The Mechanism of Shikonin Biosynthesis in
*Lithospermum* Cell Cultures

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**Introduction**

Murasaki, *Lithospermum erythrorhizon* Sieb. et Zucc. (Boraginaceae), is a perrenial herbaceous species which is rarely found in mountains and fields in Japan, Korea, and China. The root of this plant is red-purple, because its cork layers contain red naphthoquinone pigments, shikonin derivatives, which have been used as a precious dye since ancient times. Shikon, the dried root, has also been used as a crude drug, particularly in the form of a red ointment, to treat skin ailments, such as wound, burn, frostbite, swelling, and anal hemorrhage\(^1\). Pharmacological studies have verified that shikonin as well as its fatty acid esters such as acetylshikonin show antibacterial\(^2-4\), anti-inflammatory\(^5\), wound-healing\(^5\), and tumor-inhibiting\(^6\) activities.

The chemical structure of shikonin (Fig. 1), which had first been proposed by Kuroda\(^7\), was finally determined by Brockmann\(^8\) as an optical isomer of alkannin, a red pigment isolated from an European species, *Alkanna tinctoria*. It was later confirmed by Arakawa & Nakazaki\(^9\) that the absolute configuration of shikonin is of the R-form, whereas that of alkannin is of the S-form, indicating that they are enantiomers. Our stereochemical studies\(^10-12\) have revealed that each shikonin derivative isolated from the roots as well as callus cultures of *Lithospermum* is a mixture of a large proportion of the R-form (84-93%) and a small proportion of the S-form (7-16%). However, the two enantiomers showed no significant difference in the antiinflammatory activity in animal experiments\(^13\).

This paper will give a brief review of studies on the regulatory mechanisms of shikonin biosynthesis with special reference to the subcellular localization of biosynthesis and transport of shikonin in *Lithospermum* cell cultures.

![Fig. 1 Chemical structures of shikonin and its optical isomer alkannin.](image-url)
Shikonin production in callus cultures

Tabata et al.\textsuperscript{14} demonstrated that callus cultures derived from seedlings of \textit{L. erythrorhizon} produce the same shikonin derivatives as those accumulated in the cork layers of roots, when grown on the Linsmaier-Skoog (LS) agar medium\textsuperscript{15} containing indole-3-acetic acid (IAA) and kinetin in the dark. However, the production of these pigments was completely inhibited by either replacing IAA with 2, 4-dichlorophenoxyacetic acid (2, 4-D) or by irradiating cultured cells with either white light or blue light. Similarly, \textit{Lithospermum} roots irradiated with light hardly produced shikonin, suggesting that the darkness of the underground is essential to shikonin production.

Selection of high-yielding cell lines

Repeated cell selection, which had been shown to be effective in improving the capabilities of plant cell cultures to produce such secondary metabolites as nicotine\textsuperscript{16, 17} and indole alkaloids\textsuperscript{18}, was applied to \textit{Lithospermum} callus cultures to establish a high-yielding cell line, M18, whose shikonin content reached 1.2 mg/g fresh weight of cells, showing a 20-fold increase over the original strain\textsuperscript{19}. Furthermore, selection of 478 single-cell clones from a single callus gave 8 relatively stable cell lines whose average shikonin content was 2.3 mg/g fresh wt (4.8% of dry wt), which was higher than that of the intact roots (ca. 1.3%)\textsuperscript{20}.

Main factors controlling shikonin production

**Light:** Since blue light specifically inhibits shikonin formation without affecting cell growth, it might inactivate a labile flavoprotein regulating shikonin biosynthesis by decomposing its riboflavin component, which absorbs blue light (440-540 nm) to degrade itself to lumiflavine (7, 8, 10-trimethylisoalloxazine) in an alkaline condition. Actually, adding lumiflavine to cultured cells did inhibit shikonin production, and studies on the effects of synthetic analogs of flavin confirmed that the isoalloxazine skeleton is responsible for the inhibition\textsuperscript{21}. It is likely that the expression of a gene controlling the biosynthesis of shikonin might be repressed through the inactivation of a hypothetical flavoprotein functioning as a photoreceptor, since the \textit{de novo} synthesis of a key enzyme (PHB-geranyltransferase) involved in the formation of an intermediate leading to shikonin is inhibited while cell cultures are being irradiated with blue light, as will be mentioned later.

