in that of deoxyshikonin. Furthermore, ^{14}C-labelled deoxyshikonin supplied to cell cultures was incorporated into shikonin derivatives, e.g. acetyl- and β-hydroxyisovaleryl-shikonin, although ^{14}C-labelled shikonin was hardly converted into shikonin derivatives. ^{14}C-labelled deoxyshikonin incubated with a cell-free extract was also converted into esterified shikonin derivatives including acetylshikonin. These data strongly suggested that shikonin derivatives are biosynthesized from deoxyshikonin by hydroxylation and esterification at the C-1 position of its side chain both in cultured cells and in cell-free extracts of *L. erythrorhizon*.

INTRODUCTION

Cultured cells of *Lithospermum erythrorhizon* Sieb. et Zucc. are used in Japan for the commercial production of red naphthoquinone pigments, i.e. shikonin derivatives [1]. Shikonin derivatives occur in the plant root, as well as in cell cultures mostly as fatty acid esters, such as acetyl-, β,β-dimethylacryl-, isobutyl-, β-hydroxyisovaleryl-, isovaleryl- and α-methyl-*n*-butyl-shikonin, except for deoxyshikonin (**5**) which cannot form any esters due to lack of a hydroxyl group at the C-1 position of the side chain [2–5]. Tracer experiments [6, 7] have demonstrated that shikonin (**6**) is biosynthesized through the condensation of *p*-hydroxybenzoic acid (**2**), which is derived from L-phenylalanine (**1**), with geranylpyrophosphate (GPP) which is itself formed from mevalonic acid. The resulting intermediate, *m*-geranyl-*p*-hydroxybenzoic acid (**3**), is converted into a key intermediate, geranylhydroquinone (**4**), which is subsequently transformed to **6**. Although various physical and chemical factors regulating shikonin production have been investigated [8–26], no study has been made on the biosynthetic process leading to the esterification of **6**. In this paper, we report on the formation of shikonin derivatives via **5** from L-[U-^{14}C]phenylalanine (**1**) by cell cultures and cell-free extracts of *L. erythrorhizon*.

RESULTS

Ca 53–64% of the L-[U-^{14}C]phenylalanine (**1**, 3.7 kBq) administered to production medium (M9) [27, 28] at various days after inoculation was absorbed by cultured *Lithospermum* cells within 24 hr of incubation at 25° (Table 1). Radio TLC analysis of seven-day-old cell cultures after 24 hr of inoculation with **1** showed that the radioactivity had been incorporated into various shikonin derivatives including **5** (Fig. 1). Figure 2 shows the time course of the incorporation of radioactive **1** into shikonin derivatives in seven-day-old cultured cells. The appearance of radioactive **5** was first detected 5 min after the administration of labelled **1**. The radioactivity present in **5** then increased rapidly to reach the maximum value within 90 min. Thereafter it gradually decreased to reach a constant value at 24 hr. By contrast, radioactive acetylshikonin was first observed 10 min after the administration of **1**, then increased gradually to reach a maximum after 24 hr. These results suggest that **5** is biosynthesized from **1** prior to the formation of **6** and its esters.

In order to ascertain the above postulate, that **5** is converted to **6** and its esters, ^{14}C-labelled **5** was administered to four-day-old *Lithospermum* cell cultures producing shikonin derivatives in M9 medium. Analysis of the cells after a three-day incubation in the dark showed that the radioactivity was efficiently incorporated into various shikonin derivatives, suggesting the occurrence of hydroxylation of **5** to form **6**, which is then followed by the esterification of **6** with specific fatty acids, in particular acetic acids (Table 2). By contrast, ^{14}C-labelled **6** administered to cell cultures under the same conditions was hardly converted to its esters apart from a small amount of acetylshikonin. These results strongly

Table 1. The uptake of L-[U-^{14}C] phenylalanine (1) [3.7 kBq test tube^{-1}] by cultured cells of *L. erythrorhizon* during an incubation period of 24 hr

Pre-culture period in M9 medium (day)	Uptake of ^{14}C-phenylalanine (3.7 kBq test tube^{-1}) (%)
0	53.7
4	60.3
7	64.8
10	56.8

suggest that **5** is an intermediate in the biosynthetic pathway leading to **6** (Scheme 1). The validity of this hypothesis is supported by the following experiments using cell-free extracts, which would contain a multi-enzyme system [2] catalysing the biosynthetic reactions for the formation of shikonin derivatives from the substrate **5**.

