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ACCUMULATION OF *p*-*O*-*β*-D-GLUCOSYLBENZOIC ACID AND ITS RELATION TO SHIKONIN BIOSYNTHESIS IN *LITHOSPERMUM* CELL CULTURES

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Key Word Index—*Lithospermum erythrorhizon*; Boraginaceae; *p*-*O*-*β*-D-glucosylbenzoic acid; *p*-hydroxybenzoic acid; plant cell culture; biosynthesis.

Abstract—*p*-Hydroxybenzoic acid, which is one of the precursors in shikonin biosynthesis, and its glucoside (*p*-*O*-*β*-D-glucosylbenzoic acid) were isolated from the cell cultures of *Lithospermum erythrorhizon*. The glucoside was accumulated by the cells producing no shikonin in LS liquid medium, but it decreased rapidly when the cells were transferred to 'production medium' to induce shikonin synthesis. These results suggest that the precursor *p*-hydroxybenzoic acid is stored in the form of a glucoside when the cells are not synthesizing shikonin.

INTRODUCTION

Cell suspension cultures (strain M18) of *Lithospermum erythrorhizon*, which are capable of producing a large amount of shikonin (**1**) in 'production medium' M9 [1–3], fail to synthesize it in Linsmaier–Skoog (LS) medium [4] mainly because of ammonium ion supplied as a nitrogen source. However, it remains to be clarified as to which reaction step in shikonin biosynthesis is blocked and what kind of intermediate or metabolite might accumulate in the cells growing in LS medium without producing shikonin. In this paper we report the accumulation of *p*-*O*-*β*-D-glucosylbenzoic acid in such cells, and will discuss its implication in the regulation of shikonin production.

RESULTS

Isolation of p-*hydroxybenzoic acid and its glucoside*

The methanol extract of the fresh cells of culture strain M18 grown in LS medium for 3 weeks was concentrated and extracted with benzene, ethyl acetate and *n*-butanol successively. The ethyl acetate and *n*-butanol extracts contained rosmarinic acid and lithospermic acid, respectively, as we reported previously [5]. Further analysis of these extracts by TLC indicated the presence of two other UV-absorbing substances. Thus, the ethyl acetate extract was chromatographed on a Sephadex LH-20 column and eluted with 80% ethanol to give **2**. This compound gave R_fs 0.77 and 0.24 on TLC (silica gel) when developed with solvent systems I and II (see Experimental), and UV absorption maxima at 208 and 253 nm in methanol. Its ^{1}H NMR spectrum showed an AB pattern at δ6.92 (*d*, J = 9 Hz) and 7.92 (*d*, J = 9 Hz), suggesting the presence of a 1,4-substituted benzene ring. These data were identical with those of *p*-hydroxybenzoic acid, and the final identification of **2** was achieved by direct comparison with an authentic sample.

The *n*-butanol extract was also chromatographed on a column of Sephadex LH-20 and eluted with the same solvent as described above to give **3**, R_f 0.20 on TLC (silica gel, solvent I), UV absorption maxima 205 and 246 nm (MeOH). The ^{1}H NMR showed an AB pattern in the aromatic region at δ7.13 (2H, *d*, J = 9 Hz) and 7.98 (2H, *d*, J = 9 Hz). These signals were assigned to two pairs of vicinal protons in a 1,4-substituted benzene ring. In addition, there appeared a doublet at δ5.01 (1H, *d*, J = 7 Hz) assignable to H-1 of glucose and six broad proton signals between δ3.35 and 3.97. These results suggested that **3** was an *O*-glucoside of **2**. The possibility of an ester linkage was eliminated by the fact that the anomeric proton of the synthetic glucosyl ester appeared at δ5.50 in its ^{1}H NMR spectrum. The configuration of the glucoside bond was determined to be of *β*-form on the basis the coupling constant and the hydrolysis by *β*-glucosidase. These data indicated that **3** was *p*-*O*-*β*-D-glucosylbenzoic acid, and its identity was confirmed by direct comparison with an authentic specimen [6, 7].

Contents of **2** *and* **3** *in cultured cells and intact roots*

Table 1 shows the contents of **2** and **3** in various cell suspension cultures of *L. erythrorhizon*, including variant strains M130 and LY that are incapable of producing shikonin even in M9 medium.

All the cultures that produced no shikonin were found to accumulate a relatively large quantity of **3** (1.0–1.3 mg/g fr. wt or 18–23 mg/g dry wt) together with a small quantity of **2** (0.01–0.02 mg/g fr. wt or 0.18–0.35 mg/g dry wt), irrespective of culture strain. By contrast, the shikonin-producing strain M18 cultured in M9 medium contained only small quantities of **2** and **3** which were approximately one hundredth of the contents in the cultures without shikonin production.