**Ammonium ion:** Fujita et al.\textsuperscript{22} found that \textit{Lithospermum} cell suspension cultures, which failed to produce shikonin in LS liquid medium, restored the activity when transferred to White's liquid medium\textsuperscript{23}. The failure of shikonin production in LS medium proved to be due to the presence of ammonium ion, a major nitrogen source, which is absent but replaced with nitrate ion in White medium. Based on this finding, they devised the production medium M9 containing no ammonium salt but nitrate, a liquid medium optimal for shikonin production\textsuperscript{24}. We showed later that the inhibition of shikonin production is not due to the ammonium ion itself but is caused by a high level of glutamine that is accumulated by the cells supplied with such ammonium salt as \textit{NH}_4\textit{NO}_3\textsuperscript{25}. This is an interesting case where the overflow of a specific primary metabolite plays a role of remote control over the biosynthesis of a specific secondary product in plant cells.

Chemical inducer of shikonin biosynthesis

In connection with a peculiar phenomenon that \textit{Lithospermum} cells can produce shikonin on LS agar medium despite the presence of ammonium salt, Yoshikawa\textsuperscript{26} found that shikonin production is inducible by adding a small amount of agar powder (0.05% w/v) to LS liquid medium. Thus the
agar, which had long been regarded as a physiologically inactive substance, was shown to be a potent inducer of shikonin biosynthesis. Subsequently, Fukui et al.\textsuperscript{27} demonstrated that the agaropeptin component of the agar was responsible for the activity, suggesting that an intrinsic acidic polysaccharide of the cell might play an important role in initiating shikonin biosynthesis. This possibility seemed to be supported by the fact that an addition of Pectolyase (polygalacturonase) to cell suspension cultures in LS medium induced shikonin production\textsuperscript{28}. After many attempts to purify acidic polysaccharides from shikonin-producing cells in M9 medium, Tani et al.\textsuperscript{29} have finally isolated an active principle that was identified as $\alpha$-1,4-oligogalacturonide (Fig. 2), whose degree of polymerization (DP) was 12 or more (average: 18). The addition of the oligogalacturonides (DP = 12-20) to LS medium resulted in a rapid increase of PHB geranyltransferase of cultured cells, followed by the biosyntheses of shikonin and dihydroechinofuran\textsuperscript{30}. These results suggested that Lithospermum cells would release $\alpha$-1,4-oligogalacturonide as an "intrinsic inducer" of shikonin biosynthesis probably through the enzymatic hydrolysis of pectin, by a cause unlike plant pathogen-induced elicitation of phytoalexin production. The action mechanism of these oligosaccharides is not clear, but they might bind with a receptor on the cell membrane to alter cell physiology.

\begin{center}
\textbf{Fig. 2} Endogenous inducer of shikonin biosynthesis.
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\textbf{Fig. 3} Biosynthetic routes to shikonin and its related compounds.
1: L-phenylalanine, 2: p-coumaric acid, 3: p-hydroxybenzoic acid (PHB), 4: PHB-O-glucoside, 5: geranylpyrophosphate (GPP), 6: m-geranyl-p-hydroxybenzoic acid (GBA), 7: geranylhydroquinone (GHQ), 8: shikonin, 9: dihydroshikonofuran, 10: dihydroechinofuran.
\end{center}
Biosynthetic pathway