A reaction mixture consisting of a 100 000 g microsomal fraction ($\rho = 1.09$–1.10 g cm^{-3}) and substrate **5** or **6** was incubated at 27° for 3 hr, then extracted with Et_2O. The extract was subjected to TLC (silica gel) together with standard shikonin derivatives, and the radioactivity of each band corresponding to a specific shikonin derivative was measured by liquid scintillation counter. As shown in Table 3, ^{14}C-labelled **5** was converted to esterified shikonin derivatives. On the other hand, ^{14}C-labelled **6** was hardly converted to any esterified shikonin derivatives. These results clearly indicate that only **5** can readily be transformed to **6** and esters of **6** by enzymatic reactions *in vitro*.

DISCUSSION

The present study has demonstrated that deoxyshikonin (**5**) is an intermediate in the biosynthesis of shikonin (**6**) and its esters, suggesting that both hydroxylation of **5** and esterification of **6** are catalysed by membrane bound enzymes localized probably in vesicles ($\rho = 1.09$–1.10 g cm^{-3}), where *p*-hydroxybenzoate geranyltransferase, the key enzyme of shikonin biosynthesis [19, 22, 23], has recently been shown to be located [25]. The observation that the exogenously supplied substrate **5** was more efficiently converted into esterified shikonin derivatives than the substrate **6** by intact cells, as well as by the cell-free extracts, seems to suggest that the hydroxyl group at the C-1 position of the side chain of **6** interferes with the uptake of this molecule to the site of its enzymatic esterification. Based on the results of the foregoing studies, it is suggested that a series of biosynthetic reactions leading to shikonin derivatives from *p*-hydroxybenzoic acid might be carried out by a multi-enzyme system bound to the membrane vesicle, which is thought to be responsible for the transport and secretion of shikonin derivatives [16, 17]. Such a system would not only facilitate the biosynthesis of **5** from the precursor L-phenylalanine (**1**), which is estimated to take only 5 min in shikonin-producing cells, but also ensure the physical isolation of toxic secondary metabolites from the cytoplasm. This hypothetical model is illustrated in Scheme 2. It has been suggested that the final metabolites formed in the vesicle probably exist as naphthol compounds, which would be oxidized upon exposure to the air to form red pigments, i.e. shikonin derivatives [25].

Preliminary experiments have suggested that cytochrome P-450 may be involved in the enzymatic hydroxylation of **5**, since the production of esterified shikonin