Analysis of the dried roots of *L. erythrorhizon* containing shikonin derivatives (1.4% of dry wt) also showed that the contents of **2** and **3** in the roots (0.23 and 0.014 mg/g dry wt) were as small as those in the shikonin-producing cells. Furthermore, the cell suspension cultures of six

Table 1. Contents of 2 and 3 in the cultured cells of *Lithospermum erythrorhizon*

Culture strain	Culture medium	Cell yield (g fr. wt/30 ml)	Shikonin (mg/g fr. wt)	2 (mg/g fr. wt)	3 (mg/g fr. wt)
M18	LS	5.2	0	0.011	1.30
M130	LS	6.3	0	0.018	1.00
LY	LS	6.5	0	0.021	1.25
M18	M9	2.7	3.2	trace	0.012

other plant species that contain no shikonin were examined as regards the contents of 2 and 3. The results showed that very small amounts of 3 (4.7–8.0 μg/g fr. wt) and 2 (trace) were found in the cultured cells of *Mallotus japonicus*, *Datura innoxia* and *Paeonia albiflora* var. *trichocarpa*, but these compounds were not detected in *Gardenia jasminoides*, *Perilla frutescens*, *Catharanthus roseus* and *Bupleurum falcatum* cell sucpension cultures. It seems therefore that 2 and 3 are not commonly accumulated by plant cell cultures, but they are formed in large amounts in *Lithospermum* cells without shikonin production.

Time-course of the production of p-*hydroxybenzoic acid and its glucoside*

Figure 1 shows the time-course of the production of 2 and 3 in the cell suspension cultures of strain M18 grown in LS medium. The total amount of 3 increased almost in parallel with the cell growth, while its content per fresh weight of cells remained nearly constant at a relatively high level during the whole period of cell growth. On the other hand, the production of 2 lagged far behind that of 3 and began to increase at the linear growth stage.

When the cells precultured in LS medium were transferred to M9 medium for shikonin production, the content of 3 decreased rapidly to a very low level by 4th day of culture, as the cells began to produce shikonin (Fig. 2). And only a trace of 3 was detectable after 16 days of culture, when the shikonin content reached its maximum. The content of 2 also decreased with time in M9 medium, though at a slower rate as compared with that of 3.

DISCUSSION

The present study has demonstrated that the *Lithospermum* cells cultured in LS medium accumulate a large amount of 3 and a small amount of 2, without

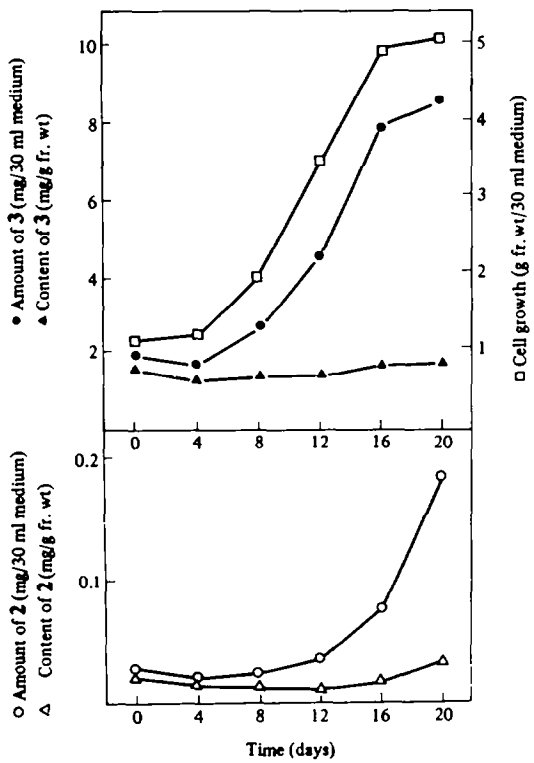


Fig. 1. Time-course of *p*-hydroxybenzoic acid (2) and its glucoside (3) in the cultured cells of *Lithospermum erythrorhizon* grown in LS medium.

