

Note

Tracer experiment revealed that (*E*)-3''-hydroxygeranylhydroquinone is not an intermediate of the shikonin/alkannin and shikonofuran biosynthetic pathways in *Lithospermum erythrorhizon*

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Abstract *Lithospermum erythrorhizon* (Boraginaceae) produces shikonin/alkannin, an enantiomeric pair of red naphthoquinone pigments with diverse biological activities. For the industrial production of shikonin/alkannin derivatives, a cell suspension culture system of *L. erythrorhizon* has been established. To produce shikonin/alkannin derivatives more efficiently in cultured cells, it is essential to understand the shikonin/alkannin biosynthetic pathway, which has not been fully elucidated. A previous study suggested that a conversion of (*Z*)- to (*E*)-3''-hydroxygeranylhydroquinone (3''-OH-GHQ) is a branching point of the shikonin/alkannin biosynthetic pathway and the shikonofuran biosynthetic pathway in *L. erythrorhizon* cell cultures. However, it is not clear whether (*E*)-3''-OH-GHQ is an intermediate of both pathways. This study performed a feeding assay with three deuterium-labeled compounds including (*E*)-3''-OH-GHQ and its (*Z*)-isomer, and showed that (*E*)-3''-OH-GHQ was not involved in the shikonin/alkannin and shikonofuran biosynthetic pathways.

Key words: alkannin, 3''-hydroxygeranylhydroquinone, *Lithospermum erythrorhizon*, shikonin, shikonofuran.

Shikonin and its enantiomeric pair, alkannin, are red naphthoquinone pigments produced in the root bark of several Boraginaceae plants, including *Lithospermum erythrorhizon*. Because shikonin/alkannin derivatives have various biological activities, plants producing these derivatives have been used as crude drugs in traditional medicine in European and Asian countries (Yadav et al. 2022). This property of *L. erythrorhizon* as a crude drug led to the establishment of cell culture systems for the industrial production of shikonin derivatives (Yazaki 2017). These cultured cells have been used to study the shikonin/alkannin biosynthetic pathway (Supplementary Figure S1). The first step of shikonin/alkannin biosynthesis is a coupling reaction of *p*-hydroxybenzoic acid (PHB) and geranyl diphosphate (GPP) to form *m*-geranyl-*p*-hydroxybenzoic acid (GBA) (Yazaki et al. 2002). This intermediate is decarboxylated to geranylhydroquinone (GHQ), which is then hydroxylated at the 3'' position to form (*Z*)-3''-hydroxygeranylhydroquinone ((*Z*)-3''-OH-GHQ) (Wang et al. 2019). (*Z*)-3''-OH-GHQ appeared to be cyclized to deoxyshikonin, followed by hydroxylation and acylation at the side chain to various shikonin/alkannin

derivatives (Oshikiri et al. 2020; Song et al. 2021). Although several studies have attempted to identify the enzymes that catalyze the reaction between (*Z*)-3''-OH-GHQ and deoxyshikonin, no enzyme has been identified to date. An enzyme assay using crude cell-free extract of cultured *L. erythrorhizon* cells reported that (*E*)-3''-OH-GHQ is produced from (*Z*)-3''-OH-GHQ (Yamamoto et al. 2020). In *Arnebia euchroma*, a plant of the Boraginaceae family, an enzyme of the cinnamyl alcohol dehydrogenase family (AeHGO) catalyzes this reaction (Wang et al. 2023). It has been suggested that this (*Z*)- to (*E*)-form conversion is a branching point of the two pathways leading to shikonin/alkannin and shikonofuran (Yamamoto et al. 2020), though it is not clear whether (*E*)-3''-OH-GHQ is an intermediate of both pathways and other biosynthetic pathways. Identification of the intermediates and the reaction mechanisms of these biosynthetic pathways is strongly desired to establish more efficient production of shikonin/alkannin derivatives in cultured cells. In this study, a feeding assay with deuterium-labeled (*E*)-3''-OH-GHQ was performed on *L. erythrorhizon* cells to investigate the involvement of this compound in the shikonin/alkannin

Abbreviations: GBA, *m*-geranyl-*p*-hydroxybenzoic acid; GHQ, geranylhydroquinone; GPP, geranyl diphosphate; PHB, *p*-hydroxybenzoic acid; 3''-OH-GHQ, 3''-hydroxygeranylhydroquinone.