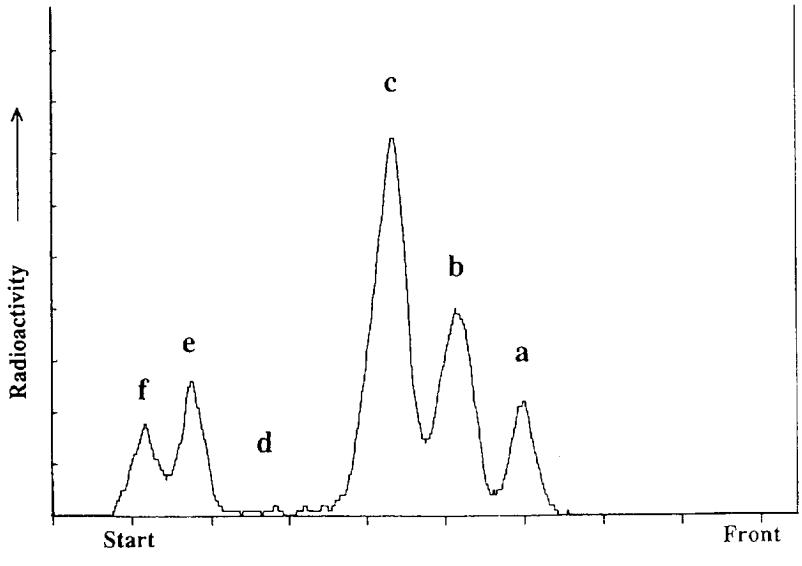


Fig. 1. Radiochromatogram of the red pigment extracted from seven-day-old cell cultures of *L. erythrorhizon* incubated with L-[U-^{14}C]phenylalanine (**1**) [3.7 kBq test tube^{-1}] for 24 hr. (a) Deoxyshikonin (**5**), (b) the mixture of β,β-dimethylacryl-, isobutyl-, isovaleryl-, α-methyl-*n*-butyl-shikonin and echinofuran B, (c) acetylshikonin, (d) shikonin (**6**), (e) β-hydroxyisovaleryl-shikonin, (f) unidentified.

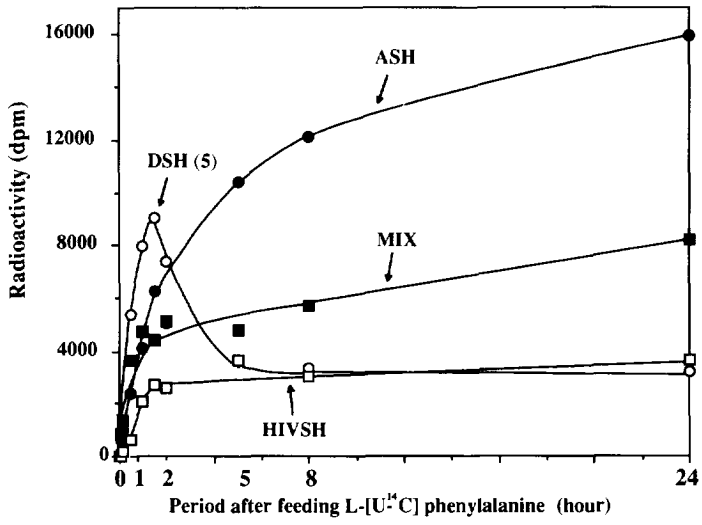


Fig. 2. Time course of the incorporation of radioactivity into shikonin derivatives in cultured cells of *L. erythrorhizon* incubated with L-[U-^{14}C] phenylalanine (**1**) [3.7 kBq test tube^{-1}]. ○: deoxyshikonin (**5**), ●: acetylshikonin, □: β-hydroxyisovaleryl-shikonin, ■: the mixture containing β,β-dimethylacryl-, isobutyl-, isovaleryl-, α-methyl-*n*-butyl-shikonin and echinofuran B.

Table 2. Incorporation of ^{14}C-labelled deoxyshikonin (**5**) and shikonin (**6**) into their shikonin derivatives in *Lithospermum* cell suspension cultures

		Radioactivity (dpm) of shikonin derivatives				
Substrate	Total activity (dpm)	DSH (**5**)	ASH	SH(**6**)	HIVSH	MIX
^{14}C-DSH (**5**)	6000	230.8	1783.3	66.8	310.9	312.8
Incorporation ratio (%)	(100)	—	(29.7)	(1.1)	(5.2)	(5.2)
^{14}C-SH (**6**)	6000	—	168.5	2680.8	—	—
Incorporation ratio (%)	(100)	—	(2.8)	—	—	—