Using *Lithospermum* callus cultures, Inouye *et al.*\(^{31}\) conducted tracer experiments to demonstrate that shikonin is biosynthesized through the prenylation of \(p\)-hydroxybenzoic acid (PHB, 3) derived from \(L\)-phenylalanine (1) with two molecules of mevalonic acid, as reported for the biosynthesis of alkannin, an optical isomer of shikonin, in *Plagiobotrys arizonicus*\(^{32}\) (Fig. 3). They also detected the formation of two important intermediates, \(m\)-geranyl-\(p\)-hydroxybenzoic acid (GBA, 6) and geranylhydroquinone (GHQ, 7) in minute amounts\(^{31}\). These results have been confirmed by Yazaki *et al.*\(^{33}\) who isolated GBA (71 mg) and GHQ (73 mg) as colorless oils from shikonin-producing cell suspension cultures (400 g dry wt) in M9 medium, together with two unusual metabolites of GHQ, *i. e.* shikonofuran E and deoxyshikonofuran. Furthermore, it has been shown that shikonin and its ester derivatives are biosynthesized from deoxyshikonin via hydroxylation and esterification at the C\(_1\)-position of the side chain by tracer experiments administering \(^{14}\)C-labeled compounds of \(L\)-phenylalanine and deoxyshikonin\(^{34}\).

On the other hand, shikonin-free cell cultures in LS liquid medium gave only a trace amount of GBA in addition to a novel compound, dihydroshikonofuran (10). This suggested that the activity to synthesize the intermediates was strongly suppressed in the white cells\(^{35}\), which directed phenylpropanoid precursors to syntheses of such phenolic acids as rosmarinic acid and lithospermic acid in place of shikonin\(^{36}\). Unexpectedly, the addition of activated carbon to the white cells in LS medium caused, for some unknown reason, extracellular release of an oily orange pigment which was identified as echinofuran B, an oxidized form of deoxyshikonofuran derived from the intermediate, GHQ\(^{37}\). Apparently, the treatment of cells with activated carbon activates the biosynthesis of GHQ in LS liquid medium, but it cannot open up the subsequent pathway leading to shikonin so that GHQ will be abnormally metabolized to form a furan ring on its side chain. This would mean that the cells would require some special conditions for carrying out a series of reactions to synthesize shikonin from GHQ along the normal pathway.

Enzymes participating in shikonin biosynthesis

1) **PHB-geranyltransferase**: The activity of this key enzyme (geranylpyrophosphate: \(p\)-hydroxybenzoate geranyltransferase) was first detected in cell-free extracts of *Lithospermum* cell suspension cultures by Heide & Tabata\(^{38,39}\). This enzyme that catalyzes the condensation of PHB (3) and geranylpyrophosphate (GPP, 5) to yield GBA (6) requires divalent cation, in particular, Mg\(^{2+}\) for the reaction, and the optimal pH for its activity ranges widely from 7.1 to 9.3. It is highly specific for both substrates, PHB and GPP, and its activity in shikonin-producing cells was 35 times as high as that in non-producing cells. This enzyme was detectable only in the microsomal fraction. Separation of the microsomal components by continuous sucrose density gradient centrifugation confirmed that the enzyme was localized in a membrane having a density of \(\rho = 1.09 - 1.10 \, \text{g} \cdot \text{cm}^{-3}\), which was distinctly different from the densities of the plastid, plasma membrane and tonoplast, but was similar to that of the endoplasmic reticulum\(^{40}\). We could liberate PHB geranyltransferase from the membrane by the use of a surface active agent (cholic acid or CHAPS) and obtained a relatively stable, solubilized enzyme, which has been partially purified by a series of column chromatography.