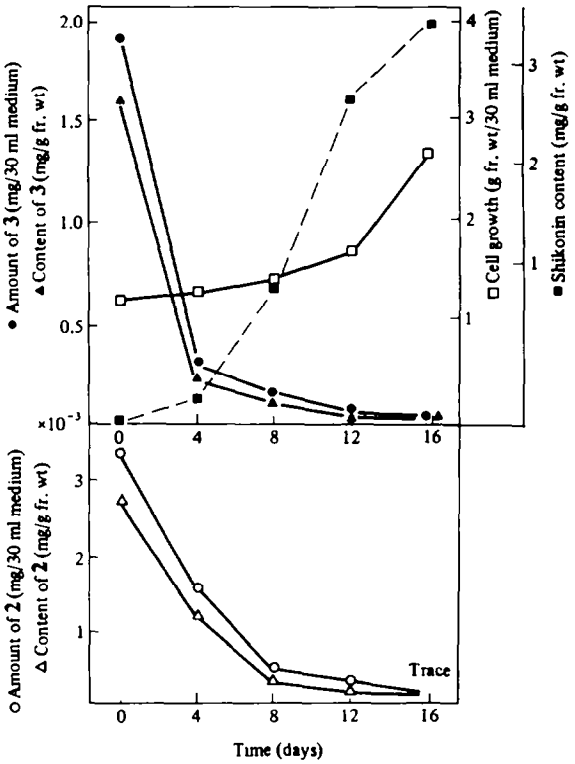


Fig. 2. Time-course of *p*-hydroxybenzoic acid (2) and its glucoside (3) in the cultured cells of *Lithospermum erythrorhizon* grown in M9 medium.

producing shikonin. When the cells were transferred from LS to M9 medium, 2 and 3 disappeared rapidly while the shikonin content increased gradually. In addition, preliminary experiments using cell cultures of various species have shown that 2 and 3 are not usually accumulated by the cultured cells of other plant species examined.

It has been reported by Zenk *et al.* [8] and Matsumura *et al.* [9] in *Plagiobotrys arizonicus* plants and *L. erythrorhizon* callus cultures, respectively, that 2 which is derived from L-phenylalanine (4) through *trans*-cinnamic acid is converted into alkannin or its enantiomer 1 via *m*-geranyl-*p*-hydroxybenzoic acid (5) and geranylhydroquinone (6). Furthermore, the tracer experiments by the latter workers have suggested that shikonin synthesis is blocked by the auxin 2,4-D at the step of decarboxylation of 5. The present work has shown that the *Lithospermum* cells synthesizing no shikonin in the LS medium still keep producing an excess of 3 which will mostly be glucosylated and probably stored in the vacuoles (Scheme 1).

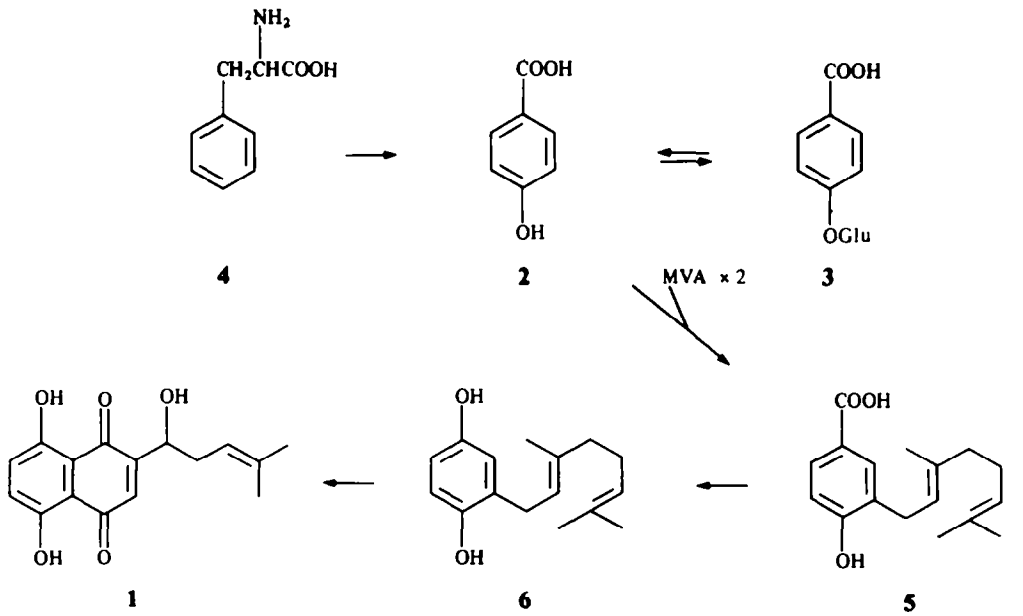
The observation that the content of 3 decreased rapidly with the induction of shikonin synthesis in the M9 medium seems to suggest that 3 would be utilized as a biosynthetic precursor of shikonin through hydrolysis.