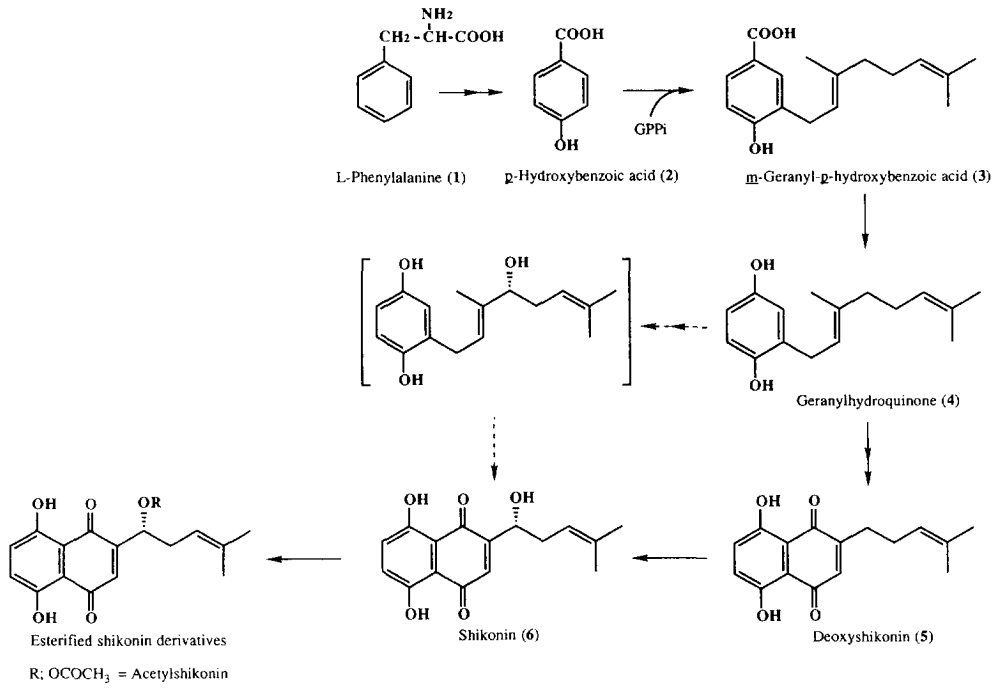
The substrate **5** or **6** (40 μg 100 μl^{-1} Et_2O: 6×10^3 dpm) was administered to four-day-old cell cultures (250 mg fr. wt) in M9 medium for three days at 25° before harvest. DSH: deoxyshikonin. ASH: acetylshikonin. HIVSH: β-hydroxyisovaleryl-shikonin. SH: shikonin. MIX: a mixture of β, β-dimethylacryl-, isobutyl-, isovaleryl-, α-methyl-*n*-butyl-shikonin and echinofuran B. Two replicates.

derivatives in *Lithospermum* cell cultures was found to be inhibited by ketoconazole (10^{-5} M), an inhibitor of cytochrome P-450 (data not shown).

EXPERIMENTAL

Culture methods. Cell culture strain M18 [11] of *L. erythrorhizon* strain M18 [11] was grown in a 500 ml-flask containing 120 ml of Linsmaier–Skoog (LS) liquid medium [29], supplemented with 10^{-6} M 3-indoleacetic acid and 10^{-5} M kinetin, on a reciprocal shaker (100 strokes · min^{-1}) at 25° in the dark. The cultures were subcultured in the same medium at intervals of 2 weeks. For production of shikonin derivatives, 200 mg fr. cells were inoculated into a test tube (180 × 20 mm) containing 7 ml of M9 medium [27, 28] containing the same hormones as above and agitated in the dark at 25°.

Administration of L-[U-^{14}C] *phenylalanine* (**1**). To each cell culture, 3.7 kBq test tube^{-1} of **1** (15.7 MBq μmol^{-1}, Amersham) was administered 0, 4, 7 or 10 days after inoculation into M9 medium and the cultures were agitated in the dark at 25°. After harvesting, the cells were extracted with 4 ml of Et_2O, twice. The medium was mixed with Scintisol 500 (Wako Chemicals), and a toluene soln containing 2,5-diphenyloxazole and 1,4-bis [2-(5-phenyloxazoyl)] benzene as a scintillator was added to an aliquot of the ethereal extract for the measurement of radioactivity by liquid scintillation counter. The remaining part of each mixt. was applied to a TLC plate (silica gel 60 F-254, Merck), developed with *n*-



Scheme 1. Proposed biosynthetic pathway leading to esterified shikonin derivatives via deoxyshikonin (**5**) in cultured cells of *L. erythrorhizon*.