2) **Geranylpyrophosphate synthase**: GPP synthase, a constitutive soluble enzyme (molecular wt: 73,000) extracted from *Lithospermum* cell suspension cultures by Heide\(^{41,42}\), catalyzes the synthesis of GPP from isopentenyl pyrophosphate (IPP) and 3,3-dimethylallyl diphosphate...
(DMAPP) in the presence of Mg$^{2+}$ or Mn$^{2+}$. This was a new discovery of the highly specific GPP synthase that takes only DMAPP and IPP as the substrates to synthesize GPP as the sole product. The purified GPP synthase was applied as an excellent catalyst to the synthesis of the position-specifically labeled[1-$^{14}$C]GPP from [1-$^{14}$C]IPP and DMAPP with a high yield of 50%\(^{43}\). It has been shown that GPP synthase is localized in the cytosol of Lithospermum cells by Sommer \textit{et al}.\(^{44}\)

3) \textit{“PHB synthase”}: Yazaki \textit{et al}.\(^{45}\) found that $p$-coumaric acid(2) incubated in a cell-free extract prepared from Lithospermum cell cultures was chiefly converted into $p$-hydroxybenzaldehyde, which subsequently was oxidized to form PHB in the presence of NAD\(^{+}\). The former reaction was substrate-specific, so that no phenylpropanoids but $p$-coumaric acid could be converted into any benzaldehyde. On the other hand, Lösher & Heide\(^{46}\) have shown the existence of another main pathway from $p$-coumaric acid to PHB, namely, $p$-coumaroyl-CoA is oxidized and cleaved by NAD-requiring soluble enzymes to give acetyl-CoA and $p$-hydroxybenzoyl-CoA which is rapidly hydrolyzed to PHB.

4) \textit{PHB-glucosyltransferase}: It is known that shikonin-free cells cultured in LS medium accumulate a substantial amount of PHB-$O$-glucoside(4)\(^{47}\) but little PHB. Bechthold \textit{et al}.\(^{48}\) purified UDPG: $p$-hydroxybenzoate glucosyltransferase (molecular wt: 47,500) from Lithospermum cell cultures grown in LS medium. This enzyme required no metal but UDPG and PHB for the reaction. We recently have shown that the reaction product, PHB-$O$-glucoside, is localized exclusively in the vacuoles isolated from the protoplasts of cultured cells\(^{49}\).

\textbf{Photocontrol of the key enzyme}

The investigation on the photocontrol of shikonin at the enzymatic level has shown that white or blue light blocks the prenylation of PHB by inhibiting the \textit{de novo} synthesis of PHB-geranyltransferase, while it promotes the activity of PHB-glucosyltransferase to glucosylate the accumulating PHB\(^{50}\). As a result, the PHB is transported into the vacuole where it is stored in the form of the non-toxic, water-soluble glucoside. However, when the cells are returned to the dark, they soon recover PHB-geranyltransferase activity to prenylate PHB supplied through the hydrolysis of PHB-$O$-glucoside by PHB-$O$-glucosidase, resuming the biosynthesis of shikonin. Thus, the light-induced dramatic change in the intermediary metabolism of PHB is achieved by a simultaneous regulation of the two enzymes, PHB-geranyltransferase and PHB-glucosyltransferase.

\textbf{Biosynthetic site and transport of shikonin}

Where in the cell is shikonin synthesized and how will it be secreted from the cell? To answer to these questions, cytological and biochemical examinations have been carried out. The electron microscopic observation revealed remarkable changes in subcellular structures that occurred in sequence after the transfer of white cells from LS medium to M9 medium to induce shikonin biosynthesis\(^{51,52}\). Many electron-dense, spherical swellings(0.1–0.2 $\mu$m in diameter) appeared along layers of elongated rough endoplasmic reticulum (rER), probably by frequent local expansions of the ER membranes. Subsequently, these rERs disintegrated into short fragments to release vesicular bodies(0.1–0.2 $\mu$m) into the cytoplasm. These vesicles then moved successively toward the periphery of the cytoplasm to fuse with the cell membrane and secreted their contents to the outside of the cell (\textbf{Fig. 4}). Gelled exudates discharged from the vesicles accumulated all over the surface of the cell wall as well as in the medium as numerous granules consisting of shikonin derivatives(27.2%), protein(21.5%), lipid(29.6%), and unidentified substance(22.7%).