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and shikonofuran biosynthetic pathways.

As substrates for the feeding assay, we synthesized a deuterium-labeled GHQ and (Z)-3''-OH-GHQ in addition to (E)-3''-OH-GHQ (GHQ- d_3 , (Z)-3''-OH-GHQ- d , (E)-3''-OH-GHQ- d) (Figure 1A) as described in Supplementary Protocol S1. *L. erythrorhizon* cell cultures, maintained as previously reported (Takanashi et al. 2019), were inoculated into M9 medium (Fujita et al. 1981) and cultured in the dark for 3 days. Subsequently, 200 μ M of deuterium-labeled compound in methanol was added to the cells and cultured for another 3 days. The same volume of methanol was added as a control. After the pigment-producing cells were collected by suction filtration, 100 mg of each were ground with metal beads, and extracted with a 10-fold (v/w) of methanol for 1 h at room temperature. The supernatant was filtered and analyzed by HPLC-mass spectrometry using an LCMS-8040 system (Shimadzu) equipped with a COSMOSIL 2.5C18-MS-II column (2.0 mm I.D. $\times$ 100 mm; Nacalai Tesque) at a flow rate of 0.2 ml min⁻¹ at 30°C. The mobile phase consisted of water containing 1% (v/v) formic acid and acetonitrile under the following gradient condition: 80/20 (v/v) at 0 min, 33/67 at 18 min, 0/100 at 19.5–24 min, and 80/20 at 24–30 min. Mass spectra were obtained in both positive

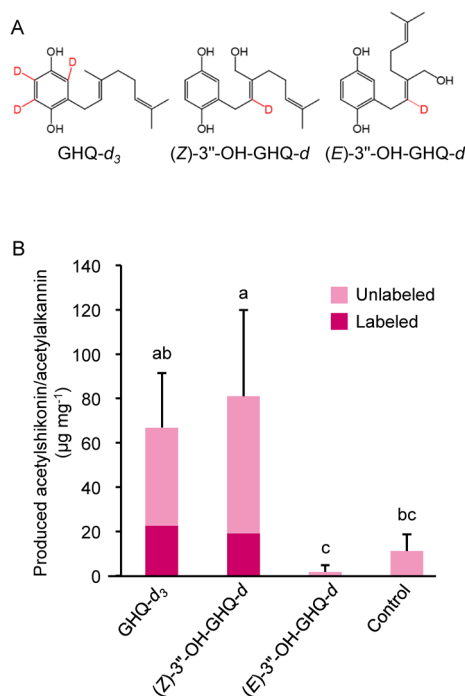


Figure 1. Production of acetylshikonin/acetylalkannin by *L. erythrorhizon* cells treated with labeled compounds. (A) Deuterium-labeled compounds used in this study. The method of synthesis of these compounds is described in Supplementary Protocol S1. (B) Comparison of acetylshikonin/acetylalkannin production and presence of labeled compounds. The labeled compounds were detected when treated with GHQ- d_3 or (Z)-3''-OH-GHQ- d . Significant differences ($p < 0.05$) of means $\pm$ SD ($n = 3$) were determined by Turkey-Kramer test and are indicated by different lowercase letters.

and negative modes. To evaluate the effect of labeled compounds on the production of shikonin/alkannin derivatives and shikonofuran derivatives, the uptake of labeled compounds in both pathways was measured.

Because cultured *L. erythrorhizon* cells mainly produce acetylated shikonin/alkannin derivatives, we first investigated their production and conversion rates of labeled compounds. The production of acetylshikonin/acetylalkannin was increased when GHQ- d_3 or (Z)-3''-OH-GHQ- d was added (Figure 1B). The conversion of applied GHQ- d_3 and (Z)-3''-OH-GHQ- d was observed from the mass shifts of molecular ion of acetylshikonin/acetylalkannin, m/z 330 to m/z 331 (Supplementary Figure S2A), and the percentage of the labeled form in the total production was on average 33.9% and 23.4%,

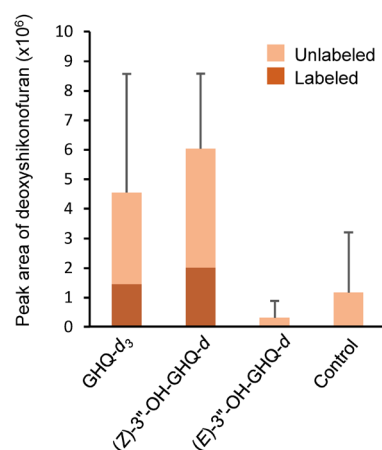


Figure 2. Production of deoxyshikonofuran. The presence of labeled compounds was shown. The labeled compounds were detected when only GHQ- d_3 or (Z)-3''-OH-GHQ- d was treated. Significant differences ($p < 0.05$) of means $\pm$ SD ($n = 3$) were calculated by Turkey-Kramer test and no significant differences were detected.