Table 3. Incorporation of ^{14}C-labelled deoxyshikonin (**5**) and shikonin (**6**) into shikonin derivatives in a microsomal fraction ($\rho = 1.09$–1.10 g cm^{-3}) prepared from cultured cells (four-day-old) of *Lithospermum*

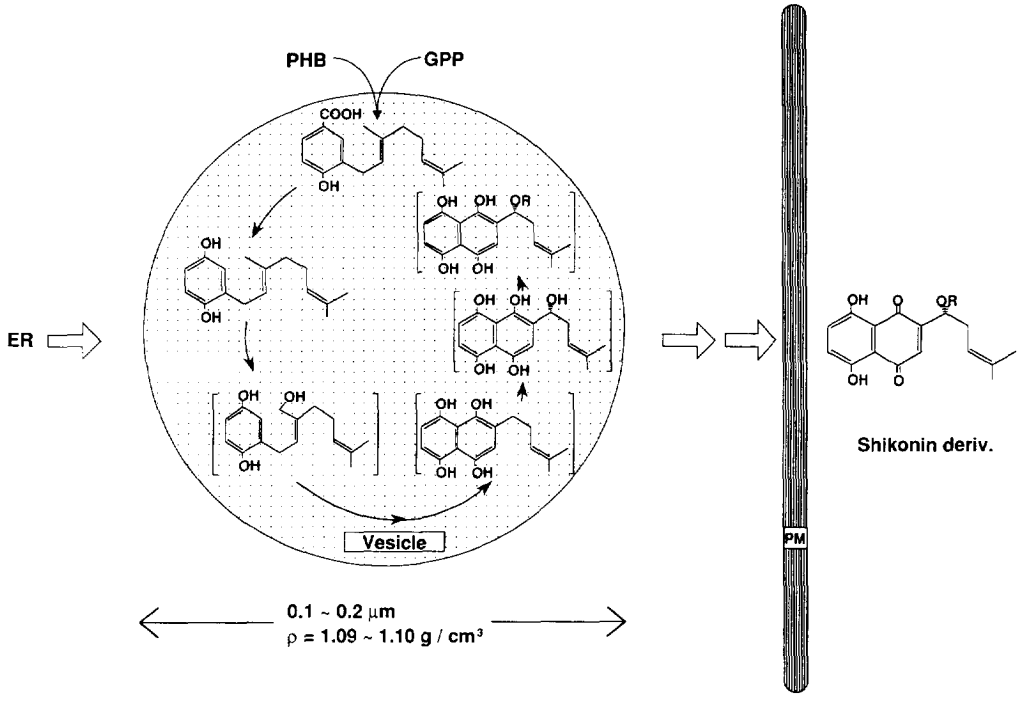
Substrate	Total activity (dpm)	Radioactivity (dpm) of shikonin derivatives				
		DSH (**5**)	ASH	SH(**6**)	HIVSH	MIX
^{14}C-DSH (**5**)	6000	52.8	246.8	30.2	61.1	166.0
Incorporation ratio (%)	(100)	—	(4.1)	(0.5)	(1.0)	(2.8)
^{14}C-SH (**6**)	6000	—	20.1	5.9	10.4	10.2
Incorporation ratio (%)	(100)	—	(0.3)	—	(0.2)	(0.2)

The reaction mixture was incubated for 3 hr at 27°. Two replicates.

hexane–C_6H_6–Me_2CO–HCO_2H (20:80:3:1) or $CHCl_3$ [5, 8], and then scanned with a radiochromanizer (Aloka JTC/ACM 505).

Tracer experiments using cultured cells. ^{14}C-labelled **5** or **6** (40 μg dissolved in 100 μl of Et_2O, 6×10^3 dpm test tube^{-1}) was administered to cell suspension cultures, which were agitated for 3 days in the dark at 25°. The ethereal extract of the treated cells was subjected to TLC, followed by radio-TLC analysis of the radioactivity present in the bonds corresponding to shikonin derivatives.