The possibility that the vesicle also might be the site of shikonin biosynthesis has been suggested
by the following observations⁴⁰: (1) the microsomal fraction (\( \rho = 1.09 - 1.10 \, \text{g} \cdot \text{cm}^{-3} \)) having PHB-geranyltransferase activity contain numerous vesicles of 0.1-0.5 \( \mu \text{m} \) in diameter, (2) the distribution pattern of marker enzymes specific to the vesicle membrane was different from those of the membranes of ER, vacuoles, and plastids as well as of the plasma membrane, and (3) the density of the vesicle was very similar to that of the ER. In addition, the high resolution cryoelectron microscopic analysis at an extremely low temperature (4.2 K) indicated that the vesicle is covered by a phospholipid monolayer instead of a lipid bilayer (Fig. 5). In a monolayer keeping its stability under the balance between the outer and the inner pressure, it is possible that the bound enzyme such as PHB geranyltransferase is partly exposed to the outside of the lipid layer where the enzymatic reaction with a water-soluble substrate would take place.

On the basis of these findings and the intracellular localization of the soluble GPP synthase within the cytosol⁴⁴, we have postulated that the first reaction step carried out by the vesicle could be the

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**Fig. 4** Diagram of shikonin production.
CW: cell wall, ER: endoplasmic reticulum, M: mitochondria, N: nucleus, P: plastid, PL: plasma membrane, V: vacuole, Vs: vesicle.

**Fig. 5** A hypothetical model for the secretion of shikonin by a membrane vesicle.
prenylation of PHB by the PHB-geranyltransferase on the phospholipid monolayer, which then would be followed by a series of enzymatic reactions including decarboxylation, hydroxylation, cyclization, and oxidation leading to the formation of shikonin. The validity of this hypothesis has been supported by the following observations: (1) the activity of GBA decarboxylase, which will convert the reaction product of PHB geranyltransferase, GBA, into the next intermediate GHQ, was also detected in the vesicle fraction; (2) 14C-labeled deoxyshikonin incubated with vesicles in vitro was transformed to shikonin and its esters including acetyl- and β-hydroxyisovaleryl-shikonin within 3 hours at 27°C, indicating the presence of enzymes responsible for both hydroxylation and acylation of deoxyshikonin in the vesicles; and (3) a yellowish liquid of the fresh vesicle fraction turned red when left overnight at the room temperature, and this change was promoted at a higher temperature or by a forced aeration, suggesting the formation of shikonin pigments from precursors in the vesicles (unpublished data). In the living cells, however, the final reaction products in the vesicles are thought to be accumulated as colorless compounds (probably unstable naphthols), which would be oxidized to yield red naphthoquinones (shikonin pigments) when excreted from the cells and exposed to the air.

Concluding remarks

It has been demonstrated that the vesicles derived from rER participate not only in the biosynthesis but also in the transport of shikonin derivatives, which are secreted from the cells by exocytosis. Such a transport system for secondary products as that found for the first time in Lithospermum cells has also been reported for the extracellular release of the alkaloid, berberine, from Thalictrum minus cell cultures and of the triterpene, bryonolic acid, from Luffa cylindrica cell cultures. Apparently, some plant species have developed unique systems not only for carrying out the biosynthesis of either lipophilic or cytotoxic secondary products in isolated compartments, but also for discharging them safely from the cells, making good use of vesicles.

Moreover, the compartmentation of a series of enzymes would facilitate orderly biosynthetic reactions, as was demonstrated for the localization of enzymes involved in the final steps of berberine biosynthesis in the vesicles (0.1–1.0 μm, ρ = 1.14 g · cm⁻³) of cultured cells of Berberis stolonifera and Coptis japonica. Actually, both membraneous differentiation and systematization of biosynthesis in the form of vesicles in Lithospermum cells made it possible to convert the labeled precursor L-phenylalanine administered to the cells into shikonin derivatives within 5 minutes. The remarkably efficient biosynthetic ability of Lithospermum cells has been applied successfully to the industrial production of shikonin with a high yield of more than 2 g/L of M9 medium in a two-week culture in a 4 kL-fermentor.

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