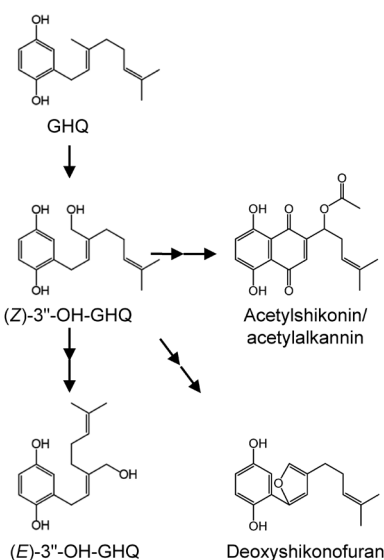


Figure 3. Biosynthetic pathway proposed by this study. (E)-3''-OH-GHQ is not an intermediate of both shikonin/alkannin and shikonofuran biosynthetic pathways.

respectively (Figure 1B).

In contrast, acetylshikonin/acetylalkannin production in (*E*)-3''-OH-GHQ-*d* treated cells was smaller than that in the GHQ-*d*₃ and (*Z*)-3''-OH-GHQ-*d* treated cells, and no labeled acetylshikonin/acetylalkannin was detected (Supplementary Figure S2A), indicating that (*E*)-3''-OH-GHQ is not a precursor of acetylshikonin/acetylalkannin. Next, we measured the conversion rates of the labeled compounds to deoxyshikonofuran, a product of the shikonofuran biosynthetic pathway (Yazaki et al. 1986). The labeled deoxyshikonofuran was observed when GHQ-*d*₃ or (*Z*)-3''-OH-GHQ-*d* was applied with the mass shift of the molecular ion, *m/z* 259 to *m/z* 262 or *m/z* 260, respectively (Supplementary Figure S2B). The conversion rates of GHQ-*d*₃ and (*Z*)-3''-OH-GHQ-*d* were on average 32.0% and 33.3%, respectively (Figure 2). When (*E*)-3''-OH-GHQ-*d* was added, deoxyshikonofuran tended to decrease and no labeled form was detected (Figure 2, Supplementary Figure S2B), similar to the result for acetylshikonin/acetylalkannin.

This study clearly demonstrated that (*E*)-3''-OH-GHQ-*d* is not an intermediate of shikonin/alkannin and shikonofuran biosynthetic pathways, since no applied deuterium-labeled compound was converted to them (Figure 3). In contrast, detection of labeled acetylshikonin/acetylalkannin and deoxyshikonofuran indicated that GHQ and (*Z*)-3''-OH-GHQ are the biosynthetic intermediates of these products (Figure 3). To increase the production of shikonin/alkannin derivatives in cultured cells, the addition of GHQ and/or (*Z*)-3''-OH-GHQ would be efficient, especially GHQ can be easily synthesized from inexpensive hydroquinone and geraniol via single chemical reaction (Baeza et al. 2012). Characterization of the enzymes that catalyze the cyclization of (*Z*)-3''-OH-GHQ to deoxyshikonin would also be effective.

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Author contribution

K.T. and B.W. conceived the research plan and designed the experiments; M.M. and H.O. performed feeding experiments; B.W. performed chemical syntheses; M.M., B.W. and K.T. wrote the article.

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Conflict of interest

The authors declare that they have no conflict of interest.

Description of Supplementary Files

Supplementary Figure S1. Proposed biosynthetic pathways of shikonin/alkannin and shikonofuran derivatives before this study. Supplementary Figure S2. Mass spectra of products treated with labeled compounds.

Supplementary Protocol S1. Synthesis of deuterium-labeled GHQ, (*Z*)-3''-OH-GHQ, and (*E*)-3''-OH-GHQ.

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