Preparation of cell-free extracts. Cultured cells (1 g fr. wt) of *L. erythrorhizon* were transferred to 100 ml flasks containing 30 ml of M9 medium [27, 28] to induce shikonin production. In addition, 5 ml liquid paraffin (ampoule quality, Merck) was added to the medium to extract excreted red pigments (shikonin derivatives) from the cell surface into the paraffin layer [25] during a culture period of 4 days. At the time of harvest, the paraffin layer was removed by pipetting after first adding 5 ml *n*-hexane to reduce the viscosity, and the cells (10 g) collected on Miracloth (Calbiochem) by filtration were suspended in 20 ml 0.1 M K-Pi buffer (pH 6.5) containing 10 mM dithiothreitol (DTT) and 1.5 g PVPP. The suspended cells were homogenized in a Potter homogenizer, then centrifuged successively at 4000 *g* for 10 min and at 10000 *g* for 15 min (RS-18, TOMY). The supernatant was



Scheme 2. A hypothetical model for the biosynthesis and secretion of shikonin (**6**) by a membrane vesicle. PHB: *p*-hydroxybenzoic acid, GPP: geranylpyrophosphate, ER: endoplasmic reticulum.

then centrifuged at 100000 *g* for 40 min. The pellet (microsome fraction) was resuspended in 1 ml of the same buffer and placed on the top of a sucrose density gradient solution (10 ml; 20, 25, 32, 43, 47% w/v sucrose in 0.1 M K-Pi buffer containing 10 mM DTT, pH 6.5) [25, 30, 31] and centrifuged for 30 min at 100000 g (RSP-4-T, Hitachi). The fr. with a density of $\rho = 1.09$–$1.10\ g\,cm^{-3}$ (sucrose concentration: 20%) that corresponds to the fr. containing vesicles [25] was collected and resuspended in 2.5 ml 0.2 M Tris–HCl buffer (pH 7.5). The resuspended fr. was applied to Sephadex G-25 (PD-10 column, Pharmacia) which had been equilibrated with a reaction buffer containing 10 mM DTT. All the procedures were carried out at 4°. Protein content was estimated by the use of BSA as a standard according to ref. [32].

Tracer experiments using cell-free extracts. The incubation mixt. (1.0 ml) contained 0.5 ml cell-free extract and 0.5 ml buffer. The assay mixt. was incubated at 27° for 3 hr after addition of ^{14}C-labelled **5** or **6** [100 μg (6×10^3 dpm) dissolved in 100 μl 0.2 M Tris–HCl buffer, pH 7.5, containing Triton X-100 (Sigma; the final concn. in the incubation mixt. was 0.3%)]. The reaction was stopped by adding 100 μl 4 N $HClO_4$ and 100 mg NaCl to the soln, which was then extracted with 0.5 ml Et_2O, 3 ×. The extract was applied to a silica gel TLC plate and developed with the solvent system described earlier, together with standard shikonin derivatives. Bands corresponding to shikonin derivatives were individually scraped off from the TLC plate, and extracted with Et_2O to measure their radioactivity content by the above-mentioned method.

Identification of ^{14}C-labelled shikonin derivatives. The radioactive compounds sepd by TLC were identified as deoxyshikonin (**5**), shikonin (**6**), acetylshikonin, β-hydroxyisovaleryl-shikonin, and a mixt. containing β, β-dimethylacryl-, isobutyl-, isovaleryl-, α-methyl-*n*-butyl-shikonin and echinofuran B by co-chromatography (TLC and HPLC) with authentic samples. The radioactive shikonin derivatives were confirmed by hydrolysing them with 2.5% KOH to yield **6**. ^{14}C-labelled **5** was unaffected by the alkaline treatment [8].

HPLC conditions. Integrator: 807-IT (JASCO), Pump: 880-PU(JASCO), Detector: 875-UV/VIS(JASCO), Column: TSK-Gel ODS 120 A (10 μm, Toyo Soda, Japan), 150 × 4.6 mm. Solvent: AcCN–H_2O–Et_3N–HOAc (70:30:0.3:0.3). Flow rate: 0.5 ml min^{-1}. Detection: 520 nm [13].

Amount of shikonin derivatives. The quantity of each shikonin derivative was determined spectrophotomerically by measuring the adsorption at 620 nm after hydrolysis with 2.5% KOH. The amount of each pigment was expressed as that of **6** [8].

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