EXPERIMENTAL

Plant material and culture method. The culture strain M18 [10] of *Lithospermum erythrorhizon*, which was originally derived from the germinating seed, has been maintained in Linsmaier–Skoog's liquid medium [4] containing 10^{-6} M IAA and 10^{-5} M kinetin. The cell suspension cultures (inoculum size: 1.1 g fr. wt) in 100 ml flasks with 30 ml medium were agitated on a reciprocal shaker (100 strokes/min) at 25° in the dark and subcultured at intervals of 3 weeks. These cells were harvested for chemical analysis 3 weeks after inoculation. For induction of shikonin synthesis, the nonpigmented cells precultured for 3 weeks in LS medium were transferred to the M9 medium [1–3] containing the same growth hormones and cultured for 2 weeks before harvest. In addition to strain M18, two variant strains M130 and LY, which are incapable of producing shikonin in M9 medium, were cultured in the media containing the same growth hormones and only 10^{-6} M 2,4-D, respectively. The cell suspension cultures of *Gardenia jasminoides*, *Perilla frutescens* var. *crispa*, *Mallotus japonicus*, *Catharanthus roseus*, *Bupleurum falcatum*, *Datura innoxia* and *Paeonia albiflora* var. *trichocarpa* were also cultured in the LS medium containing 10^{-6} M 2,4-D under the same culture conditions. The dried roots of *Lithospermum erythrorhizon* were purchased from the market.

Isolation of 2 *and* 3. The fresh cells (468 g fr. wt) of strain M18 cultured in LS liquid medium were extracted with MeOH (3 l.). The extract was concd *in vacuo* and extracted successively with C_6H_6, EtOAc and *n*-BuOH. The EtOAc extract (460 mg) was chromatographed on a column of Sephadex LH-20 (2 × 43 cm) and eluted with 80% EtOH to obtain a series of fractions (5 g each). The combined fractions 10–13 were subjected to prep. TLC (solvent system: $CHCl_3$–MeOH, 4:1) to isolate 2 (11 mg) as colourless needles, mp 212–213°. Fractions 17–40 contained mainly rosmarinic acid (180 mg). The *n*-BuOH extract (1.43 g) also was fractionated by the above-mentioned method and fractions 11–15 were re-chromatographed on a Sephadex LH-20 column and eluted with the same solvent as above. Treatment of the eluate with Amberlite XAD-2 gave 3 (200 mg) as colourless needles, mp 210–211°. Fractions 23–25 contained crude rosmarinic acid (49 mg), while fractions 28–40 yielded lithospermic acid (66 mg). Compounds 2 and 3 were identified by direct comparison with authentic samples.

HPLC and TLC. The pigmented cells of culture strain M18 grown in M9 medium as well as the nonpigmented cells of culture strains M18, M130, and LY grown in LS medium were collected on Miracloth filtration and homogenized with 5-fold volume of MeOH. The suspension cultures of the other species also were treated similarly. The MeOH extracts were then subjected to HPLC analysis; column: TSK-GEL ODS 120A, 10 μm (Toyo Soda, Japan), 150 × 4.6 mm, solvent system: H_2O–MeOH–HOAc (385:10:5), flow rate: 1.5 ml/min, pressure: 100 kg/cm², detection: absorption at 250 nm. The quantities of 2



Scheme 1.

and 3 were estimated from the corresponding peak areas recorded by Chromatopac C-R1A (Shimadzu). TLC analysis of the extracts were carried out using Kieselgel 60F-254 (Merck) and the solvent system (I) EtOAc–MeOH–H_2O (100:16.5:13.5) or (II) $CHCl_3$–MeOH (9:1).

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REFERENCES

1. Fujita, Y., Tabata, M., Nishi, A. and Yamada, Y. (1982) *Plant Tissue Culture Proc. 5th Intl. Cong. Plant Tissue and Cell Culture* (Fujiwara, A., ed.) p. 399. Maruzen, Tokyo.
2. Fujita, Y., Hara, Y., Ogino, T. and Suga, C. (1981) *Plant Cell Rep.* **1**, 59.
3. Fujita, Y., Hara, Y., Suga, C. and Morimoto, T. (1981) *Plant Cell Rep.* **1**, 61.
4. Linsmaier, E. F. and Skoog, F. (1965) *Physiol. Plant.* **18**, 100.
5. Fukui, H., Yazaki, K. and Tabata, M. (1984) *Phytochemistry* **23**, 2398.
6. Sabalitschka, T. and Schwetzer, F. L. (1929) *Arch. Pharm.* **267**, 675; *Chem. Abstr.* **24**, 1703 (1930).
7. Gaind, K. N. and Chopra, K. S. (1976) *Planta Med.* **30**, 174.
8. Schmid, H. V. and Zenk, M. H. (1971) *Tetrahedron Letters* 4151.
9. Matsumura, H. (1981) Ph.D. Thesis, Kyoto University.
10. Mizukami, H., Konoshima, M. and Tabata, M. (1978) *Phytochemistry* **17**, 95.